

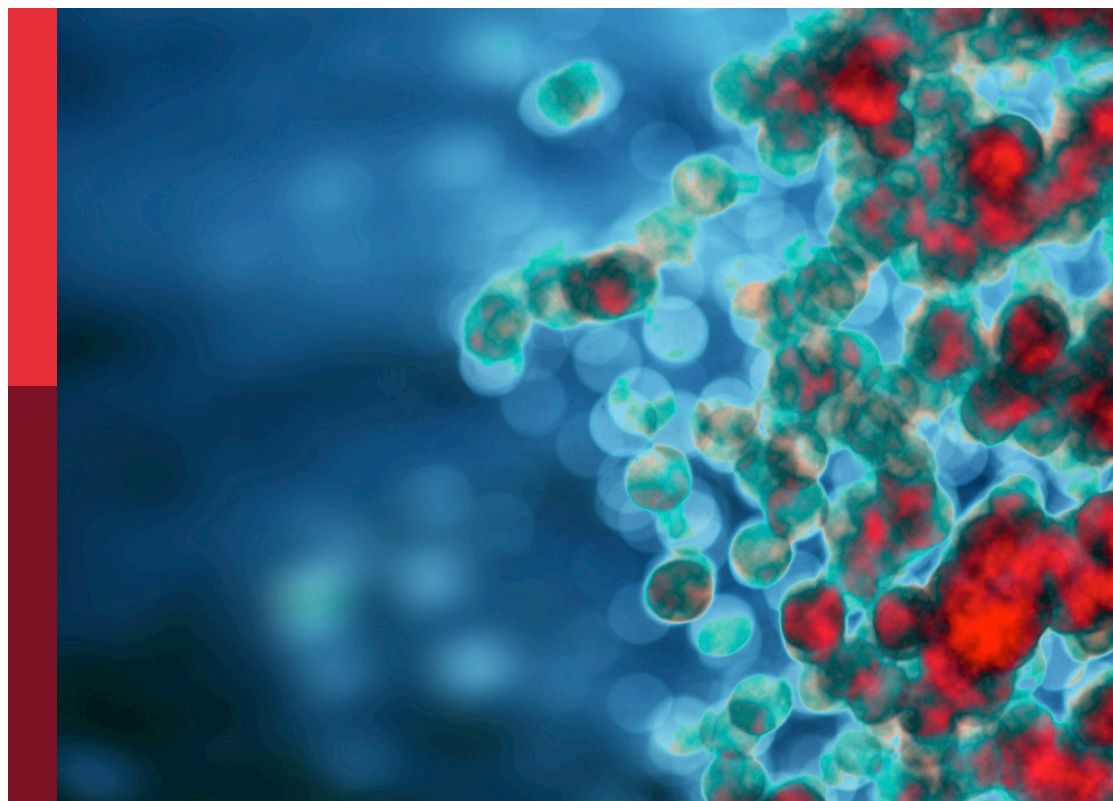
Community series in the role of complement in health and disease, volume II

Edited by

Maciej Cedzynski, Thomas Vorup-Jensen, Anna Swierzko
and Marcin Okrój

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Community series in the role of complement in health and disease, volume II

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Editorial: Community series in the role of complement in health and disease, volume II

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Editorial on the Research Topic

Community series in the role of complement in health and disease, volume II

The complement system (C') is a crucial mediator of the innate immune response, interacting with other innate mechanisms protecting the body and with factors of acquired immunity. It also contributes significantly to cell homeostasis, tissue development and repair, reproduction, and interacts with other endogenous cascades, such as the coagulation system. Each of the three major complement activation pathways (classical, CP; alternative, AP; and lectin, LP) employs specific recognition molecules and initiating serine proteases. All of them converge into a common pathway, leading to the formation of the biologically highly active anaphylatoxin C5a and the C5b-9 membrane attack complex (MAC). The latter forms transmembrane channels, either inducing “sub-lytic” activation of the cell or resulting in target cell lysis. Although the complement system was discovered in 1888, it remains the subject of intense investigation, as demonstrated by the present volume. Significant advances are ongoing in various aspects, including its clinical significance and its role in regulating the immune response.

Generally, as an essential branch of first-line defense, complement protects the host from pathogens and abnormal self-derived components. This protection can be achieved through several mechanisms: (i) opsonization by activated factors, (ii) attracting immune cells and enhancing phagocytosis, or (iii) direct lysis after incorporation of the MAC into the cell envelope of the invading pathogen. Therefore, complement plays key roles in (i) preventing the spread of infection to other cells and tissues, (ii) participating in the clearance of damaged cells and tissues, and (iii) preventing the development of chronic inflammation and/or cancer. On the other hand, uncontrolled complement activation may lead to life-threatening effects such as systemic inflammation and shock, dysregulation of coagulation/fibrinolysis, and auto-aggression. Furthermore, under certain conditions, deregulated complement activation may also promote tumor growth and development, also known as tumorigenesis.

The eighteen articles of this Research Topic summarize recent achievements and provide timely reviews in the field of complement research.

Complement: basic mechanisms, interactions, and cross-talks

Foreign agents that attack the host's body, including pathogens, their products released naturally or in effect of treatment/immune response as well as broadly understood poisons, both natural (such as animal venoms or plant/fungal toxins) or being the result of intentional human activity, commonly are potent activators of the complement system. Infective microorganisms have developed a variety of evasion strategies, protecting them from C'-dependent opsonization and/or lysis, such as surface-exposed long carbohydrate chains, the release of extracellular vesicles, or binding complement inhibitors, e.g. factor H (FH). On the other hand, it is not uncommon that an "evolutionary action" has a corresponding reaction. An example of such a relationship was presented by [González-Alsina et al.](#), who demonstrated an elongation factor-Tu-mediated interaction of *Pseudomonas aeruginosa* with factor H-related protein-3 (FHR-3), one of the FH antagonists. As elevated concentration of FHR-3 was associated with higher serum bactericidal activity, it was suggested that it offers protection against *Ps. aeruginosa* infection.

Involvement of complement factors is not necessarily associated with pattern recognition and cascade activation. [Bally et al.](#) confirmed (reported elsewhere) the interaction of MASP-2 proenzyme with SARS-CoV-2 nucleocapsid protein. However, no subsequent complement activation was found. On the other hand, incubation of the N protein with active protease resulted in its direct cleavage. The clinical significance of this process remains an open question.

As mentioned, complement system activation may be triggered by toxins or venoms produced by higher organisms. A commonly known (and widely used in research) example is cobra venom factor, a component of the venom of *Naja* species. Although non-toxic itself, it facilitates the penetration of other venom components into the victim's body, via C' activation. The *in vitro* study performed by [Silva de França et al.](#) demonstrated that Africanized honeybee (*Apis mellifera*) venom activates complement in human serum via all three canonical pathways (AP predominantly), potentially inducing the release of anaphylatoxins and the generation of sC5b-9. This potent stimulation is another example where complement may contribute to life-threatening immunopathological events through envenomation with multiple stings or hypersensitivity. The complement, coagulation, and kallikrein-kinin systems are known to interact to maintain homeostasis and, conversely, contribute to pathology when excessively activated. Their cross-talks reflect the ability of involved proteases to cleave various substrates. Results presented by [Lopatko Fagerström et al.](#) indicate that kallikrein-kinin system activation induces C' activation on primary glomerular endothelial cells via bradykinin B1 receptor (B1R) signalling pathway. Data

from wild-type and B1/B2 receptor knockout mice demonstrated that the cross-talk is associated with vascular inflammation in the kidney. Collectively, the papers published by [Silva de França et al.](#) and [Lopatko Fagerström et al.](#) suggest novel possibilities for therapeutic inhibition of the complement system and can serve as an inspiring point for further investigations.

Recently, the complosome (intracellularly expressed components of the complement system) has been gaining growing attention. Using single-cell RNA sequencing, [Jarczak et al.](#) demonstrated expression of multiple innate immunity-related genes, including those associated with C' in human haematopoietic stem/progenitor cells. They suggested that the complosome contributes to the regulation of haematopoiesis via the C5a-C5aR-NOD-like receptor protein 3 (NLRP3) inflammasome axis. Moreover, the identified expression of a variety of immune response genes was supposed to be potentially helpful in providing targets for regenerative medicine.

Complement: clinical associations

The clinical associations of the complement system are complex and multifaceted, ranging from a life-saving multitool to a perpetrator of life-threatening events, from therapeutic agents to therapeutic targets and disease markers. The majority of papers included in this Research Topic have been related to this issue.

Complement in women's diseases, pregnancy pathology, and premature newborns

Endometriosis (EM) is a common, chronic, inflammatory gynaecological disorder affecting >10% of women of childbearing age. It is associated with the proliferation of endometrial-like tissue outside the uterus. Apart from such symptoms as dysmenorrhea, pelvic pain, dyspareunia, and dyschezia, it may lead to infertility. Although the etiopathogenesis of EM is still not precisely understood, complement activation is considered to play a role in its development. [Agostinis et al.](#) investigated the involvement of C1q in the pathology. The expression of *C1QA*, *C1QB*, and *C1QC* genes appeared higher in endometrial lesions compared with normal endometrium, which was probably due to the presence of CD68+ cells in the EM microenvironment. C1q was demonstrated to promote angiogenesis in endothelial cells isolated from endometrial ovarian cysts as well as from healthy ovaries and to induce cell motility through gC1qR (receptor for the globular head of C1q). From this perspective, the C1q-gC1qR axis was suggested as a potential therapeutic target in EM. On the other hand, C1q may contribute to the physiological angiogenesis in the ovary.

Dysregulation of the complement system, or its overactivation, for example, as the result of intrauterine infection, is often associated with complications of pregnancy, preterm births, and adverse effects of prematurity. Data summarized and discussed in the review by [Balduit et al.](#) confirm the key role of C' in pre-eclampsia (PE). Based on the literature, the Authors underlined the

role of both excessive activation (as evidenced by lower C3 and C4 but higher C4d, C3a, C5a, factor D, Bb, and C5b-9 levels in cases compared with controls) and impaired regulation (lower factor H in PE). Again, the complement system was suggested as a target for treatment.

Preterm newborns (approx. 11% of live births occur prematurely) are at high risk of perinatal complications. Gajek et al. investigated clinical associations of complement-activating collectins in that group. Low concentrations of collectin-10 and collectin-11 (CL-10, CL-11) in cord serum were associated with birthweight <1.5 kg, low Apgar score, and need for prolonged hospitalisation. Low CL-10 was additionally related to the gestational age ≤32 weeks, fetal growth restriction, and need for intensive care >4 days. Decreased concentrations of both CL-10 and CL-11, as well as mannose-binding lectin (MBL), appeared to be risk factors for respiratory distress syndrome (RDS). Similar relationships were found for some *COLEC11* and *MBL2* polymorphisms. The data presented suggest an important role of complement-activating collectins in maintaining homeostasis in preterm neonates.

Complement in infections

The complement system is considered to constitute the first-line antimicrobial defence branch, responsible for serum microbicidal activity and cooperation with other mechanisms of innate and acquired immunity. In the paper cited above, Gajek et al. demonstrated an association between low CL-10 concentration in cord serum and perinatal early-onset infections.

As mentioned, González-Alsina et al. suggested FHR-3 to protect against *Ps. aeruginosa* systemic infections, as it enhanced the killing of clinical strains isolated from blood. On the other hand, it appeared ineffective against highly serum-resistant bacteria, therefore it cannot be considered “the perfect killer”.

The retrospective analysis performed by Koami et al. revealed that a low CH50 value (<25 U/ml) in patients hospitalised due to infections (CH50 determined within 1 week of admission) was associated with multiple organ failure and coagulopathy, and predicted fatal outcome. The highest mortality rate was noted in patients having both a low CH50 value and C3 concentration. The Authors concluded that early recognition of low complement activity may be useful for risk identification and the prevention of organ failure, thereby improving the outcome.

Complement in age-related macular degeneration

Associations between the complement system and AMD were investigated by Armento et al. and Omori et al., using retinal pigment epithelium (RPE) cells and mice as models, respectively. In the first-mentioned study, it was found that RPE cells homozygous for the H variant, corresponding to the factor H (*CFH* gene) Y402H polymorphism, are more susceptible to

oxidative stress induced by hydroquinone compared with Y-homozygous counterparts. However, normal serum or purified FH had no greater effect on the cell response (Armento et al.). The other study demonstrated that MASP-1-, MASP-3-, and MASP-1/3-null mice exhibited milder symptoms of macular degeneration (RPE depigmentation and destruction, trophy of the photoreceptor layer (PL), and thinning of the outer nuclear layer) after intravenous injection of NaIO₃, compared with WT animals. Significantly lower C3 activation was observed in MASP-3- and double-knockout mice, suggesting that MASP-3 (and consequently the alternative pathway) is the key player and a candidate therapeutic target in dry AMD (Omori et al.). The role of complement-related gene polymorphisms in AMD pathogenesis was further discussed in a review by Alic et al. In addition to the well-characterized *CFH* Y402H variant, they highlighted several other variants (both risk-associated and protective) of genes encoding complement factors, such as *FB*, *C2*, *C3*, *FI*, *CFHR1*, *CFHR3*, and *C9*.

Complement and innate autoimmunity

The involvement of complement in the pathogenesis of renal diseases has been documented in many investigations. In this Research Topic, Xu et al. focused on membranous nephropathy (MN). They performed differential expression protein (DEP) analysis, based on proteomic data from patients' urine specimens. It revealed significant correlations between the concentrations of 27 complement-related proteins and the presence of proteinuria. Furthermore, C1s and collectin-12 (CL-12) correlated with tubular atrophy/interstitial fibrosis and monocyte infiltration, while CD59 appeared to be a predictor of disease remission. The urine levels of specific complement components differed between MN patients and healthy controls, as well as between MN patients and those with IgA nephropathy. It seems likely that some of these components may be considered candidate markers of disease progression or remission.

Systemic sclerosis is an autoimmune disease of connective tissue that affects numerous organs, including the skin, lungs, cardiovascular system, kidneys, and alimentary tract. Yin et al. studied the association of C3 in a murine model, using angiotensin II receptor type 1 (AT1R) as an antigen. The C3^{-/-} mice experienced more severe pulmonary inflammation, accompanied by a higher rate of apoptosis, compared with WT animals. The study suggested an anti-apoptotic and rather unexpected anti-inflammatory role of C3 in the lung during the autoimmune response.

Complement and the complosome in cancer

As mentioned, the complement system may protect against carcinogenesis or, on the other hand, promote it, depending on multiple endogenous and exogenous factors and conditions. That problem, with emphasis on complosome and non-canonical

complement pathways (extracellular activity with no direct involvement of canonical pathways), was discussed in a review published by [de Freitas Oliveira-Tore et al.](#) Based on available literature, they concluded that various complement components regulate the tumour microenvironment *via* receptor signalling (*e.g.* C3a-C3aR and C5a-C5aR axis) and cross-talk with stromal cells.

Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is widely used in the treatment of haematologic malignancies, such as leukaemias, multiple myeloma, or lymphomas. [Fageräng et al.](#) investigated complement functional activity and sC5b-9 levels in patients with acute myeloid leukaemia undergoing allo-HSCT. Data from multiple samples (the first was taken before conditioning chemotherapy, while the last – 4 weeks after transplantation) revealed that the C' was fully active within the study period in patients who did not experience complications. In turn, marked changes were observed in cases of infections and endotheliopathy. The challenge of post-HSCT blood with bacteria resulted in a hyperinflammatory cytokine response, which appeared to be reducible *via* C3 inhibition. This finding was suggested to have therapeutic potential.

Complement in cardiovascular and metabolic diseases

The review published by [Kong et al.](#) focuses on the multiple disease associations of AP serine protease, factor D, including arrhythmia, aortic aneurysm, hypertension, coronary heart disease, ischemia/reperfusion injury, heart failure, obesity/dyslipidemia, insulin resistance, and diabetic cardiomyopathy. Thoroughly discussed literature data suggest that FD is a prospective marker of cardiovascular and metabolic diseases and/or a therapeutic target. Another review, by [Alic et al.](#), concerns the association of the complement system in general with lipid-mediated pathologies. Apart from the AMD as mentioned above, an involvement of C' in atherosclerosis, metabolic syndrome, and metabolic dysfunction-associated steatotic liver disease was described. Both papers demonstrate the crucial role of the complement system in systemic metabolism.

Complement and smoking-related diseases

As pointed out above, C' may be activated by the intentional introduction of certain products to the body. Adverse effects of both active smoking/vaping and second-hand tobacco smoke exposure are a reason for millions of severe disease cases and premature deaths worldwide. The review by [Alarabi et al.](#) focuses on the role of C' activation, which is often an underestimated relationship in morbidity and mortality. An involvement of C' in smoking-induced

respiratory, cardiovascular, inflammatory, mucosal, and macular degeneration diseases was discussed in detail. The Authors also draw attention to the contribution of thromboinflammation to the pathology associated with the aforementioned complement-coagulation interplay. Such smoke ingredients as tobacco glycoprotein (TGP), particulate matter (PM), and heavy metals were considered to induce C' activation. It was summarized that the interaction between smoking and complement involves a variety of genetic, biochemical, and environmental factors, affecting the risk of disease development and its outcome.

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Revisiting the interaction between complement lectin pathway protease MASP-2 and SARS-CoV-2 nucleoprotein

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Complement activation is considered to contribute to the pathogenesis of severe SARS-CoV-2 infection, mainly by generating potent immune effector mechanisms including a strong inflammatory response. Involvement of the lectin complement pathway, a major actor of the innate immune anti-viral defense, has been reported previously. It is initiated by recognition of the viral surface Spike glycoprotein by mannose-binding lectin (MBL), which induces activation of the MBL-associated protease MASP-2 and triggers the proteolytic complement cascade. A role for the viral nucleoprotein (N) has also been reported, through binding to MASP-2, leading to protease overactivation and potentiation of the lectin pathway. In the present study, we reinvestigated the interactions of the SARS-CoV-2 N protein, produced either in bacteria or secreted by mammalian cells, with full-length MASP-2 or its catalytic domain, in either active or proenzyme form. We could not confirm the interaction of the N protein with the catalytic domain of MASP-2 but observed N protein binding to proenzyme MASP-2. We did not find a role of the N protein in MBL-mediated activation of the lectin pathway. Finally, we showed that incubation of the N protein with MASP-2 results in proteolysis of the viral protein, an observation that requires further investigation to understand a potential functional significance in infected patients.

KEYWORDS

complement system, lectin pathway, MASP-2, SARS-CoV-2, nucleoprotein

1 Introduction

As is well known, SARS-CoV-2, the human coronavirus associated with severe acute respiratory syndrome (SARS), is responsible for the COVID-19 pandemic. Evidence for involvement of an excessive inflammatory immune response in the disease severity has emerged (1). The complement system is a major actor of anti-microbial host innate immune defense. Complement activation orchestrates immune and inflammatory processes aiming at pathogen elimination but that might contribute to excessive inflammation and tissue injury if not properly regulated. Clinical studies have provided evidences for strong complement activation in the serum and lungs of critically ill COVID-19 patients (2–4) and a few case reports have shown a beneficial effect of blocking complement at the levels of C3 or C5 components, which are involved in the generation of the pro-inflammatory C3a and C5a mediators (5–7).

Complement can be activated through three pathways, classical, lectin and alternative, which involve different recognition proteins but converge at the level of C3 complement component cleavage. The lectin complement pathway is of particular importance in the context of viral infection. It is initiated by recognition of viral surface glycoproteins by soluble oligomeric proteins of the collectin family, including mannose-binding lectin (MBL) and ficolins, which associate with MBL-associated serine proteases (MASPs) to trigger the complement cascade. In line with clinical studies reporting that MBL likely contributes to complement activation and uncontrolled inflammation (8–11), a few case reports showed successful treatment of COVID-19 patients with Narsoplimab, a human monoclonal antibody against MASP-2, the effector protease of the lectin pathway, known to inhibit complement activation and with anticoagulant effects (12–14). Inhibition of the lectin pathway activation by a monoclonal antibody targeting MASP-2 has also been shown to reduce acute respiratory distress syndrome (ARDS) severity in mouse models of SARS-CoV-2 infection (15, 16).

The molecular determinants of the lectin pathway activation have been previously investigated and MBL has been shown to interact with SARS-CoV-2 Spike surface glycoprotein and to trigger complement activation (13, 17). In addition, *in vitro* studies have provided evidence for blockage of viral entry by MBL, suggesting a protective function (17, 18). A role for the SARS-CoV-2 N protein (alias nucleoprotein or nucleocapsid) has also been reported through interaction with the catalytic domain of MASP-2, leading to MBL-dependent overactivation of this protease and potentiation of the lectin pathway (13, 15). However, the participation of the N protein in activation of the lectin pathway has been questioned in a recent conference presentation abstract (19).

The aim of the present study was to reinvestigate the molecular interactions of the SARS-CoV-2 N protein with the MASP-2 protease of the lectin pathway and its possible effect on complement activation. We took advantage of our capacity to express recombinant MASP-2 as a full-length protease, in addition to its catalytic domain, and used two forms of recombinant viral N protein, either expressed in bacteria or secreted by mammalian cells. Using *in vitro* settings, we could not

confirm the interaction of the viral N protein with the catalytic domain of MASP-2 and a role of N protein in activation of the lectin complement pathway. We also identified N protein as a substrate of the MASP-2 protease, which, to our knowledge, had not been reported before.

2 Materials and methods

2.1 Proteins

2.1.1 Recombinant MASP-2

Recombinant human MASP-2 with a C-terminal Flag tag was produced by stable transfection of 293-F cells using a pcDNA3.1_MASP-2-Flag expression vector as described by Lorvellec et al. (20). The expression plasmid coding for recombinant MASP-2 stabilized in a proenzyme form by mutation of the active site Ser 618 into Ala (mature protein numbering) was generated with the QuickChange II XL site-directed mutagenesis kit (Agilent Technologies), using the mutagenic primers described by Zundel et al. (21) and the pcDNA3.1_MASP-2-Flag plasmid as template. Each plasmid was used for stable transfection of Freestyle 293-F cells (ThermoFisher Scientific) and the recombinant proteins were purified from cell culture supernatants by anti-FLAG and C1q affinity chromatography, as described by Lorvellec et al. (20). MASP-2 molar concentration was estimated using a M_r value of 75,100 and an absorbance (0.1%, 1 cm) at 280 nm ($A_{0.1\%, 1\text{ cm}}$) of 1.57. SDS-PAGE analysis of purified MASP-2 showed that it was recovered in an active form, as assessed by the presence of the A and B chains under reducing conditions (Supplementary Figure S1A), whereas the S618A mutant exhibited a single band corresponding to the proenzyme form of the protease.

The expression plasmid coding for the catalytic domain of MASP-2 encompassing the CCP1, CCP2 and serine protease (SP) domains (amino acids 282–671 of mature protein, named CCP1,2-SP) was generated from the pcDNA3.1_MASP-2-Flag plasmid by deletion of the N-terminal CUB1-EGF-CUB2 sequence using the QuickChange II XL site-directed mutagenesis kit. This plasmid served as a template to generate the expression plasmid coding for the S618A mutant of MASP-2 catalytic domain. Both catalytic domains were purified from cell culture supernatant on an anti-FLAG M2 agarose column (Sigma-Aldrich) as described by Lorvellec et al. (20). Most of the recombinant proteins were recovered in the flow through, dialyzed against 20 mM Na_2HPO_4 , 5 mM EDTA, pH 8.6 and loaded onto a Q-Sepharose Fast Flow column (Cytiva) equilibrated in the same buffer. Elution was carried out by applying a linear gradient from 0 to 350 mM NaCl in the same buffer, as described previously (22). Fractions containing the recombinant proteins were identified by Western blot analysis, dialyzed against Tris-buffered saline, pH 7.5 (TBS, EuroMedex) and concentrated by ultrafiltration up to 0.34 mg/ml. The concentration of MASP-2 catalytic domain was estimated using M_r and $A_{0.1\%, 1\text{ cm}}$ values of 43,540 and 1.84, respectively. As observed for full-length MASP-2, the catalytic domain of the protease was produced in an activated form whereas its S618A

mutant was stabilized in the proenzyme form (Supplementary Figure S1A). The purity of the fragment was estimated to be >85%.

2.1.2 Other human complement proteins

Activated C1r and C1s proteases were purified from human serum as described by Arlaud et al. (23), and their concentration estimated using M_r and $A_{0.1\%, 1\text{ cm}}$ of 86,300 and 1.24 (C1r), and 78,900 and 1.45 (C1s).

Recombinant mannose-binding lectin (MBL) was produced in 293-F cells and purified by affinity chromatography on N-acetylglucosamine-agarose as described previously (24). Its molar concentration was estimated using $A_{0.1\%, 1\text{ cm}} = 0.78$ and $M_r = 305,000$, assuming a majority of tetrameric species present in the samples.

2.1.3 Recombinant SARS-CoV-2 N and S proteins

Two recombinant forms of N protein were used, expressed either in bacteria (N-Bact) or in mammalian Expi293 cells (N-Expi). N-Bact (aa 1–419 with an N-terminal His6-tag) was expressed in *Escherichia coli* BL21 (DE3) and purified by Ni-affinity and size exclusion chromatographies as described by Bessa et al. (25). N-Bact concentration was estimated using a M_r of 44,000 and an $A_{0.1\%, 1\text{ cm}}$ of 0.95. SDS-PAGE analysis showed a major band at 55 kDa and a minor degradation product (Supplementary Figure S1B). A pcDNA3.4 plasmid encoding N-Expi (aa 1–419 with mouse IgG2 Heavy Chain signal peptide and a C-terminal His6-tag) was kindly provided by Dr S. Simon, CEA- I2BC, Saclay. The recombinant protein was produced using the Expi293 transient expression system as described by the manufacturer (ThermoFisher Scientific). The cell culture supernatant was loaded using recommended procedure on a Hitrap Chelating HP column (Cytiva) equilibrated in TBS containing 5 mM imidazole and the recombinant N protein was eluted using a 5–500 mM imidazole gradient in TBS. The protein was dialyzed against TBS and concentrated to 0.36 mg/ml. N protein concentration was estimated using $M_r = 47,000$ and $A_{0.1\%, 1\text{ cm}} = 0.95$. N-Expi analysis by SDS-PAGE showed a large band at around 60 kDa likely reflecting the heterogeneous glycosylated state of the protein, with some degradation products (Supplementary Figure S1C), as reported previously for a commercial protein produced with a proprietary signal peptide sequence (26).

The trimeric ectodomain of prefusion stabilized S protein was expressed in Expi293F cells and purified by Ni-affinity and size exclusion chromatography as described by Thépaut et al. (27). S protein was quantified using $M_r = 420,000$ and $A_{0.1\%, 1\text{ cm}} = 1.04$.

2.2 Characterization of N interaction with complement proteins by surface plasmon resonance (SPR)

SPR interaction analyses were performed at 25°C on a T200 instrument (Cytiva). N proteins were captured through their His-tag using a penta-His antibody (Qiagen) immobilized on a CM5

Series S sensorchip (Cytiva). The antibody was diluted to 20 µg/ml in 10 mM sodium acetate pH 4.5 and 6,300–7,300 RU were covalently coupled to two adjacent flow cells using the amine coupling chemistry in HBS-EP+ (Cytiva). N proteins (1,200–2,300 RU) were captured on the Penta-His antibody by injecting N-Bact (4 µg/ml) or N-Expi (18 µg/ml) in TBS buffer, pH 7.4, Tween 20 0.05% (TBS-T). Binding was measured at a flow rate of 20 µl/min in TBS-T containing 2 mM CaCl_2 (TBS-Ca-T). The specific binding signal was obtained by subtracting the signal over the reference surface (flow cell with immobilized Penta-His antibody and without N captured). Regeneration of the surfaces was achieved by 10 µl injections of 10 mM glycine pH 2.

Kinetic data were recorded in single cycle kinetics mode, using five concentrations of MASP-2 S618A (ie 37.5, 75, 150, 300 and 600 nM). Buffer blanks were subtracted from the data sets (double referencing). Global fitting of the data to the 1:1 Langmuir binding model was performed using the Biacore T200 evaluation 3.2 software (Cytiva). The apparent equilibrium dissociation constants (K_D) were calculated from the ratio of the dissociation and association rate constants (k_d/k_a). χ^2 values were below 1 in all cases. Kinetic values represent means $\pm$ SD ($n = 2$ or 3, as indicated).

2.3 N proteolysis by MASP-2 and homologous proteases

Recombinant SARS-CoV-2 N and S proteins were incubated with complement serine proteases using an enzyme/substrate molar ratio of 1/10 in TBS for 90–120 min at 37°C. The samples were transferred to ice and immediately reduced with 50 mM dithiothreitol in 0.1 M Tris-HCl, pH 8.0 containing 4 M urea and 1% SDS for 60 min at 37°C and alkylated with 140 mM iodoacetamide for 20 min at 37°C. Samples were analyzed by SDS-PAGE and staining with Instant Blue dye reagent (Expedeon) or Quick Coomassie Stain (Clinisciences).

2.4 Activation of the lectin complement pathway

Complement activation via the MBL-dependent lectin pathway was investigated using a C4b deposition ELISA, as described by Lacroix et al. (28). S, N and control mannan (at concentrations of 10, 20 and 50 µg/ml, respectively, in PBS) were coated on microplates and incubated with either 10% human MBL-deficient human serum (Statens Serum Institute, Copenhagen, Denmark) reconstituted with 5 µg/ml recombinant human MBL, or 4% normal human serum (NHS). NHS was obtained from the Etablissement Français du Sang Rhône-Alpes (agreement number EFS AURA 21–001 regarding its use in research). The potential contribution of viral N protein to mannan- or S protein-mediated complement activation was assayed by adding soluble N protein (10 µg/ml) in the serum.

2.5 Statistical analysis

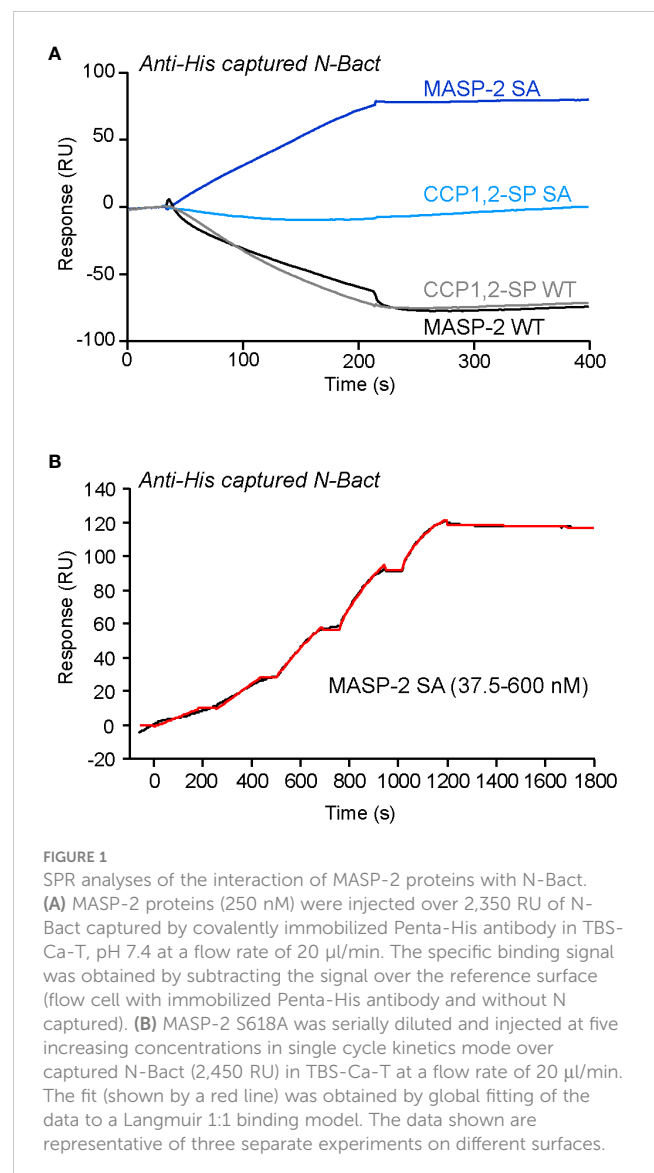
Where applicable, Prism GraphPad software was used for the statistical analyses of data. Comparisons among groups were performed using one or two-way ANOVA followed by Bonferroni's or Tukey's multiple comparison tests.

3 Results

Contradictory results have been reported about the interaction of the N protein of SARS-CoV-2 and complement proteins and its consequences, more particular its capacity to enhance activation of the lectin complement pathway through interaction with the MASP-2 protease (15). Published experiments have been performed using different sources of recombinant N (commercial or home-made), including a fusion protein with a signal peptide, secreted as a glycosylated protein by eukaryotic cells, or a non-glycosylated form produced in eukaryotic cells without signal peptide (26) or by bacteria. We produced both forms (N-Expi and N-Bact) to reinvestigate the capacity of N protein to interact with the MASP-2 protease of the lectin complement pathway. The full-length protease was expressed in 293-F cells, either as a wild-type active protease or stabilized in a proenzyme form by mutation of the active site Ser by Ala, and its catalytic domain (active or proenzyme) was also produced using the same cells (Supplementary Figure S1).

3.1 N protein interaction with MASP-2

We investigated the interaction of N with MASP-2 by SPR spectroscopy, using oriented capture of both N protein forms through their His-tag and injection of the soluble protease. As illustrated for N-Bact in Figure 1A, injection of proenzyme MASP-2 (S618A mutant) resulted in a signal increase, reflecting binding of the protease, whereas no significant binding was detected for the proenzyme form of MASP-2 catalytic domain. In contrast, a decrease of the signal was observed reproducibly when injecting the wild-type forms of both MASP-2 and its catalytic domain (Figure 1A). Comparable results were obtained using recombinant N-Expi protein, except that a weak binding of wild-type MASP-2 was observed (Supplementary Figure S2A). Kinetic analysis of proenzyme MASP-2 binding to captured N-Bact (Figure 1B) yielded an association rate constant (k_a) of $1.39 \pm 0.35 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, a dissociation rate constant (k_d) of $1.50 \pm 0.83 \times 10^{-4} \text{ s}^{-1}$, with a resulting apparent equilibrium dissociation constant (K_D) of $12.3 \pm 9.3 \text{ nM}$ ($n = 3$), reflecting high affinity. Similar values were obtained using captured N-Expi (Supplementary Figure S2B), with $k_a = 1.44 \pm 0.63 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $k_d = 1.72 \pm 0.29 \times 10^{-4} \text{ s}^{-1}$ and $K_D = 13.7 \pm 4.0 \text{ nM}$ ($n = 2$). The observed decrease of the signal when injecting the active proteases was intriguing and led us to hypothesize that N protein might be cleaved by MASP-2, which would result in loss of captured ligand over time.



3.2 N protein cleavage by MASP-2

In order to check the above hypothesis, N proteins were incubated with full-length MASP-2, either activated (wild-type) or stabilized in proenzyme form (S618A mutant). Incubation of N-Bact with 10% active MASP-2 (molar ratio) for 90 min at 37°C resulted in disappearance of the band corresponding to the intact N protein with concomitant appearance of several degradation fragments of lower molecular weights (Figure 2A). Incubation of N-Bact with proenzyme MASP-2 or buffer did not induce any change, indicating that the observed proteolysis was due to the enzymatic activity of MASP-2. Incubation with the catalytic domain of MASP-2, either activated or stabilized in a proenzyme form, under the same molar ratio and reaction conditions, resulted in similar patterns (Figure 2B), thus confirming the capacity of MASP-2 enzyme to cleave N protein. Proteolysis of N-Expi by the wild-type MASP-2 proteases could also be observed (Supplementary Figure S3A), although the pattern was less clear due to the

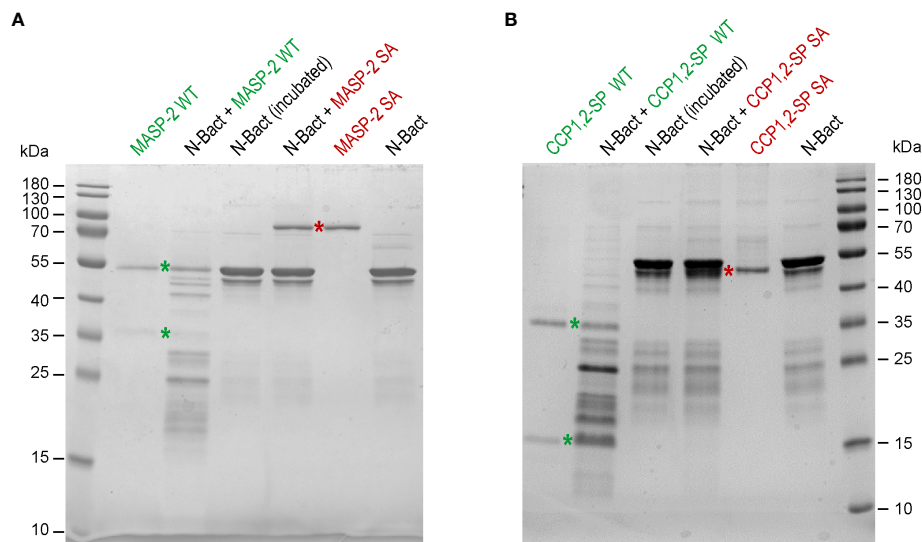


FIGURE 2

MASP-2 proteolytic activity on N-Bact protein. N-Bact was incubated with wild-type activated MASP-2 (A) or its catalytic CCP1,2-SP fragment (B), their proenzyme S618A counterparts or TBS for 90 min at 37°C. The digestion products and the control proteases and substrate were loaded on 12.5% acrylamide gels and SDS-PAGE analysis was performed under reducing conditions. The bands corresponding to the two chains of activated wild-type MASP-2 or its catalytic domain and to their proenzyme counterparts are indicated by green and red stars, respectively. The molecular masses (kDa) of the markers are indicated. Each gel shown is representative from three experiments.

glycosylated and partially proteolysed state of the purified protein. We also investigated MASP-2 capacity to cleave the Spike protein of SARS-CoV-2. As shown in [Supplementary Figure S3B](#), no detectable cleavage of S was observed following incubation for 2 h at 37°C with 10% enzyme: substrate (recombinant trimeric Spike ectodomain) ratio. The activity of MASP-2 was further compared with that of the two homologous proteases of the classical complement pathway, C1r and C1s. C1s has a substrate specificity similar to MASP-2 by cleaving complement proteins C4 and C2 whereas C1r only activates zymogen C1s. As shown in [Figure 3](#), incubation with C1s resulted in very partial proteolysis of N-Bact protein, characterized by generation of fragments similar to those observed in the case of MASP-2. A similar profile was observed with C1r, although the cleavage was even less efficient than with C1s. These results indicate that complement proteases C1s and C1r are much less efficient than MASP-2 in cleavage of N protein.

3.3 N protein and activation of the lectin complement pathway

It has been demonstrated that SARS-CoV-2 Spike protein is able to activate the lectin complement pathway ([13](#), [15](#), [17](#)) but the role of the viral N protein is controversial ([13](#), [19](#)). Activation of the lectin complement pathway was measured by C4b deposition in wells coated with either mannan or the viral S protein, following incubation with either MBL-deficient HS reconstituted with MBL or normal HS (NHS). As expected, control mannan and S protein triggered MBL-dependent activation of the lectin complement pathway ([Figures 4A, B](#)). Addition of either N-Bact or N-Expi to the sera (NHS or MBL-deficient HS reconstituted with MBL) did

not induce statistically significant differences in the level of C4b deposition ([Figures 4A, B](#)). The capacity of the N protein to trigger complement activation complement was also assayed directly by coating N-Bact or N-Expi. We observed weak, although significant C4b deposition using both sera (NHS and MBL-deficient HS), with no effect of reconstitution of the MBL-deficient serum with MBL ([Figures 4C, D](#)). This result indicates that C4b deposition on coated N proteins was clearly not the result of MBL-dependent activation of the lectin pathway. Comparison between C4b deposition induced by SARS-CoV-2 S and both N protein forms confirmed that the Spike protein was much more efficient in triggering activation of the lectin complement pathway ([Figure 4E](#)).

4 Discussion

In the present study, we revisited the complement activating capacity of the N protein and the molecular interactions between the MASP-2 protease of the lectin complement pathway and the nucleoprotein of SARS-CoV-2, using home-made and well characterized recombinant SARS-CoV-2 N and human complement MASP-2 proteins. It seemed important to use the N protein either produced in bacteria or secreted by mammalian cells since differences have been reported between both proteins, mainly because the secreted protein contains numerous post-translational modifications, including N- and O-glycosylations ([26](#)). Contradictory results have been reported regarding the recombinant N protein produced in HEK293 cells without signal sequence, which was characterized as a non-glycosylated ([26](#)) or glycosylated protein ([29](#)). Our assumption of the relevance of the bacterial recombinant N protein is based on the fact that the native

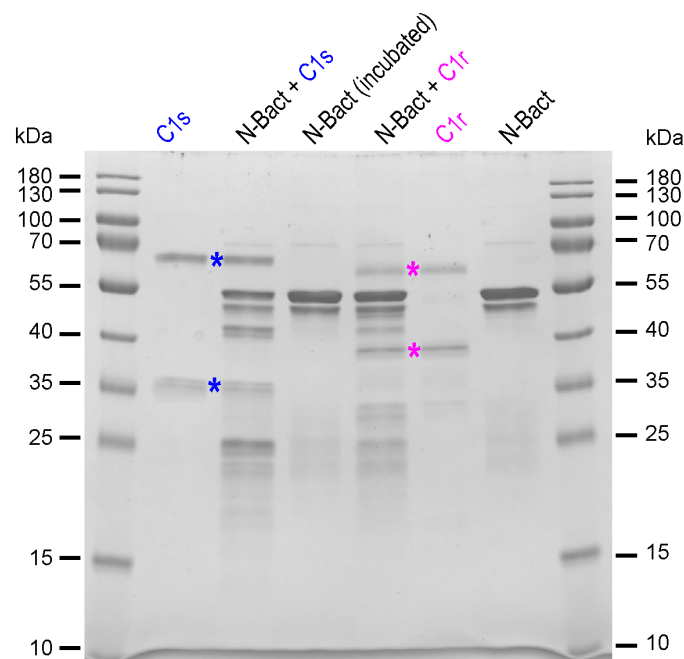


FIGURE 3

Proteolytic activity of complement C1s and C1r proteases on N-Bact protein. N-Bact was incubated with activated C1s, activated C1r or TBS for 90 min at 37°C. The digestion products and the control proteases and substrate were loaded on a 12.5% acrylamide gel and SDS-PAGE analysis was performed under reducing conditions. The bands corresponding to the two chains of activated C1s and C1r are indicated by blue and pink stars, respectively. The molecular masses (kDa) of the marker are indicated. A gel representative from two experiments is shown.

viral N protein is an intracellular protein lacking a signal sequence, which is expected to prevent some post-translational modifications such as N-glycosylation. In addition, using the recombinant protein either produced in *E. coli* or secreted by CHO cells, Rump et al. have demonstrated that glycosylation of the N-protein masks some of its immunodominant antibody epitopes (30).

Several studies have demonstrated that interaction of the Spike glycoprotein of SARS-coronaviruses with MBL triggers activation of the lectin complement pathway (13, 17, 18), which was confirmed in the present study. The N protein was proposed to potentiate lectin pathway activation (15) or to directly trigger complement activation (13), but we could not detect an effect of soluble N protein, produced either in bacteria or in Expi293 cells, on mannan- or SARS-CoV-2 Spike protein-mediated complement activation. Although weak C4b deposition was observed on coated N proteins, it did not arise from MBL-dependent activation of the lectin complement pathway. Interestingly, this observation is in accordance with data reported recently by Kocsis et al. in a conference presentation abstract (19), who concluded that the N protein activates the alternative complement pathway but not the lectin or classical pathways. The fact that we detected weak C4b deposition on coated N proteins likely reflects some activation of the classical pathway, which might be caused by the presence of anti-N protein antibodies. Indeed our NHS was collected after the pandemic and, given the known high immunogenicity of N protein, the presence of antibodies is highly plausible. It should be mentioned that our data are not in contradiction with the reported therapeutic benefit of MASP-2 specific inhibition by

Narsoplimab. Indeed, since this protease is essential to trigger the lectin complement pathway, its inhibition will anyway reduce complement activation and its potential noxious consequences.

A new result of the present study resides in the observed capacity of the MASP-2 protease to cleave the N protein, which was assessed by comparing proenzyme and activated forms of the full-length protease and its catalytic domain. This cleavage property implies transient interaction between the active protease and its substrate, which could not be detected in our SPR measurements due to the proteolysis of N protein. However, we observed high affinity interaction of the viral protein (either produced in bacteria or secreted by Expi293 cells) with full-length MASP-2 stabilized in the proenzyme form. The K_D values determined by SPR were in the 10 nM range, lower than those determined by Gao et al. (15) for SARS-CoV-1 and SARS-CoV-2 N proteins (268 and 514 nM, respectively). However, direct comparison is not possible due to the lack of experimental details of the latter study, regarding the nature of immobilized ligands and soluble analytes, the protease activation state and the immobilization procedure. In the case of N-Expi, a weak interaction was observed with wild-type MASP-2, which might explain previously reported N protein-MASP-2 interactions (15). The possible biological significance of the interaction between proenzyme MASP-2 and the N-protein is difficult to predict from *in vitro* experiments using isolated recombinant proteins. The concentration of MASP-2 in serum is very low (0.5 µg/ml) (31) which corresponds to a molar concentration of 6 nM, lower than the determined K_D value for the interaction with the N protein (between 10 and 20 nM). In

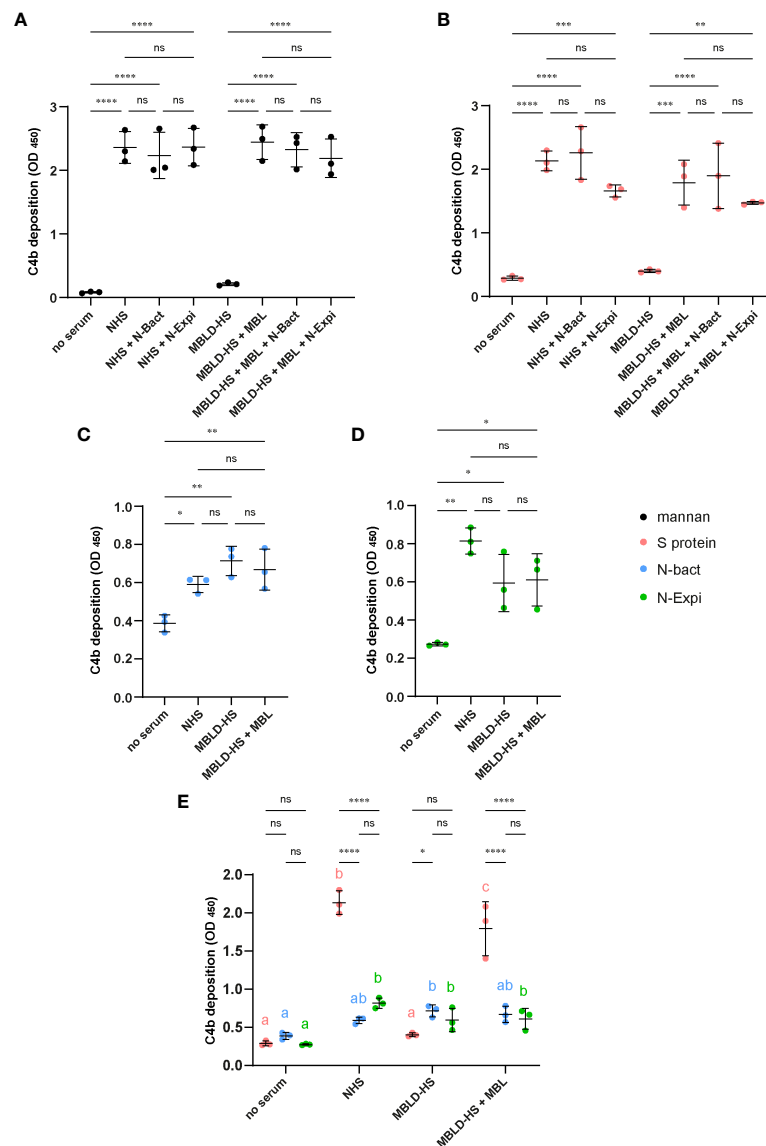


FIGURE 4

(A, B) Effect of N proteins on mannan- and S protein-mediated complement activation. Normal human serum (NHS) or MBL-deficient serum (MBLD-HS) reconstituted with MBL were added to microwells coated with 50 $\mu\text{g/ml}$ mannan (A) or 10 $\mu\text{g/ml}$ S protein (B) in the presence or absence of 10 $\mu\text{g/ml}$ N-Bact or N-Expi. Incubation without serum or with MBLD-HS served as controls. Complement activation was measured by a C4b deposition ELISA. (C, D) N protein-mediated complement activation. Normal human serum (NHS) or MBL-deficient serum (MBLD-HS) reconstituted with MBL were added to microwells coated with 20 $\mu\text{g/ml}$ N-Bact (C) or N-Expi (D) and complement activation was measured as in (A, B). All data represent mean and SD of three independent experiments performed in duplicate. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's (A, B) or Tukey's (C, D) multiple comparisons. (E) Comparison of the data obtained with the different sera and coated S protein (B), N-Bact (C) and N-Expi (D). Statistical analysis was performed by two-way ANOVA, followed by Tukey's multiple comparison test. Simple effects within sera are presented using compact letter display. Different letters with the same color indicate statistically significant differences ($p < 0.05$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, no statistically significant differences.

addition, all proenzyme MASP-2 in serum forms complexes with multiple complement pattern recognition proteins such as MBL, ficolins and collectin-11 (31–33), which likely restricts its interaction capacities.

In contrast to the two studies reporting interaction of the catalytic domain of MASP-2 with the N proteins of the SARS-CoV-2, SARS-CoV and MERS-CoV (13, 15), we could not detect any interaction of SARS-CoV-2 N protein with the catalytic domain of MASP-2 (activated or proenzyme). This observation is in accordance with recent data reported in an abstract presented at the last complement

International Workshop in Newcastle (19). However, the lack of interaction of the proenzyme catalytic domain is somehow surprising because of the capacity of its active counterpart to cleave the N protein, which implies transient enzyme-substrate interaction. It may indicate that the MASP-2/N protein interaction involves additional N-terminal domains of the protease. It should be mentioned that co-authors of Gao et al. (15) reported previously that the SARS-CoV-1 N protein interacts with MAP19, a splicing product of MASP-2 encompassing the N-terminal CUB1-EGF segment of the protease and thus devoid of enzymatic activity (34). These former data are in

apparent contradiction with the 2022 study where the authors found no interaction between SARS-N proteins and the N-terminal CUB1-EGF-CUB2 fragment of MASP-2 (15). We were not able to check the interaction of the N protein with this fragment by SPR because the purified recombinant fragment was not available to us. It may also be considered that the presence of the N-terminal domains of MASP-2 could stabilize the interaction of the N protein with the full-length protease.

The susceptibility of the N protein to proteolysis is not unexpected given its heterogeneous structure. Indeed, it comprises two globular RNA binding domains and three intrinsically disordered regions, located at the N- and C-terminal extremities and in the central region connecting the two structured domains (35). It has been found that the N protein undergoes proteolysis in the vicinity of the linker region, giving rise to several proteoforms (36). Notably, the flexible linker sequence contains several arginine residues corresponding to potential cleavage sites for trypsin-like proteases. MASP-2 has trypsin-like specificity and is known to cleave itself (autoactivation), complement C2 and C4 proteins, C1-Inhibitor, prothrombin (22, 37, 38) and more recently the HMGB1 alarmin (20). It is homologous to the C1s protease of the classical complement pathway and has been shown to have higher catalytic efficiency than C1s (39). It is therefore not surprising that C1s was also found to cleave the N protein, although less efficiently than MASP-2. A possible role for MASP-2 in the cleavage of the Spike protein of SARS-CoV-1 had also been suggested, based on comparison of data using recombinant MBL and serum-derived MBL (contaminated with MASPs) (18), which was not verified in the present study using recombinant SARS-CoV-2 Spike protein. It should be mentioned that the possible functional significance of N proteolysis by MASP-2 remains to be investigated. It may impact *in vitro* experiments including incubation steps with isolated recombinant proteins, which however do not reflect the complex physiological situation of infection. Importantly, serum MASP-2 is activated in the context of complexes with different pattern recognition proteins and we did not detect an effect of N protein on activation of the MBL-dependent lectin complement pathway. It has also been shown recently that the N protein synthesized during SARS-CoV-2 infection is released from cells and binds to both infected cells and bystander non-infected cells through association with heparan sulfate/heparin (40). In addition, cell-bound N protein was shown to interact with several chemokines and to modulate the host immune response.

In conclusion, we could not verify N protein-mediated activation of the lectin complement pathway (13) or potentiation of complement activation (15), in accordance with a recently published conference presentation abstract (19). We did not find essential differences between non-glycosylated N-Bact protein and the N-Expi glycosylated protein secreted by mammalian cells, in the context of our experiments. We also did not confirm the interaction of N protein with the catalytic domain of MASP-2, which had been reported in previous studies (13, 15). However, we could show that the N protein interacts with full-length MASP-2, but only with the protease in the proenzyme state. Finally, we provide evidence for the capacity of active MASP-2 to cleave the N protein, although the

physiological significance of this cleavage in the serum of infected patients remains to be elucidated.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Etablissement Français du Sang Auvergne Rhône Alpes 21-001. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from Etablissement Français du Sang Rhône-Alpes (agreement number EFS AURA 21-001 regarding its use in research). Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

IB: Writing – review & editing, Conceptualization, Formal analysis, Investigation, Methodology. GD: Formal analysis, Investigation, Methodology, Writing – review & editing. VR: Investigation, Writing – review & editing. SG: Resources, Writing – review & editing. MBo: Resources, Writing – review & editing. J-BR: Investigation, Writing – review & editing. MT: Resources, Writing – review & editing. SDD: Formal analysis, Validation, Writing – review & editing. CD-P: Funding acquisition, Writing – review & editing. CG: Validation, Writing – review & editing. FF: Resources, Writing – review & editing. MBL: Resources, Writing – review & editing. PP: Funding acquisition, Writing – review & editing. NT: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The function of the complement system remains fully intact throughout the course of allogeneic stem cell transplantation

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Introduction: Hematopoietic stem cell transplantation (HSCT) is associated with immune complications and endothelial dysfunction due to intricate donor-recipient interactions, conditioning regimens, and inflammatory responses.

Methods: This study investigated the role of the complement system during HSCT and its interaction with the cytokine network. Seventeen acute myeloid leukemia patients undergoing HSCT were monitored, including blood sampling from the start of the conditioning regimen until four weeks post-transplant. Clinical follow-up was 200 days.

Results: Total complement functional activity was measured by WIELISA and the degree of complement activation by ELISA measurement of sC5b-9. Cytokine release was measured using a 27-multiplex immuno-assay. At all time-points during HSCT complement functional activity remained comparable to healthy controls. Complement activation was continuously stable except for two patients demonstrating increased activation, consistent with severe endotheliopathy and infections. *In vitro* experiments with post-HSCT whole blood challenged with *Escherichia coli*, revealed a hyperinflammatory cytokine response with increased TNF, IL-1 β , IL-6 and IL-8 formation. Complement C3 inhibition markedly reduced the cytokine response induced by *Staphylococcus aureus*, *Aspergillus fumigatus*, and cholesterol crystals.

Discussion: In conclusion, HSCT patients generally retained a fully functional complement system, whereas activation occurred in patients with severe complications. The complement-cytokine interaction indicates the potential for new complement-targeting therapeutic strategies in HSCT.

KEYWORDS

HSCT, immunosuppression, innate immunity, complement, cytokines

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a highly effective treatment for various hematologic disorders (1). Despite significant advancement and improved survival rates, the transplant procedure remains linked to a considerable incidence of treatment-related morbidity and mortality (2). This association is attributed to immune-mediated complications, notably graft-versus-host disease (GVHD) (3), as well as infectious complications and endothelial syndromes including transplant-associated thrombotic microangiopathy (TA-TMA) (4, 5).

The early immunological responses following HSCT are typically split into three overlapping phases (6). The first phase is characterized by an inflammatory milieu with increased pathogen- and damage-associated molecular pattern (PAMP/DAMP) levels and inflammatory mediators induced by the conditioning regimen (3, 6). The proinflammatory state leads to the activation of antigen-presenting host cells, initiating the activation of donor T-cells (7), neovascularization and endothelial dysfunction (8, 9). A third, effector phase, GVHD, may occur upon sufficient alloreactivity. Tissue damage is caused by direct CD8-mediated cytotoxicity and release of inflammatory mediators (3, 10).

Much research has focused on the cellular immune system during HSCT (11), whereas less is known about the humoral response, such as the complement system. The complement system is an innate immunity sensor, functioning as an upstream recognition alarm system that discerns threats from exogenous and endogenous sources (12). The specific functions of the complement system include microbial defense by phagocytosis, with the opsonization of the microbe by iC3b binding to the CR3 receptor. Other main effector functions are mediated through the formation of the highly potent C5a molecule, which induces inflammation, and the lytic terminal C5b-9 complex, which can penetrate bacterial and cellular membranes (12). Importantly, inappropriate or excessive activation of complement can damage the host severely.

The complement system is highly relevant for HSCT, as these patients are at higher risk for infections (5) and endothelial disorders (13), which are linked to complement function. TA-TMA is characterized by endothelial cell activation, microvascular hemolytic anemia, and complement dysregulation (13, 14). Several studies have shown that increased complement activation as

measured by the soluble C5b-9 complex (sC5b-9) predicts the development of TA-TMA (13, 15–17). Therapeutic inhibition targeted at complement component C5, preventing C5a and C5b-9 formation, seems promising as reported in a large pediatric cohort with TA-TMA (18). Furthermore, a multi-center study has previously indicated a correlation between complement activation and outcome after HSCT (19). In this study we aimed to investigate the innate immune profile, including neutrophil and platelet activation, the cytokine network, and, in particular, the function and activation of complement in HSCT.

Methods

Study design, patients, transplantation procedure and acquisition of blood samples

Acute myeloid leukemia (AML) patients (n=17) undergoing allogeneic HSCT were recruited by the Hematology Department at Oslo University Hospital.

A summary of the various conditioning regimens is provided in [Supplementary Table 1](#). All patients were treated with cyclosporine-based GVHD prophylaxis which consisted of post-transplant cyclophosphamide, mycophenolate mofetil, and cyclosporin (starting on day +5) for haploidentical donors or donors with HLA-A or HLA-B mismatches, or anti-thymocyte globulin, methotrexate, and cyclosporine (starting on day -1) for other donors. All patients received antifungal prophylaxis with posaconazole from day 0 until engraftment, and fluconazole from engraftment until day +75 post-transplant. Antibacterial prophylaxis was not routinely administered, and growth factors were only administered in patients with haploidentical donors. All patients received ursodeoxycholic acid from the start of conditioning regimens until day 100. The total observation time for the patients was 200 days post-transplant.

Samples were obtained six days before transplantation (day -6), one day before transplantation (day -1), one day after transplantation (day +1), and weekly following transplantation (day +7, day +14, day +21 and day +28). The HSCT transplantation process commences with six days of

chemotherapy; the day -6 sample was obtained before the first chemotherapy dose and was considered as baseline sample. The day -6 sample was compared with samples from healthy median age and gender-matched controls (n= 10) recruited during the same period. There were only nine samples in the whole material that were not obtained; this due to administrative difficulties.

Three tubes of blood (4.5 mL) were drawn: 1) Vacuette K₂EDTA tubes (Greiner Bio-One, Kremsmünster, Austria), 2) silica-clotted tubes for preparing of serum (Greiner Bio-One), and 3) endotoxin-free NUNC tubes (Thermo Fischer Scientific, Roskilde, Denmark) prefilled with the specific thrombin inhibitor lepirudin. All tubes were stored in an ice-water bath and processed within two hours after collection. Immediately after receiving the samples, EDTA plasma was prepared by centrifugation (3000 × g, 15 min, at 4°C). Blood for serum preparation was incubated at room temperature until clotting and centrifuged as for the EDTA plasma. Plasma and serum were aliquoted and immediately stored at -80°C.

The lepirudin whole blood model

Whole blood was sampled into sterile NUNC cryo tubes containing lepirudin (Refludan® Pharmion, Copenhagen, Denmark) at a final concentration of 50 µg/mL for anticoagulation. The whole blood model was performed as previously described (20). Lepirudin blood was incubated with PBS (negative control), 10⁷ *E. coli*/mL, 10^{7.5} *E. coli*/mL, 10⁷ *S. aureus*/mL, 10⁷ *C. albicans*/mL, 10⁷ *A. fumigatus*/mL, or 1 mg/mL cholesterol crystals for 0, 30, and 120 min. Complement activation was stopped, by adding 20 mM of EDTA (final concentration) at the indicated timepoints. Whole blood samples were centrifuged at 3000 × g, 15 min, at 4°C to obtain plasma, which was stored at -80°C until further analysis. Incubation with microbes was performed with heat-inactivated *E. coli* (ATCC-33572, American Type Culture Collection, Manassas, VA), heat-inactivated *S. aureus* (Cowan, ATCC-12598, American Type Culture Collection), heat inactivated *C. albicans* (SC5314, ATCC MYA-287, American Type Culture Collection), and heat-inactivated *A. fumigatus* (clinically isolated strain 6881 cultivated at Copenhagen University Hospital - Rigshospitalet, Copenhagen). Cholesterol crystals were prepared as described by Samstad et al. (21). Inhibition of the complement system was performed using 10 µM (final concentration) of the C3-inhibitor Cp40 (compstatin) (22).

Complement function and activation

Complement function was measured in fresh-frozen serum in the WIESLAB® Total Complement System Screen kit (SVAR, Malmö, Sweden), an enzyme immunoassay previously described (23). Results are given in percent of a normal serum pool defined to contain 100% complement function. Complement activation was measured in plasma by an ELISA detecting the sC5b-9, as originally described (24), and performed as later modified (25). sC5b-9 was detected by the mAb aE11 reacting with a neoepitope expressed in C9 when incorporated into C5b-9 and not expressed in native C9

(26). The results are expressed as complement arbitrary units (CAU) per mL as defined by Standard #2 (25).

Activation of platelets and neutrophils, and release of cytokines

Platelet β-thromboglobulin (BTG) (Human CXCL7/NAP-2 DuoSet ELISA, R&D Systems, Minneapolis, MO) and neutrophil myeloperoxidase (MPO) (Human Myeloperoxidase DuoSet ELISA, R&D Systems) were measured by ELISA and performed according to the manufacturer's instructions. Cytokines were analyzed using a Bio-plex Human Cytokine 27 multiplex assay (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions.

Hematology and microbiology

Samples for hematology and microbiology were analyzed in the routine laboratories at Oslo University Hospital.

Data analysis

Statistical analyses were performed with the Mann-Whitney U test and Kruskal-Wallis tests using GraphPad Prism 9.5.1 (San Diego, CA). Data are presented as medians with interquartile ranges or individual dots for all data. A p<0.05 was considered significant.

Ethical considerations

Ethical approval was provided by Regional Committee for Medical and Health Research Ethics of South-East Norway (REK 285790). Informed and written consent was provided from patients before inclusion in the study.

Results

Study cohort and demographics

The patient cohort demographics are presented in Table 1. All 17 included patients had AML in remission at the start of the conditioning regimen (baseline). Fourteen patients were alive at the end of the 200-day observation period; 12 of them in complete remission. Patient comorbidities encompassed cardiac conditions, including one case with atrial fibrillation and another with previous mitral valve plasty; and autoimmune conditions, including vitiligo and diabetes mellitus type I. Bacteremia was detected in 47% of patients, with the causative agents being *Enterobacter*, *Streptococcus*, *Stenotrophomonas*, *E. coli*, *Klebsiella*, *S. capitis*, and fungi included including *C. glabrata* and *A. fumigatus*. Eight patients developed acute GVHD and one of these developed chronic GVHD.

TABLE 1 Demographic data, comorbidities, complications and outcome.

Pretransplant patient characteristics	
Included patients (n)	17
AML ¹ diagnosis (n)	17
Gender (n)	
Male	11 (65%)
Female	6 (35%)
Age (median and range)	
Male	59 (25–73)
Female	68 (53–73)
BMI ² in Kg/m ² (median and range)	
Male	26 (19–31)
Female	24 (21–30)
Comorbidities	
Cardiac	2 (13%)
Autoimmune	3 (18%)
Neurological	0 (0%)
Posttransplant outcomes	
Acute GVHD grad III–IV ³	
Yes	8 (47%)
No	9 (53%)
Engraftment	
Days to ANC ⁵ 0.5x10 ⁹ /L	DAYS
Days to PLT ⁶ 20 x10 ⁹ /L	DAYS
Bacteremia	
Yes	8 (47%)
No	9 (53%)
EBV or CMV reactivation	
Yes	7 (41%)
No	10 (59%)
Disease status at day 200	
Remission	12 (86%)
Relapse	2 (14%)
Outcome at day 200	
Alive	14 (82%)
Dead	3 (18%)

¹ AML, acute myeloid leukemia; ² BMI, Body Mass Index; ³ GVHD, graft versus host disease.
⁴ANC, neutrophils; ⁵PLT, platelets.

Changes in cell populations, activation of neutrophils and platelets, and acute phase activation in patients undergoing HSCT

As expected, all patients experienced a marked and steady fall in all cellular components from baseline to lowest value, observed

approximately one-week post-transplant (Figures 1A–E), followed by a subsequent increase to baseline levels or even higher after four weeks. At four weeks post-transplant all patients had achieved engraftment with full donor chimerism.

BTG, released from the platelets, showed a pattern virtually identical to the platelet count (Figure 1F), with a significant correlation (Spearman, rho=0.79, p=0.048) (Supplementary Figure 1A). MPO, released mainly from the neutrophils, showed a pattern virtually identical to the neutrophil count (Figure 1G), with a significant correlation (Spearman, rho=0.96, p=0.002) (Supplementary Figure 1B). C-reactive protein (CRP) increased steadily from baseline to a peak at one-week post-transplant, returning to baseline at day +28, reflecting the substantial acute phase reaction occurring during the early transplantation (Figure 1H). CRP and IL-6 correlated significantly (Spearman, rho=0.85, p=0.02).

Total complement activity and activation product sC5b-9 in patients undergoing HSCT

In contrast to the well-studied immune cell changes during HSCT, the complement function has been far less studied. Thus, we investigated whether the complement system was affected, either by being suppressed with reduced functional activity using the WIELISA test or being pathologically activated using the sC5b-9 test (Figure 2). Notably, we found that the complement function was completely preserved during the whole period, showing at least 100% function (mean of normal healthy controls) of both the classical and the alternative pathway (Figure 2A, left panel). Two of the patients, however, diverged substantially from the others. Patient A (PA) and patient B (PB) showed a substantial fall in complement activity in both pathways (Figure 2A, middle and right panel).

sC5b-9 levels were slightly elevated in the patients from admittance to the end of the study (Figure 2B, left panel). PA and PB, however, showed markedly increased complement activation (Figure 2B, middle and right panel). PB had a marked increase in sC5b-9 already at admittance, peaking at day +7, then declining and reaching a new top at day +28. The patient suffered severe complications diagnosed at day +24 as sinusoidal obstruction syndrome with high transaminases (AST 1876, ALT 1257), required dialysis, developed GVHD and TA-TMA, and died at day +105. PB had a sC5b-9 pattern similar to PA, reaching the highest level at day +28. This patient suffered from infection diagnosed as *S. maltophilia* at day +14, followed by *S. capitis* and *C. glabrata* at day +19 and *A. fumigatus* in the lungs at +day 30. The patient died at day +90 from relapse of AML. The sC5b-9 increase in these two patients is consistent with the fall in functional activity described above, consistent with consumption of the native components. PA and PB were two of the three patients that died during the 200 days observation period. A third patient died already on day +11 due to septic shock and was not included due to lack of samples. To further characterize the differences between PA/PB and the other patients with

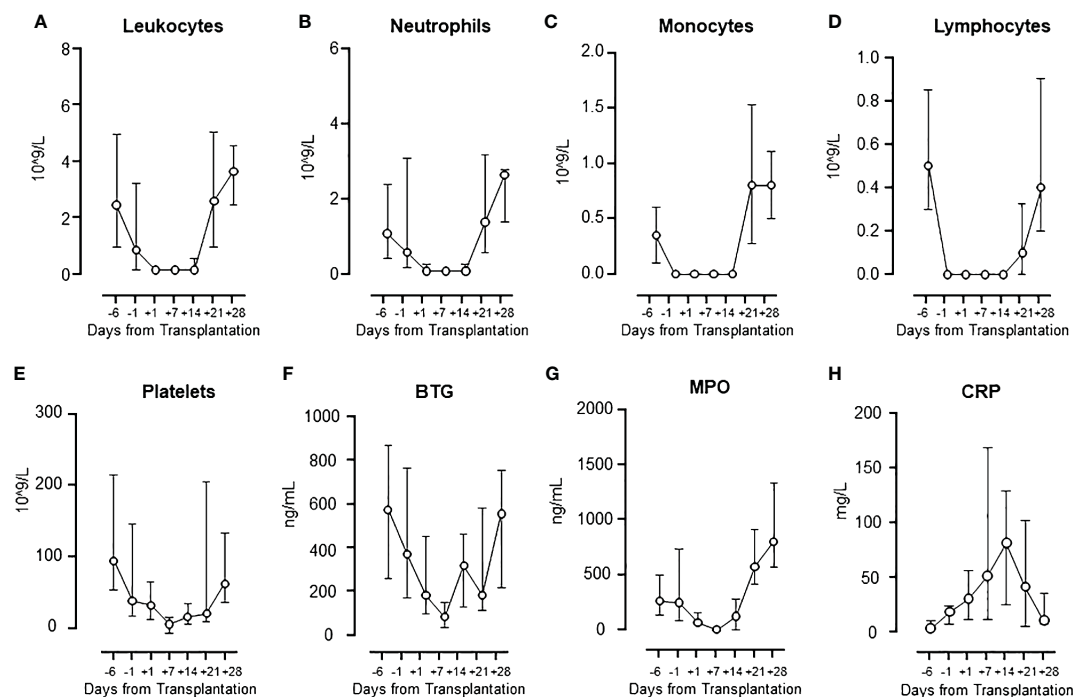


FIGURE 1

Cell population numbers and activation markers during HSCT. The changes in cell population numbers of leukocytes (A), neutrophils (B), monocytes (C), lymphocytes (D), and platelets (E), and the levels of activation markers BTG (F) and MPO (G), and CRP (H) are presented. Graphs show median $\pm$ interquartile range (n=17).

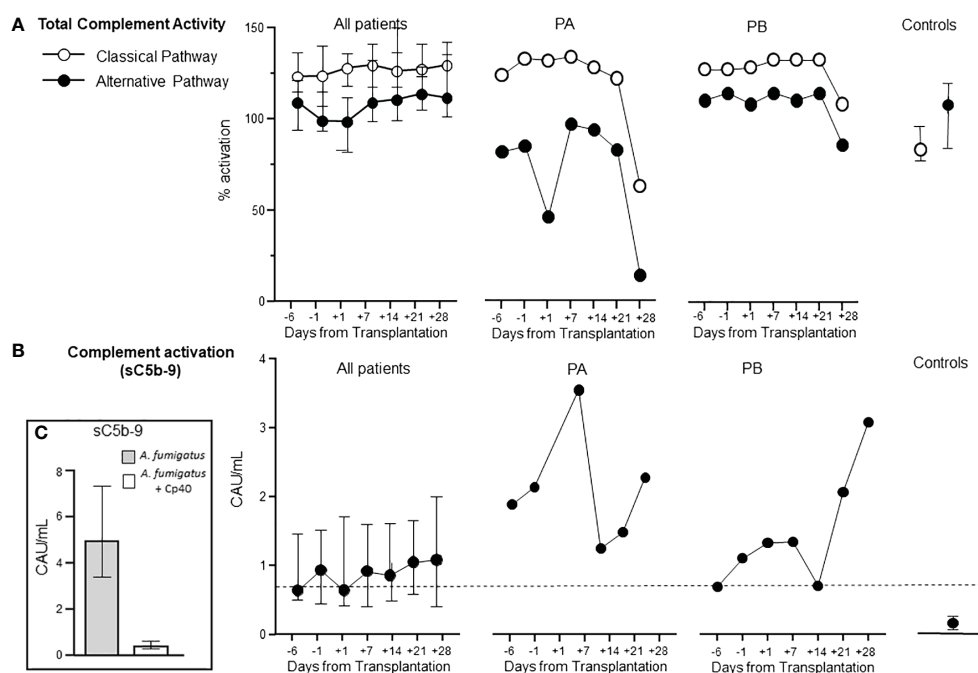


FIGURE 2

Complement function and activation during HSCT. The total functional complement activity of the classical and alternative pathway was measured using immunoassay (Wieslab®) with 100% as average reference normal value in all patients (A, left). The degree of complement activation *in vivo* was measured as sC5b-9 in all patients (B left). Two patients (PA and PB) showed clearly different patterns from the rest of the group for complement activity (A middle and right) and for complement activation (B middle and right). Healthy controls are shown to the right in (A, B). Graphs show median $\pm$ interquartile range (n=17). Dotted line in (B) represent upper reference value from normal blood donors. Panel (C) describes the effect of complement inhibition induced by *A. Fumigatus* - see below.

uneventful course, the CRP and IL-6 levels were compared (Supplementary Figure 2). A higher level of both CRP (Supplementary Figure 2A) and IL-6 (Supplementary Figure 2B) was found in the PA/PB patients, with a substantial increase during the course, as compared to the other patients.

Comparisons between plasma cytokines from AML patients pre-HSCT and healthy controls

Six cytokines representing interleukins, chemokines and interferons were selected for comparison between AML patients at hospital admission and healthy controls (Figure 3). TNF, IL-6, IL-1ra, IL-8/CXCL8, MIP-1 α /CCL3 and IFN γ (Figures 3A–F) were all statistically significantly higher in the patients than in the healthy controls, reflecting the hyperinflammatory state in AML patients.

Patterns of plasma cytokine levels in patients undergoing HSCT

The concentration of several cytokines was measured in the patients during the whole period (Figure 4). Three different patterns were observed. Pattern 1, a gradual increase in cytokine release during the post-transplant period was seen for IL-1 β and IL-13, illustrated by IL-1 β (Figure 4A). Pattern 2, a steep increase from baseline to a peak at transplantation, eventually declining to baseline levels, was seen for

MCP-1/CCL2, IP-10/CXCL10, IL-8/CXCL8, MIP-1 α /CCL3, G-CSF and FGF-basic, illustrated by MCP-1/CCL2 (Figure 4B) and IP-10/CXCL10 (Figure 4C). Pattern 3, a combination of pattern 1 and 2, showing a distinct peak at transplantation, decreasing to baseline followed by a subsequent second increase two weeks after transplantation, was seen for IFN γ and eotaxin, illustrated by IFN γ (Figure 4D). The remaining eight cytokines displayed differential patterns with TNF, IL-1ra, IL-9, MIP-1 β /CCL4 and RANTES/CCL5 showing a distinct decline at day +7, illustrated by TNF (Figure 4E), and an opposite increase at day +7 seen for IL-6 (Figure 4F).

Patterns of cytokine levels in HSCT patient incubated with *E. coli*

Whole blood samples from the observation period were incubated with *E. coli* to induce a robust cytokine release with PBS as negative control (Figure 5). Four classical proinflammatory cytokines were analyzed: TNF, IL-1 β , IL-6 and IL-8/CXCL8 (Figures 5A–D). The results showed a virtually identical pattern for all cytokines with a substantial response to *E. coli* at admittance, no response from day -1 to day +7, and then an abrupt increase in response from day +14 (Figure 5). The cytokine response followed the leukocyte count closely (Figure 1).

Notably, the expression of TNF, IL-1 β , IL-6, and IL-8/CXCL8 following incubation with *E. coli* was substantially and significantly ($p < 0.01$ for all) elevated at day +28 compared to day -6 (hospital admission) (Figure 5), consistent with a more potent inflammatory

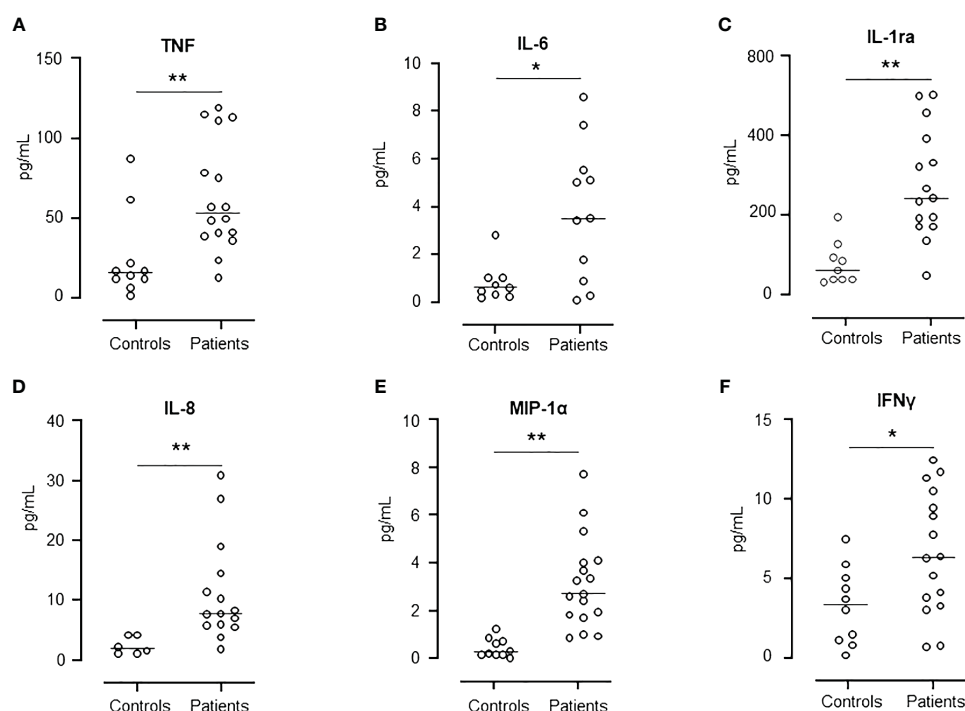


FIGURE 3

Comparison of cytokine release levels between patients at day -6 and healthy controls. Figure shows the levels of TNF (A), IL-6 (B), IL-1ra (C), IL-8 (D), MIP-1 α (E), and IFN γ (F). Graph shows median and all values, $n=10$ in healthy control group and $n=17$ in patient group. Statistical analysis was done using the Mann-Whitney U test. Significance is shown as * $p < 0.05$, ** $p < 0.01$.

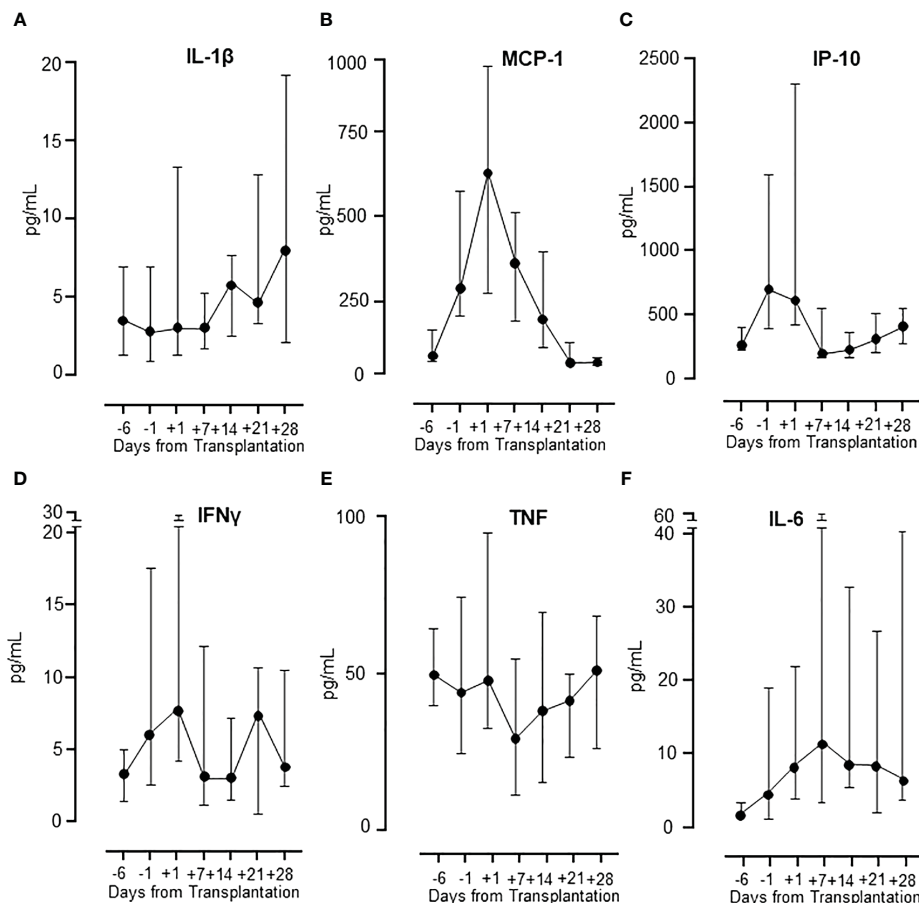


FIGURE 4

Cytokine release in patients during HSCT. The different patterns of cytokine release in patients during the whole observation period are illustrated by IL-1 β (A), MCP-1 and IP-10 (B, C), IFN γ (D) and TNF and IL-6 (E, F). Graphs show median $\pm$ interquartile range (n=17).

response from the donor cells after the engraftment compared to the cells from the patient at admission.

Comparisons of the cytokine response following challenge with PAMPs and DAMPs to whole blood from AML patients pre-HSCT as compared to healthy controls

We then compared the patients' blood at admission with healthy controls with respect to cytokine release (TNF, IL-1 β , IL-6, and IL-8/CXCL8) in response to PAMPs and DAMPs relevant for immunosuppressed patients, i.e. *S. aureus*, *C. albicans*, *A. fumigatus*, and cholesterol crystals (Figure 6). No statistical differences were seen between patients and health controls.

Patterns of cytokine levels in whole blood from patients undergoing HSCT when incubated with PAMPs and DAMPs

The pattern of cytokine release was similar for TNF, IL-1 β , IL-6, and IL-8/CXCL8 (Figures 7A–D), declining from day -6 to close to

zero and markedly increasing from day +14, reaching levels at day +28 comparable to or higher than those at day -6. Statistically significant higher levels at day +28 compared to day -6 were seen for TNF incubated with *S. aureus*, and for TNF, IL-1 β and IL-8 incubated with *C. albicans*.

Effect of complement inhibition on the expression of cytokines during HSCT

Finally, we investigated the role of complement on the cytokine response using the C3 inhibitor Cp40 to determine complement-dependent responses. First, we documented that Cp40 completely blocked the formation of sC5b-9 in blood incubated with *A. fumigatus* (Figure 2C). We then incubated blood from healthy controls and patients at day -6 and day +28 with *S. aureus*, *C. albicans*, *A. fumigatus*, and cholesterol crystals (Figure 8). The results are shown as % change of cytokine release in the samples pre-treated with Cp40 compared to PBS and significance is calculated as difference between the uninhibited sample and the Cp40 pre-inhibited sample as determined by Kruskal-Wallis. Overall, the results were similar in healthy control and patient samples, consistent with the intact complement function during

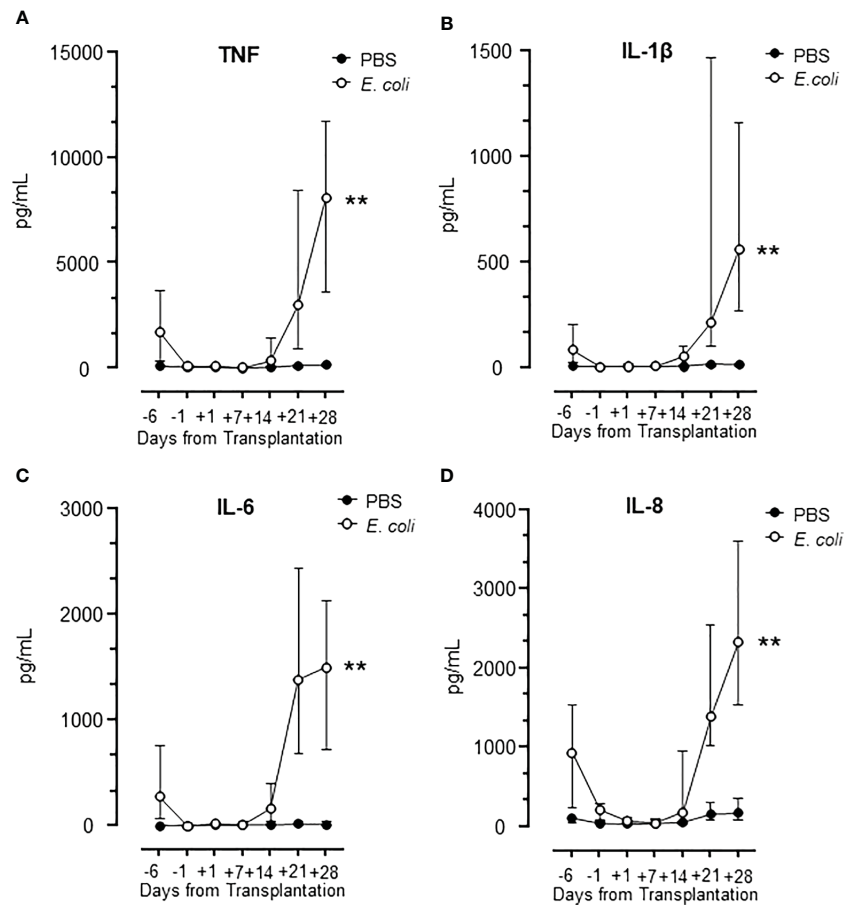


FIGURE 5
Cytokine release during HSCT in lepirudin whole blood incubated with *E. coli*. The pattern of cytokines TNF (A), IL-1 β (B), IL-6 (C), and IL-8 (D) are shown in samples obtained during the whole observation period. Graphs show median $\pm$ interquartile range (n=17). Statistical analysis was done using the Mann-Whitney U test. Significance is shown as: **p < 0.01.

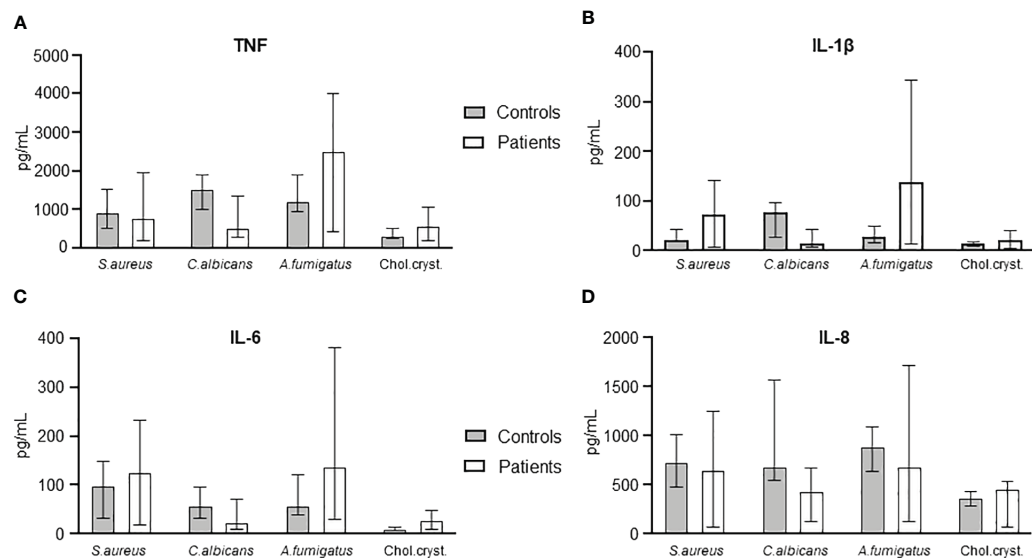


FIGURE 6
Comparison of cytokine levels in *S. aureus*, *C. albicans*, *A. fumigatus*, and cholesterol crystals between HSCT patients at day -6 and healthy controls. TNF (A), IL-1 β (B), IL-6 (C), and IL-8 (D) are shown compared between controls and patients. Graphs show median $\pm$ interquartile range (n=6). Statistical analysis was done using the Mann-Whitney U test.

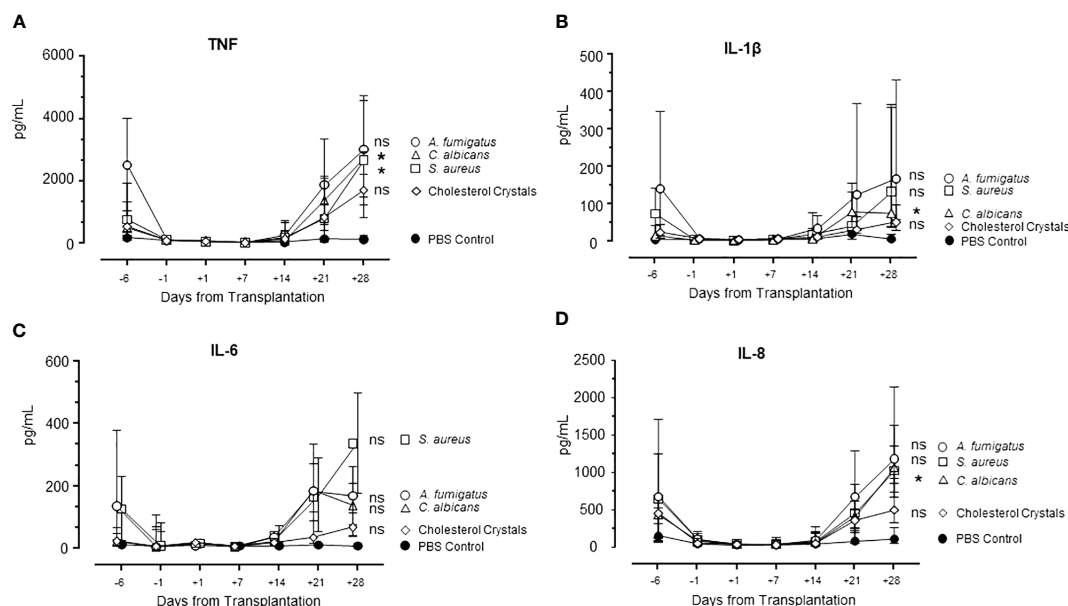


FIGURE 7

Cytokine release during HSCT in *S. aureus*, *C. albicans*, *A. fumigatus*, and cholesterol crystals incubated in whole blood. Pattern of cytokines TNF (A), IL-1 β (B), IL-6 (C), and IL-8 (D) during the whole observation period of patients are shown. Graphs show median $\pm$ interquartile range (n=6). Statistical analysis was done using Mann-Whitney U test. Significance shown as: ns for $p > 0.05$, * $p < 0.05$.

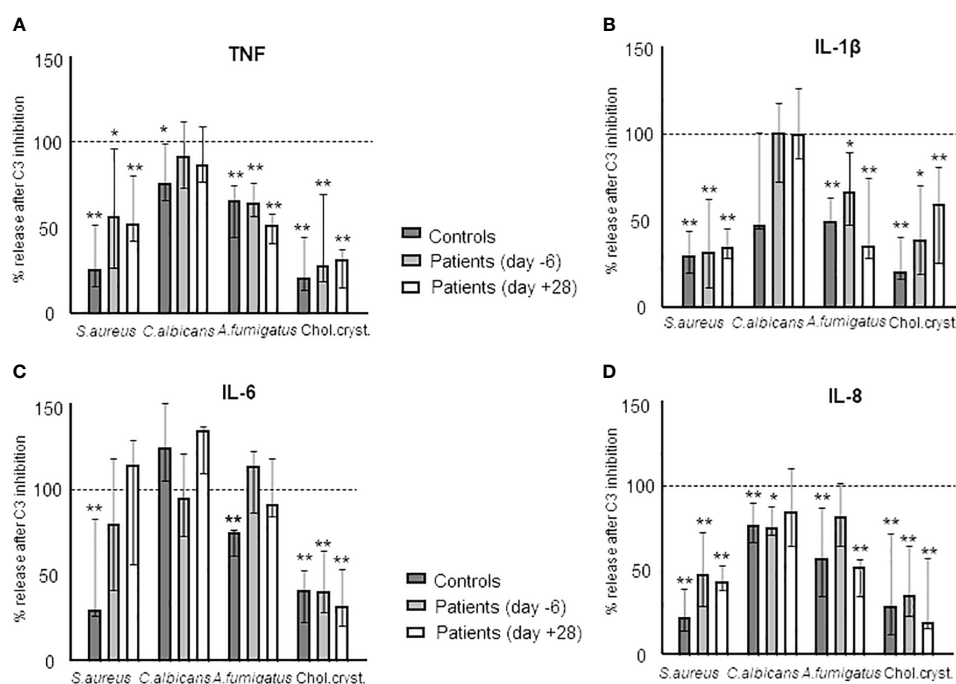


FIGURE 8

The complement dependency of the cytokine release during HSCT in samples obtained from HSCT patients *in vitro* incubated with *S. aureus*, *C. albicans*, *A. fumigatus*, and cholesterol crystals. Sample comparison is between samples incubated with the respective agents and sample pre-treated with the C3-inhibitor CP40 and then incubated with the respective agent. The effect is illustrated by the degree of inhibition in % as compared to the non-inhibited sample: TNF (A), IL-1 β (B), IL-6 (C), and IL-8 (D). Graph shows median $\pm$ interquartile range (n=6). Statistical analysis was done using the Kruskal-Wallis test. Significance shown as: * $p < 0.05$, ** $p < 0.01$.

HSCT. There was a statistically significant inhibition caused by Cp40 for TNF and IL-1 β release induced by *S. aureus*, *A. fumigatus*, and cholesterol crystals (Figures 8A, B); for IL-6 release induced by cholesterol crystals (Figure 8C); and for IL-8 release for all the activators (Figure 8D).

Discussion

This study presents novel insight into the innate immune system during HSCT. We observed that the complement system remained functionally intact during the whole period in patients with uneventful treatment courses and that the complement activity did not differ compared to an age- and sex-matched cohort. This indicates that the complement system can be activated in the same manner as in healthy individuals to protect against infection and can induce adverse inflammatory responses if activated improperly.

How the complement system changes during the early phase after transplantation is inadequately explored. Complement proteins are mainly produced in the liver and extensively consumed during infectious challenges. Despite the liver cell stress caused by conditioning regimens (27), alloreactivity, GVHD prophylaxis, antifungal azoles, and persistent systemic inflammation caused by mucous membrane inflammation and bacterial translocation, most patients maintained a fully active complement system, protecting them against infection. However, certain aspects of complement functionality, such as phagocytosis, will be affected by the lack of effector cells during HSCT. Other complement functions, such as lysis of bacteria through the terminal complement complex will be intact and of particular importance for protection against complement sensitive strains. Furthermore, an intact complement activity will be of importance when treating patients with immunotherapy that partly rely on complement for lysing cancer cells. On the other hand, an intact complement system may be a threaten to the patients by causing tissue damage through over-activation occurring during complications like TA-TMA and GVHD.

An important question is to which degree the complement system is activated during the early post-transplant period. Our data indicate that neither the conditioning regime, GVHD prophylaxis, stem cell infusion, nor the early post-transplant toxicity significantly alters the complement system. Baseline sC5b-9 levels in patients were slightly elevated compared to controls, aligning with the modest increase in inflammatory response observed in cytokines. This modest complement activation at baseline did not appear to contribute to a higher degree of post-transplant complement activation. The complement system was not investigated in the donors for logistical reasons. However, complement deficiencies in the normal population is extreme rare (28) and the use of non-parametric data handling would not have been influenced by a single deficiency.

A distinct activation pattern was observed in two patients (PA and PB), both of whom encountered severe complications and died during the observation period. Both patients showed a similar pattern of complement activation as detected by sC5b-9 with an initial increase followed by a decline and then an escalating increase

until day +28. This final increase correlated with an abrupt decrease in the complement function in both classical and alternative pathway, which is consistent with a consumption of the native component during activation. Retained complement function both of classical and lectin pathway was observed in all patients with uneventful course. This is of particular interest for the classical pathway, since C1q is one of few complement components that are not produced by the liver, but by immune cells, mainly the monocytic line (29, 30), which are virtually depleted during HSCT. However, C1q is also produced by non-immune sources such as osteoclasts (31) and endothelial cells (32). This could explain why the classical pathway remained fully active, together with the fact that a certain drop in C1q concentration would not lead to lower classical pathway activity since C2 is the rate limiting factor (33).

Patients PA and PB illustrate how complement can be activated with the same pattern despite strictly different etiology: PA displayed DAMP-induced, sterile inflammation due to sinusoidal obstruction syndrome and GVHD, while PB suffered from PAMP-induced infectious inflammation due to bacterial and fungal infection. Both PA and PB showed a marked increase in CRP and IL-6 as compared to patients with uneventful course. These results indicate that systemic complement activation is involved in the pathogenesis of different complications leading to severe morbidity during HSCT, which is in accordance with a previous study (19). Recent results indicate that complement inhibition is beneficial in pediatric TA-TMA (18) indicating that complement may play a role in developing HSCT-related complications.

The activation markers BTG and MPO, indicators of platelet (34) and neutrophil (35) activation, followed a pattern virtually identical to the cell counts of the individual cell populations. Accordingly, there was a highly significant correlation between platelet counts and BTG release, and between neutrophil counts and MPO, supporting the respective cell types to be the main source of these mediators. In contrast to BTG and MPO, the systemic marker of inflammation CRP showed a different pattern (36) with normal levels at baseline that increased substantially to peak at day +7, followed by a decrease to baseline. IL-6 has been shown to be the main promotor of CRP (37). The strong correlation between IL-6 and CRP found in this is consistent with previously published data in HSCT patients (38).

As we observed a general increase in cytokines in whole blood at day -6 compared to healthy controls, we examined the level of all cytokines included in the study throughout the observation period. Three distinct patterns were observed. *First*, IL-1 β and IL-13 were low at baseline and increased one to two weeks after transplantation, reaching a maximum level at days +21 and +28. *Second*, six cytokines, of which comprised four chemokines, showed a distinct increase from the baseline, reaching a peak at transplantation and declining thereafter to baseline. MCP-1/CCL2, IP-10/CXCL10, IL-8/CXCL8, MIP-1 α /CCL3, G-CSF and FGF-basic belonged to this group. This second pattern reflects an interesting hyperinflammatory response before and during the aplastic phase of HSCT and suggests that these cytokines are produced extra-vascularly. This has been shown for MCP-1 which can be produced by endothelial cells, epithelial cells and

smooth muscle cells (39), and IP-10/CXCL10 produced by endothelial cells, epithelial cells, and keratinocytes (40). Both MCP-1/CCL2 and IP-10/CXCL10 are released in response to IFN γ signaling (40, 41). Third, IFN γ and eotaxin showed two distinct peaks, corresponding to the peak at transplantation seen for pattern two and a late increase seen for pattern one, suggesting a contribution from extravascular cells and donor leukocytes. IFN γ is a significant mediator of the immune response and has previously been shown to be released by T-cells, NK cells, NKT cells, macrophages, dendritic cells, and B cells (42, 43).

Considering the heightened susceptibility to infections in the patients undergoing allogeneic HSCT, we aimed to delve deeper into the characterization of cytokine release by incubating their blood with *E. coli* (20). The classical proinflammatory cytokines TNF, IL-1 β , IL-6 and IL-8/CXCL8, display a typical response in normal blood incubated with *E. coli* (44). Blood from the HSCT patients at baseline responded to *E. coli* with a similar pattern as described for blood from healthy donors, whereas this response declined to undetectable levels during the aplastic phase. A post-transplant release of the cytokines was markedly increased at day +28 compared to pre-transplant indicating a more robust response to *E. coli* in the transplanted cells. The elevated cytokine levels seen at day +28 in the patients may be related to trained immunity, a concept suggesting a memory-like state in innate immune responses (45, 46). This could result from prolonged exposure to DAMPs and PAMPs during the early engraftment phase, when gastrointestinal barrier breach secondary to mucositis remains prominent.

To determine if this cytokine response was *E. coli* specific, blood was incubated with other PAMPs and DAMPs commonly observed during the early post-transplant period such as *S. aureus*, *C. albicans* and *A. fumigatus* (47). Cholesterol crystals were included to the experimental setup to simulate DAMP-induced sterile inflammation, a crucial component of the prevailing clinical manifestation following HSCT and associated toxicity (48). Cholesterol crystals have also been shown to induce TA-TMA, a common complication post-HSCT (49). Our data indicate that the augmented inflammatory responses post-transplant is not *E. coli* or pathogen specific. Previous studies on the cytokine levels post-transplant have shown a similar trend with an increase in cytokine release (50, 51), and confirmed this to be an increase compared to levels observed prior to transplantation (51).

The final goal of this study was to determine the role of complement in the cytokine release in HSCT patients. The effect of complement inhibition, using a C3 inhibitor, differed between the different agents used. *S. aureus*, *A. fumigatus* and cholesterol crystal-induced cytokine release were more efficiently reduced by C3 inhibition than cytokines induced by *C. albicans*. Overall, the effect of complement inhibition was comparable in samples from patients and from healthy controls. These results indicate a retained complement-dependent effect in patients undergoing HSCT, which is efficiently blocked by a complement inhibitor with the same effects on the secondary induced cytokine release as observed in normal controls.

In conclusion, patients undergoing HSCT have a fully intact complement system, which is generally was not activated during the

early post-transplant period. However, two patients who died due to opportunistic infection and TA-TMA, respectively, underwent a substantial complement activation. The cytokine inflammatory response studied *in vitro* was reduced by inhibiting complement activation. Thus, we provide novel insights into the innate immune responses during HSCT and new avenues for potential complement therapies during HSCT.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethical approval was provided by Regional Committee for Medical and Health Research Ethics of South-East Norway (REK 285790). Informed and written consent was provided from patients before inclusion in the study. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

BF: Conceptualization, Investigation, Project administration, Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Methodology. LC: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. CS: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. KM: Data curation, Formal analysis, Methodology, Writing – review & editing. CL: Data curation, Methodology, Writing – review & editing, Project administration. JH: Data curation, Methodology, Writing – review & editing, Formal analysis. TG-D: Writing – review & editing, Conceptualization, Project administration, Supervision. RW: Conceptualization, Project administration, Supervision, Writing – review & editing, Data curation. TE: Conceptualization, Data curation, Writing – review & editing, Methodology. GT: Conceptualization, Writing – review & editing, Investigation, Resources, Supervision. PG: Conceptualization, Investigation, Supervision, Writing – review & editing, Funding acquisition, Methodology. AB-D: Conceptualization, Investigation, Supervision, Writing – review & editing, Resources. TT: Conceptualization, Investigation, Supervision, Writing – review & editing, Methodology, Project administration. TM: Conceptualization, Investigation, Project administration, Supervision, Writing – review & editing, Funding acquisition, Resources, Validation, Visualization, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1422370/full#supplementary-material>

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Systematic review of the complement components as potential biomarkers of pre-eclampsia: pitfalls and opportunities

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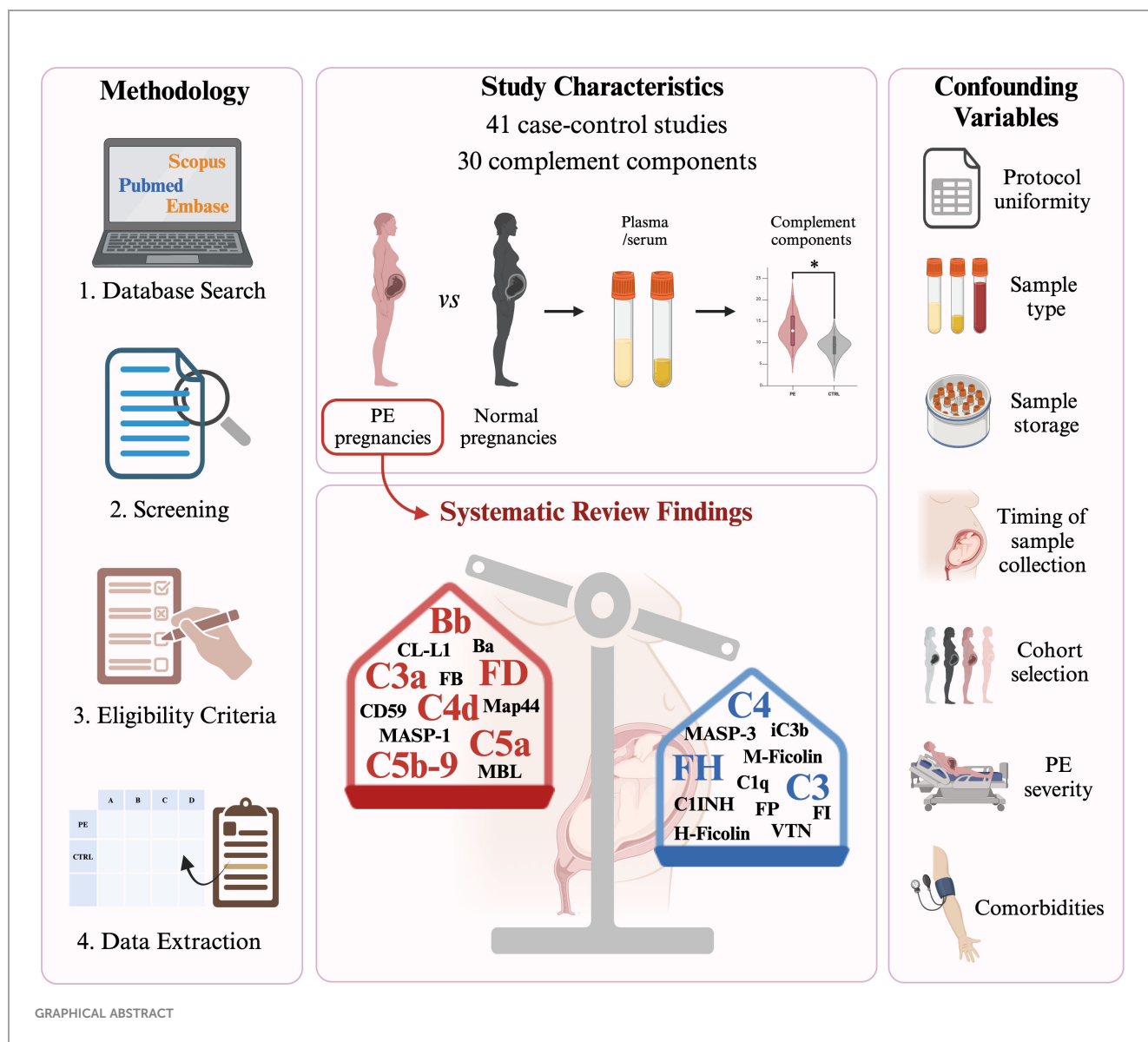
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The complement system (C) is a crucial component of the innate immune system. An increasing body of research has progressively shed light on the pivotal role of C in immunological tolerance at the feto-maternal interface. Excessive C activation or impaired C regulation may determine the onset of pregnancy-related pathological conditions, including pre-eclampsia (PE). Thus, several studies have investigated the presence of C components or split products in blood matrixes (*i.e.*, plasma, serum), urine, and amniotic fluid in PE. In the current study, we systematically reviewed the currently available scientific literature reporting measurements of C components as circulating biomarkers in PE, based on a literature search using Pubmed, Scopus, and Embase databases. A total of 41 out of 456 studies were selected after full-text analysis. Fourteen studies (34.1%) were identified as measuring the blood concentrations of the classical pathway, 5 (12.1%) for the lectin pathway, 28 (68.3%) for the alternative pathway, 17 (41.5%) for the terminal pathway components, and 16 (39%) for C regulators. Retrieved results consistently reported C4, C3, and factor H reduction, and increased circulating levels of C4d, Bb, factor D, C3a, C5a, and C5b-9 in PE compared to normal pregnancies, depicting an overall scenario of excessive C activation and aberrant C regulation. With evidence of C activation and dysregulation, C-targeted therapy is an intriguing perspective in PE management. Moreover, we also discussed emerging pitfalls in C analysis, mainly due to a lack of experimental uniformity and biased cohort selection among different studies and laboratories, aiming to raise a more comprehensive awareness for future standardization.

Systematic review registration: <https://www.crd.york.ac.uk/prospero/>, identifier CRD42024503070.

KEYWORDS

pre-eclampsia, complement system, systematic review, biomarker, pregnancy



1 Introduction

Pre-eclampsia (PE) is a pregnancy-related disorder diagnosed by onset of hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg) after 20 weeks of gestation, associated with proteinuria (≥ 300 mg in 24 hours) and/or presence of kidney or liver dysfunction, neurological complications, hemolysis, or thrombocytopenia, and/or fetal underdevelopment. It is accounted for significant maternal and neonatal morbidity and mortality (1, 2). Based on gestational age at clinical presentation, PE is usually classified into preterm (< 37 weeks of gestation), term (≥ 37 weeks of gestation), and post-partum (from 48 hours to 6 days after delivery) (3, 4). According to onset, PE has been defined as early-onset PE (EOPE; < 34 weeks) and late-onset PE (LOPE; > 34 weeks) (5). It is now widely accepted that these two entities may differ in etiology and should be accounted for as different phenotypes of the disease (6, 7). Conversely, PE classification into

mild and severe (SPE) forms has now lost significance. It can also progress to more severe complications, such as HELLP (Hemolysis, Elevated Liver enzymes, and Low Platelet count) syndrome (8) and eclampsia (9).

PE affects 5–7% of pregnant women worldwide and can occur in any pregnancy, although several associated risk factors exist. These include PE in previous pregnancies, maternal age (< 20 and ≥ 35 years), metabolic disorders, pregnancies achieved through assisted reproductive technology, and pre-existing conditions, such as chronic kidney disease, thyroid dysfunction, and systemic lupus erythematosus (SLE) (10). A family history of PE is associated with an increased risk, suggesting a genetic background (11). However, no single high-risk gene is associated with PE, reflecting the syndromic nature of the disease.

PE management involves outpatient monitoring of blood pressure and proteinuria, or potential hospitalization in more severe cases. Delivery is often the most effective intervention. Up

to now, the soluble fms-like tyrosine kinase 1 (sFlt-1)/placental growth factor (PlGF) ratio is the only predictive biomarker for short-term risk of PE and adverse pregnancy outcomes (12). Additional predictive screenings via algorithms have been extensively evaluated and validated by various institutions (13). Thus, there is a critical need for serum biomarkers for early diagnosis and screening as reliable predictors of PE in the short term.

The etiology of PE is complex and multifactorial. A consistent body of evidence supports the involvement of an abnormal placentation process mainly due to immunological dysfunctions occurring at the fetal-maternal interface (13). Dysregulation of the complement system (C) is recognized as one of the main contributors to PE pathogenesis (14, 15). The C is a crucial

mediator of the innate immune response, being also involved in cell homeostasis, tissue development and repair, and crosstalk with other endogenous systems (*i.e.*, renin-angiotensin, coagulation, and kinin-kallikrein systems). C consists of more than 50 fluid-phase and membrane-bound proteins. Its activation can be initiated by three distinct pathways (*i.e.*, classical, CP; lectin, LP; and alternative, AP) (Figure 1), converging on the common activation of the major component C3 and in the production of proinflammatory mediators, opsonization, membrane attack complex (MAC) formation, and target cell lysis (16, 17).

An accumulating body of evidence has extensively pointed out the pivotal role of C in immunological tolerance at the fetomaternal interface (18). A shift towards excessive C activation or impaired C regulation may determine the onset of pregnancy-

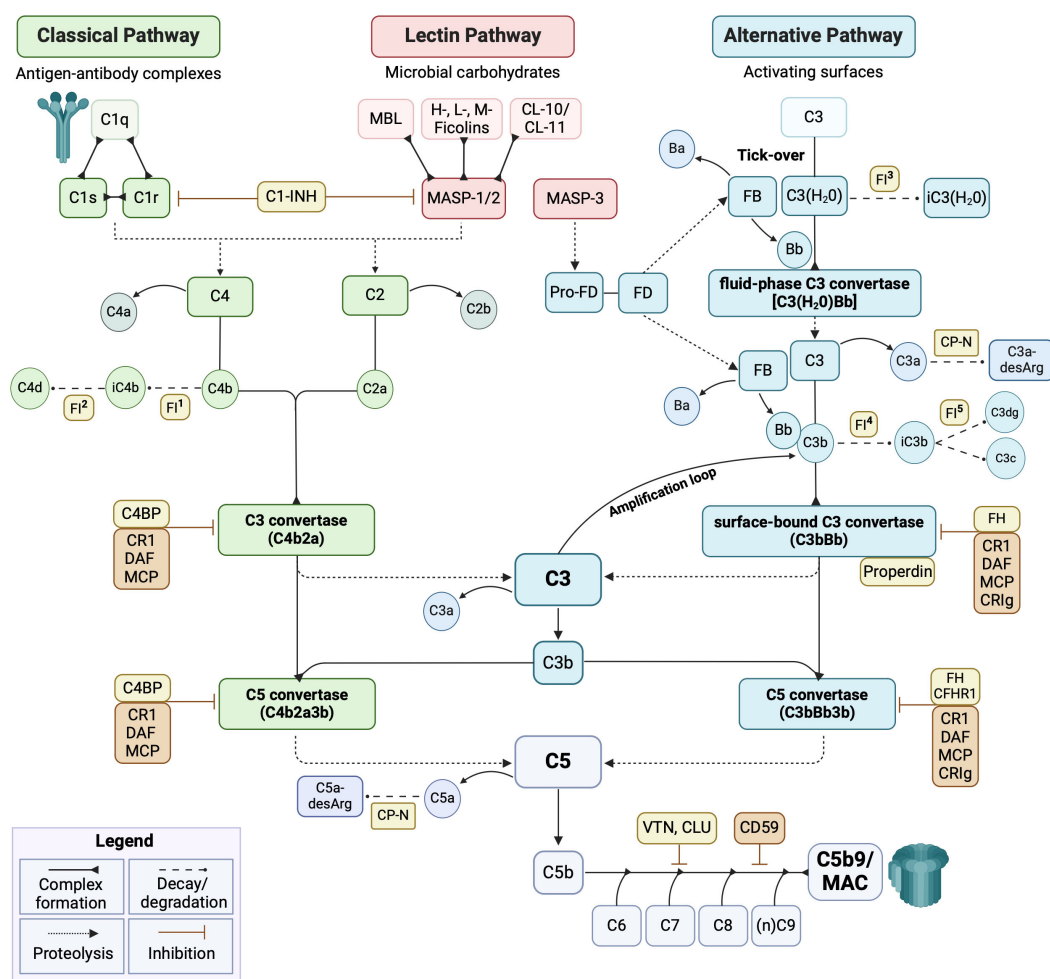


FIGURE 1

Schematic overview of the complement system cascade. The complement system (C) operates via three pathways: classical (green), lectin (pink), and alternative (cyan). The classical pathway is mainly triggered by the binding of C1q to antigen-antibody complexes. The lectin pathway is activated by mannan-binding lectin (MBL), ficolins, or collectins (CLs), which recognize microbial carbohydrates and form a complex with MASP-1 and MASP-2. The alternative pathway is constitutively active at basal levels, with C3 undergoing spontaneous hydrolysis (tick-over). All three pathways converge on the common component C3 and proceed with the terminal pathway (grey). The C activation is tightly controlled by several fluid-phase (yellow) and membrane-bound (orange) regulators. FI activity is supported by several cofactors: ¹⁻² C4BP, CD46, CR1; ³ FH/FH-L1; ⁴ FH, FH-L1, CD46, CR1; and ⁵ CD46, CR1. C1-INH, C1-inhibitor; C4BP, C4b-binding protein; CFHR1, complement factor H-related protein 1; CLU, clusterin; CP-N, carboxypeptidase-N; CR1, complement receptor 1; CRIg, complement receptor immunoglobulin; DAF, decay-accelerating factor; FB, factor B; FD, factor D; FH, factor H; FI, factor I; MAC, membrane attack complex; MCP, membrane cofactor protein; VTN, vitronectin. Image created with BioRender.com.

related pathological conditions, including PE (15). An aberrant or excessive C activation in the placenta can be responsible for PE pathogenesis, leading to inflammation and endothelial dysfunction (14, 18, 19). This may be mainly due to increased circulating anaphylatoxins, enhanced deposition of C factors in the placenta, or consumption of circulating C factors, leading to placental dysfunction. C activation products can damage endothelial cells, impairing their functions and contributing to the high blood pressure and other symptoms frequently observed in PE (20). This inflammatory condition may also impact the function of other organs, such as liver and kidney, leading to complications. Interestingly, recent research has explored the possibility of targeting the C as a novel therapeutic approach for PE (21, 22).

C proteins and their activation products have been frequently found in the placenta of women with PE (23), but the relationship between circulating C components and placental C deposition remains elusive. In addition to excessive C activation leading to a simultaneous reduction in C component levels and an increase in activation products, different levels of C components between healthy pregnancies and PE may also be caused by the different hepatic synthesis due to systemic inflammation. Furthermore, one should consider the presence of a massive quantity of circulating microvesicles in the bloodstream of PE patients; they can act as decoys for C components and contribute to an aberrant concentration of C proteins and inhibitors. Thus, research interest has been focused on the evaluation of C components as PE biomarkers before the disease onset (predictive biomarkers) or during the active disease (diagnostic biomarkers). Several studies have investigated the presence of C components or split products in blood matrixes (*i.e.*, plasma, serum), urine, and amniotic fluid in PE.

This systematic review aims to evaluate the current state of the art of C components as potential circulating biomarkers in PE women, provides a general overview of C status in PE, and raises awareness about measuring C components in a reliable and standardized manner. This may help to explore novel predictive approaches and therapeutic opportunities in PE management.

2 Methods

2.1 Search methods and data sources

A systematic literature search was carried out to identify original articles reporting the values of circulating C components in PE pregnancies compared to healthy pregnancies. The search strategy was developed following the recommendations of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (24). PubMed, Scopus, and Embase databases were interrogated for relevant articles published until the 13th March 2024. The search terms included: “preeclampsia”, “complement”, “serum”, “plasma”, “blood”, and “biomarker”. The specific search strategy is reported in the [Supplementary Material](#). The review was registered in the PROSPERO database (CRD42024503070).

2.2 Selection of studies

Removal of duplicates and screening of records were performed using Rayyan systematic review tool (25). Two independent investigators (AB and AM) screened the articles by title and abstract, followed by full-text checking for their eligibility. Relevant articles were included if they met all the following eligibility criteria: 1) English language; 2) original peer-reviewed article; and 3) study measuring the levels of at least one C component in the blood of PE *vs* healthy women. Articles were not included if they were as follows: 1) articles published in languages other than English; 2) case reports/case series; 3) reviews, systematic reviews, meta-analyses, book chapters, conference or comment papers, prospective or retrospective studies; 4) studies reporting biomarkers in urine, cord or fetal blood; 5) studies which did not report numerical values for the measurements of circulating C components; and 6) animal studies. Disparities were resolved by a third investigator (CA).

All considered articles of interest among those identified were reported in this review.

2.3 Data extraction

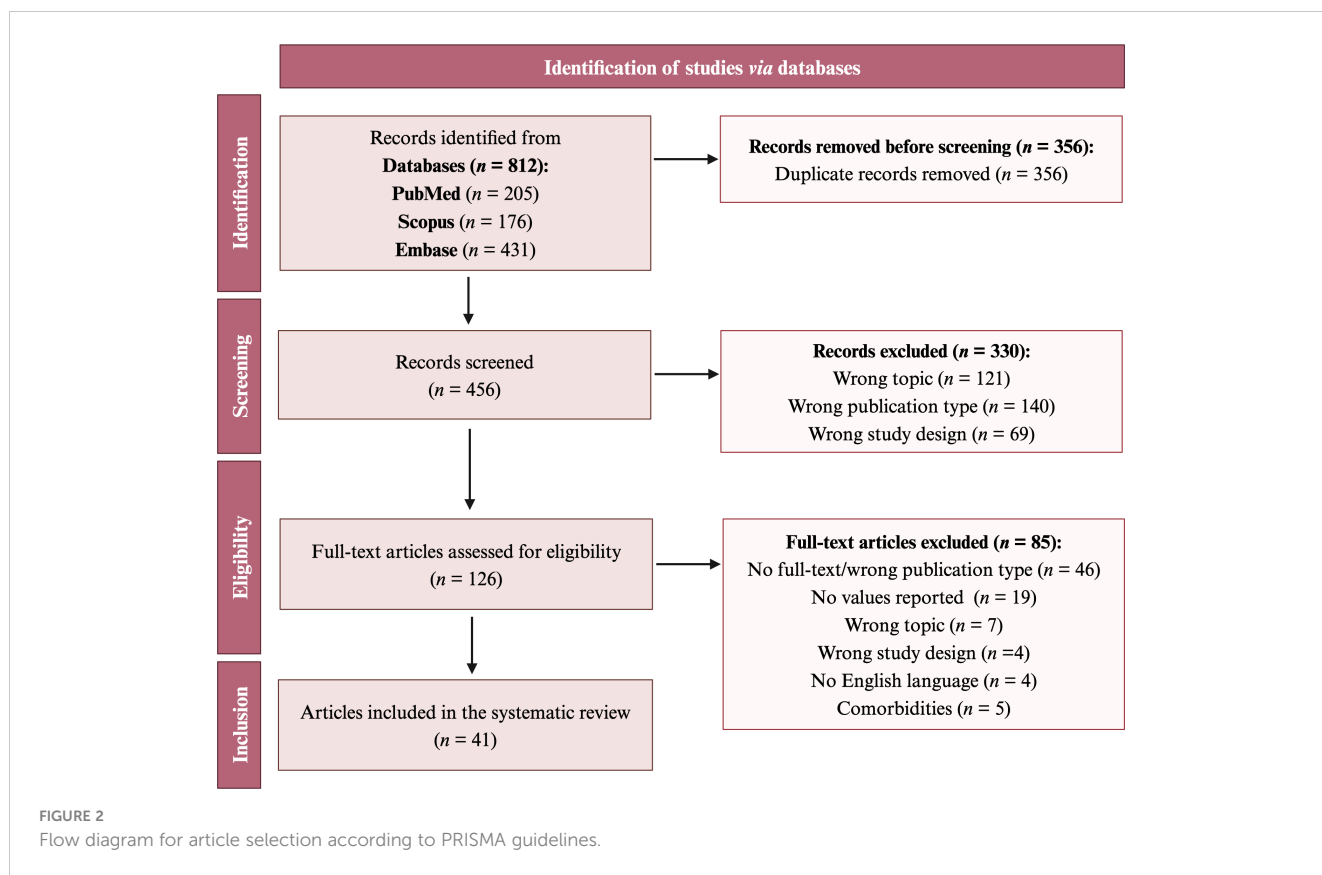
The reported variables include: the first author's name, year of publication, and reference of each study; the values of the biomarkers in PE and control groups, with sample size and units of measurement; *p-values* and the statistical test used; the time of sample collection and type of blood sample (*i.e.*, serum, plasma); the techniques used for biomarker measurement; notes about EOPE, LOPE, and/or SPE. The recorded statistical measures were reported as mean with standard deviation (mean $\pm$ SD) or median with interquartile range [median (IQR)].

Most articles reported a single value for a biomarker, which was included in the study. In a few research articles, multiple values obtained in different cohorts were reported for a single biomarker; in such cases, all cohorts were separately reported.

3 Results

3.1 Studies included for systematic review

The results of the literature search and selection process are illustrated in [Figure 2](#). A total of 812 records were retrieved after the first search in the above-mentioned databases. Duplicates ($n = 356$) were removed via Rayyan, and the remaining 456 records were screened. During the screening phase, involving the examination of the title and abstract, 330 records were excluded for the following reasons: wrong topic ($n = 121$), wrong publication type ($n = 140$), and wrong study design ($n = 69$). Full-text screening resulted in 126 articles, which were assessed for eligibility. During the eligibility phase, 85 full-text articles were excluded since they did not meet the inclusion criteria: wrong publication type ($n = 46$), no values were reported ($n = 19$), wrong topic ($n = 7$), wrong study design ($n = 4$),



languages other than English ($n = 4$), and presence of comorbidities ($n = 5$). Forty-one studies were included in this systematic review. The included papers and relative values are reported in Tables 1–5.

The C markers can be divided into categories based on their involvement in the C cascade.

3.2 Classical pathway components

Fourteen studies (34.1%) were identified as measuring the blood concentrations of CP components, including C1q, C4, C4a, and C4d, as summarized in Table 1. Five studies measured C1q levels (12.1%), nine C4 (21.9%), one C4a (2.4%), and four C4d (9.7%). Regarding C1q, two studies reported no statistically significant differences in C1q levels between PE and control pregnancies (26, 28). One study showed significantly higher C1q levels in SPE (27). Two studies analyzing two different PE cohorts (EOPE and LOPE) reported significantly lower or higher C1q levels in PE, respectively (29, 30). Conversely, the majority of the studies were coherent with reporting a significantly lower level of C4 in PE women (29, 31, 32, 34, 35, 37), also when analyzing EOPE and LOPE separately (29). Only one article measured C4a levels, not detecting any significant difference in PE women, except for those experiencing the delivery of a small-for-gestational-age newborn (38). Four studies related to C4d fulfilled the inclusion criteria and suggested an increase in C4d levels in PE women (27, 30, 33, 39), particularly in LOPE (30).

3.3 Lectin pathway components

Our systematic review of currently available research highlighted very few studies (5/41; 12.1%) analyzing blood concentrations of LP components' serum levels, as shown in Table 2. LP components were mainly measured in one study by Larsen and colleagues. They reported significantly higher serum concentrations of CL-L1, Map44, MASP-1, and MASP-3 in PE women than in healthy pregnancies, while significantly lower H- and M-ficolin levels. At the same time, no significant differences were observed in Map19, MASP-2, and MBL (41). MBL levels were further investigated in four additional studies (27, 28, 30, 42), but only one demonstrated an association between increased MBL levels and PE pregnancies (42).

3.4 Alternative pathway components

The vast majority of studies focused their interest on the assessment of AP components in serum or plasma samples (28/41; 68.3%), including Ba, Bb, C3, C3a, C3c, C3d, Factor B (FB), Factor D (FD), and iC3b, as reported in Table 3. Fragment Ba levels were measured by Blakey and colleagues in two different cohorts of PE patients matched with controls, reporting significantly higher levels of Ba in PE women only in one cohort (31). Seven studies (17.1%) related to Bb concentrations were evaluated, and they

TABLE 1 Classical pathway.

Reference	Factor	PE		Control		<i>p</i> -value	Test		Time	Sample	Technique	Notes
		Values	n	Values	n							
Wang et al., 2021 (26)	C1q	194.0 (170.0–226.0) mg/L	49	204.0 (177.0–228.0) mg/L	147	0.346	Median (IQR)	Mann Whitney U test	upon inclusion	serum	ARCHITECT ci16200 Integrated System Chemistry/Immunology Analyzer	
Chen et al., 2021 (27)	C1q	100.9 (47.1– 449.4) µg/mL	32	92.5 (54.2– 171.9) µg/mL	48	0.001	Median (IQR)	Mann Whitney U test	n.a.	plasma	home-made ELISA	SPE ¹
He et al., 2020 (28)	C1q	192.5 ± 33.1 mg/L	21	205.0 ± 33.0 mg/L	63	0.361	Mean ± SD	t-test	~36 weeks	plasma	immunoturbidimetric assay	
Jia et al., 2019 (29)	C1q	159 (142–176) µg/mL	43	187 (148–247) µg/mL	30	<0.001	Median (IQR)	Mann Whitney U test	after admission to hospital	serum	immune transmission turbidity method on an automated AU 5800 biochemical analyzer (Beckman Coulter, Brea, CA, USA)	EOPE
Jia et al., 2019 (29)	C1q	155 (129–177) µg/mL	30	194 (179–211) µg/mL	30	<0.001	Median (IQR)	Mann Whitney U test	after admission to hospital	serum	immune transmission turbidity method on an automated AU 5800 biochemical analyzer (Beckman Coulter, Brea, CA, USA)	LOPE
He et al., 2016 (30)	C1q	88.17 (48.92–154.68) µg/mL	30	88.17 (54.19–171.87) µg/mL	30	0.337	Median (IQR)	Mann Whitney U test	n.a.	plasma	home-made ELISA	EOPE
He et al., 2016 (30)	C1q	123.34 (47.10–449.36) µg/mL	30	92.06 (42.82–136.13) µg/mL	30	0.003	Median (IQR)	Mann Whitney U test	n.a.	plasma	home-made ELISA	LOPE
Blakey et al., 2023 (31)	C4	0.20 ± 0.08 g/L	33	0.31 ± 0.08 g/L	33	<0.001	Mean ± SD	t-test	within seven days prior to delivery	serum	electrochemiluminescent immunoassays (MesoScale Diagnostics (MSD, Rockville, MD))	
He et al., 2020 (28)	C4	520.5 ± 143.9 mg/L	21	572.1 ± 173.8 mg/L	63	0.454	Mean ± SD	t-test	~36 weeks	plasma	immunoturbidimetric assay	
Jia et al., 2019 (29)	C4	139 (107–178) µg/mL	43	223 (197–269) µg/mL	30	<0.001	Median (IQR)	Mann Whitney U test	after admission to hospital	serum	immune transmission turbidity method on an automated AU 5800 biochemical analyzer (Beckman Coulter, Brea, CA, USA)	EOPE
Jia et al., 2019 (29)	C4	136 (103–180) µg/mL	30	235 (188–280) µg/mL	30	<0.001	Median (IQR)	Mann Whitney U test	after admission to hospital	serum	immune transmission turbidity method on an automated AU 5800 biochemical analyzer (Beckman Coulter, Brea, CA, USA)	LOPE
Sarween et al., 2018 (32)	C4	0.23 (0.18–0.31) g/L	88	0.27 (0.22–0.35) g/L	107	0.001	Median (IQR)	Mann Whitney U test	n.a.	serum	Hitachi Cobas 6000 Turbidimeter (Roche diagnostics, West Sussex, UK)	
Derzsy et al., 2010 (33)	C4	0.31 (0.25–0.40) g/L	60	0.28 (0.24–0.35) g/mL	60	n.s.	Median (IQR)	Mann Whitney U test	n.a.	serum	radial immunodiffusion	

(Continued)

TABLE 1 Continued

Reference	Factor	PE		Control		<i>p</i> -value	Test		Time	Sample	Technique	Notes
		Values	n	Values	n							
Mellembakken et al., 2001 (34)	C4	0.16 (0.07–0.48) g/L	15	0.21 (0.10–0.30) g/L	19	<0.01	Median (IQR)	Mann Whitney U test	before labor	plasma	nephelometric technique	
Buyon et al., 1986 (35)	C4	28.9 ± 3.1 mg/dL	17	37 ± 1.8 mg/dL	24	<0.05	Mean ± SD	t-test	during labor	serum	radial immunodiffusion	
Massobrio et al., 1985 (36)	C4	32 ± 2.1 mg/dL	38	35 ± 1.5 mg/dL	30	n.s.	Mean ± SD	t-test	before labor	serum	radial immunodiffusion	
Hsieh and Cauchi, 1983 (37)	C4	19.1 ± 9.4 mg/dL	10	31.5 ± 5.5 mg/dL	30	<0.001	Mean ± SD	t-test	n.a.	plasma	radial immunodiffusion	
Soto et al., 2010 (38)	C4a	9124.3 (1020.3–25,940) ng/mL	54	10125.4 (850.7–32,640) ng/mL	134	n.s.	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Assay Designs, Inc., Ann Arbor, MI, USA)	
Chen et al., 2021 (27)	C4d	8.43 (0.47–34.49) µg/mL	32	5.23 (0.82–11.4) µg/mL	48	0.15	Median (IQR)	Mann Whitney U test	n.a.	plasma	home-made ELISA	SPE ¹
He et al., 2016 (30)	C4d	8.62 (0.35–32.52) µg/mL	30	6.31 (1.69–11.41) µg/mL	30	1.0	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel)	EOPE
He et al., 2016 (30)	C4d	10.56 (0.47–34.49) µg/mL	30	4.88 (0.82–15.58) µg/mL	30	0.014	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel)	LOPE
Halmos et al., 2012 (39) & Derzsy et al., 2010 (33)	C4d	0.16 (0.10–0.21) µg/mL	60	0.11 (0.08–0.15) µg/mL	60	<0.05	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel)	

EOPE, early-onset pre-eclampsia; IQR, interquartile range; LOPE, late-onset pre-eclampsia; n.a., not available; SD, standard deviation; SPE, severe pre-eclampsia. ¹SPE defined according to the 2013 American College of Obstetricians and Gynecologists (ACOG) hypertension in pregnancy guidelines (40).

TABLE 2 Lectin pathway.

Reference	Factor	PE		Control		<i>p</i> -value	Test		Time	Sample	Technique	Notes
		Values	n	Values	n							
Larsen et al., 2019 (41)	CL-L1	668 (602–714) ng/mL	54	551 (490–636) ng/mL	51	<0.0001	Median (IQR)	Mann Whitney U test	37 weeks (CTRL)/before delivery (PE)	serum	immunofluorimetric assay	
Larsen et al., 2019 (41)	H-ficolin	20,338 (17,793–22,134) ng/mL	54	28,412 (23,750–32,675) ng/mL	51	<0.0001	Median (IQR)	Mann Whitney U test	37 weeks (CTRL)/before delivery (PE)	serum	immunofluorimetric assay	
Larsen et al., 2019 (41)	M-ficolin	3,804 (3,179–4,978) ng/mL	54	4,780 (3,692–5,891) ng/mL	51	0.005	Median (IQR)	Mann Whitney U test	37 weeks (CTRL)/before delivery (PE)	serum	immunofluorimetric assay	
Larsen et al., 2019 (41)	MAp19	635 (523–679) ng/mL	54	626 (514–730) ng/mL	51	0.99	Median (IQR)	Mann Whitney U test	37 weeks (CTRL)/before delivery (PE)	serum	immunofluorimetric assay	
Larsen et al., 2019 (41)	MAp44	3,116 (2,704–3,462) ng/mL	54	2,707 (2,424–3,062) ng/mL	51	0.005	Median (IQR)	Mann Whitney U test	37 weeks (CTRL)/before delivery (PE)	serum	immunofluorimetric assay	
Larsen et al., 2019 (41)	MASP-1	36,057 (29,831–43,815) ng/mL	54	24,123 (17,124–29,973) ng/mL	51	<0.0001	Median (IQR)	Mann Whitney U test	37 weeks (CTRL)/before delivery (PE)	serum	immunofluorimetric assay	
Larsen et al., 2019 (41)	MASP-2	515 (391–642) ng/mL	54	556 (448–716) ng/mL	51	0.40	Median (IQR)	Mann Whitney U test	37 weeks (CTRL)/before delivery (PE)	serum	immunofluorimetric assay	
Larsen et al., 2019 (41)	MASP-3	4,848 (4,110–5,765) ng/mL	54	5,515 (4,481–6,252) ng/mL	51	0.03	Median (IQR)	Mann Whitney U test	37 weeks (CTRL)/before delivery (PE)	serum	immunofluorimetric assay	
Chen et al., 2021 (27)	MBL	3058 (130.4–23,384) ng/mL	32	3293 (654.4– 5182) ng/mL	48	0.72	Median (IQR)	Mann Whitney U test	n.a.	plasma	home-made ELISA	SPE ¹
He et al., 2020 (28)	MBL	4965.2 (3580.6–6530.4) ng/mL	21	4278.0 (1508.9–7464.8) ng/mL	63	0.6	Median (IQR)	Mann Whitney U test	~36 weeks	plasma	home-made ELISA	
Larsen et al., 2019 (41)	MBL	2094 (791–3588) ng/mL	54	1825 (701–3250) ng/mL	51	0.84	Median (IQR)	Mann Whitney U test	37 weeks (CTRL)/before delivery (PE)	serum	immunofluorimetric assay	
He et al., 2016 (30)	MBL	3000.20 (83.82–23 383.84) ng/mL	30	3361.18 (654.37– 4173.32) ng/mL	30	1.100	Median (IQR)	Mann Whitney U test	n.a.	plasma	home-made ELISA	EOPE
He et al., 2016 (30)	MBL	3176.56 (111.98–14 452.08) ng/mL	30	3039.76 (26.07–5182.46) ng/mL	30	1.210	Median (IQR)	Mann Whitney U test	n.a.	plasma	home-made ELISA	LOPE
Than et al., 2008 (42)	MBL	1128 (13.6–11870.0) ng/mL	99	886.3 (6.1–7805.1) ng/mL	187	<0.01	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Antibody-Shop A/ S, Gentofte, Denmark).	

EOPE, early-onset pre-eclampsia; IQR; interquartile range; LOPE, late-onset pre-eclampsia; n.a., not available; SD, standard deviation; SPE, severe pre-eclampsia. ¹SPE defined according to the 2013 American College of Obstetricians and Gynecologists (ACOG) hypertension in pregnancy guidelines (40).

consistently demonstrated higher levels of Bb in PE, both in EOPE and LOPE (27, 29, 30, 33, 43–45). The central component C3 was analyzed in eight articles (19.5%), with a significant decrease in PE patients (29, 31, 33), even though not all studies reached statistical significance (32, 34–36, 46). Twelve articles (29.3%) measured C3a levels in the blood of PE women compared to healthy pregnancies, detecting an overall significant increase of C3a in PE (27, 30, 33, 39, 49, 50). Conversely, neither C3c (28, 53) nor C3d (36) plasma assessment showed significant differences between PE and normal pregnancies. FB was assessed by two studies, which determined a significant increase in serum levels (26) and not significant in plasma (28) of pregnant women with PE. Four studies consistently determined a greater concentration of adipsin/FD in serum (54, 55, 57) or plasma (56) of women with PE, particularly increased in SPE (54). Only one study by Blakey *et al.* reported a significant reduction of iC3b levels in PE (31).

3.5 Terminal pathway components

Terminal pathway components were investigated in 17/41 studies (41.5%) retrieved from the literature, mainly focusing on C5a and C5b-9 (Table 4). Circulating levels of the anaphylatoxin C5a were evaluated by twelve studies (29.3%). Most studies accordingly reported higher levels of C5a in the PE group (27, 30, 38, 48–51). Only one study by He and colleagues reported a significant reduction (28), while circulating C5a levels did not differ significantly between PE and control groups in the remaining studies (47, 52, 60, 61). Circulating levels of C5b-9 were measured by thirteen research articles (31.7%). Almost all of them were consistent with significantly higher concentrations of C5b-9 in blood samples of women with PE (27, 30, 33, 39, 49, 51, 54, 60–62). Interestingly, Burwick *et al.* analyzed C5b-9 in four different PE cohorts (PE, SPE, EOPE, and EOSPE), highlighting significantly higher plasmatic levels in PE compared to matched healthy groups, except for EOPE (62).

3.6 Complement regulators

Several studies (16/41; 39.0%) assessed the circulating levels of different C regulators, mainly focusing on C1-inhibitor and Factor H (FH), as summarized in Table 5. Four studies investigated the association between circulating C1-inhibitor levels and PE (33, 34, 63), but only one reported a significant reduction of C1-inhibitor in women with PE (37). Factor H levels were evaluated in seven studies, which determined an overall reduction of this C regulator in patients with PE (26, 28, 29, 46, 47, 66) in both EOPE and LOPE (29). A statistically significant reduction in PE cohorts was observed also for Factor I (FI) (67), properdin (31), and vitronectin (68). Velasquez and colleagues reported higher plasma levels of CD59 in PE (64). Conversely, C4b-binding protein (C4BP) (33) and complement receptor 1 (CR1)/CD35 (65) did not show any statistical differences in PE and control groups.

4 Discussion

4.1 Overview of the complement components in pre-eclampsia

Even though C dysregulation may have a role in affecting placental formation before the disease onset, the contribution of C in PE pathophysiology mainly consists in a secondary mechanism of amplification of tissue injury and inflammation, following endothelial damage and local placental ischemia and hypoxia, and results in a cascade of reactions that contribute to the rapid development and symptom exacerbation of PE at the systemic level (69, 70). When discussing C testing, one should always bear in mind the complexity of C and the interdependence among its individual components. For ease of comprehension, our systematic review dissected the involvement of C components as biomarkers of PE, initially focusing on the contribution of the different C pathways as separate entities, and then moving to a more extensive overview.

As regards the CP and LP, studies were coherent in reporting lower levels of C4 and higher levels of C4d in PE compared to healthy pregnancies. This supports the hypothesis that the relatively low C4 values in PE may indicate a low grade of CP and LP activation with consumption of C4, as suggested by the increase in C4d levels. Agostinis and co-authors also demonstrated that lower levels of C4 were associated with PE (71). Consistently, excessive deposition of C4d at the placental site was frequently reported in PE (23, 31, 72) as a marker of CP and LP activation, even though C4d deposits seem more likely related to CP activation due to the co-localization with C1q (73). The vast majority of the resulting proteolytic cleavage product C4d remains at the activation site, while part of it may eventually enter the circulation as free C4d.

The CP or LP may be triggered by repeated ischemia–reperfusion injury and oxidative stress caused by defective placentation in PE (72, 73), while AP exacerbates the activation cycle via an amplification loop. In human PE, C has been shown to be locally activated via the LP triggered by H-ficolin, while MBL did not appear to be particularly involved (23). Larsen and colleagues reported that LP protein concentrations in the serum generally increased throughout the trimesters of normal pregnancy and reduced after delivery in healthy pregnant women and in women with PE (41). A study by Celik and Ozan also reported higher MBL levels in SPE patients compared to women with uncomplicated pregnancies (74), in accordance with further studies (42, 75). Conversely, herein reported results about C1q levels were inconclusive. Dijkstra and colleagues observed no significant differences in serum C1q between PE and control pregnancies in three different cohorts (76), while Agostinis *et al.* reported a decrease in PE patients, both in serum and plasma samples, maybe due to C1q binding to circulating immunocomplexes or to syncytiotrophoblast extracellular vesicles derived from PE placentas (71, 77, 78). C1q reduction is also supported by the findings of Jia *et al.*, both in EOPE and LOPE (29). In the occurrence of PE related to antibody-mediated autoimmune diseases (e.g., antiphospholipid syndrome), C1q reduction could

TABLE 3 Alternative pathway.

Reference	Factor	PE		Control		p-value	Test		Time	Sample	Technique	Notes
		Values	n	Values	n							
Blakey et al., 2023 (31)	Ba	150 (119–223) ng/mL	33	113 (89–148) ng/mL	33	0.012	Median (IQR)	Mann Whitney U test	within seven days prior to delivery	plasma	electrochemiluminescent immunoassays (MesoScale Diagnostics (MSD, Rockville, MD))	
Blakey et al., 2023 (31)	Ba	165 (117–268) ng/mL	35	151 (113–198) ng/mL	35	0.310	Median (IQR)	Mann Whitney U test	within seven days prior to delivery	plasma	electrochemiluminescent immunoassays (MesoScale Diagnostics (MSD, Rockville, MD))	
Chen et al., 2021 (27)	Bb	0.62 (0.19–3.32) µg/mL	32	0.44 (0.24–0.82) µg/mL	48	<0.01	Median (IQR)	Mann Whitney U test	n.a.	plasma	home-made ELISA	SPE ¹
Jia et al., 2019 (29)	Bb	491 (423–669) ng/mL	43	389 (312–470) ng/mL	30	0.001	Median (IQR)	Mann Whitney U test	after admission to hospital	serum	immune transmission turbidity method on an auto- mated AU 5800 biochemical analyzer (Beckman Coulter, Brea, CA, USA)	EOPE
Jia et al., 2019 (29)	Bb	503 (367–773) µg/mL	30	374 (323–418) µg/mL	30	0.003	Median (IQR)	Mann Whitney U test	after admission to hospital	serum	immune transmission turbidity method on an auto- mated AU 5800 biochemical analyzer (Beckman Coulter, Brea, CA, USA)	LOPE
He et al., 2016 (30)	Bb	0.62 (0.21–3.55) µg/mL	30	0.45 (0.24–0.59) µg/mL	30	<0.001	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel)	EOPE
He et al., 2016 (30)	Bb	0.68 (0.19–4.10) µg/mL	30	0.43 (0.26–0.82) µg/mL	30	0.027	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel)	LOPE
Velickovic et al., 2015 (43)	Bb	1.26 ± 0.60 µg/ml	38	0.98 ± 0.45 µg/ml	253	0.003	Mean ± SD	t-test	upon admission to hospital for delivery	plasma	ELISA (Quidel)	
Hoffman et al., 2013 (44)	Bb	1.45 ± 1.03 µg/ml	24	0.65 ± 0.23 µg/ml	20	<0.001	Mean ± SD	t-test	prior to delivery	plasma	ELISA (Quidel)	
Derzsy et al., 2010 (33)	Bb	0.12 (0.10–0.14) µg/mL	60	0.11 (0.09–0.15) µg/mL	60	n.s.	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel)	
Lynch et al., 2008 (45)	Bb	0.8 ± 0.3	32	0.68 ± 0.2	669	0.02	Mean ± SD	t-test	before 20 weeks' gestation	plasma	home-made ELISA	
Blakey et al., 2023 (31)	C3	1.90 ± 0.39 g/L	33	2.36 ± 0.39 g/L	33	<0.001	Mean ± SD	t-test	within seven days prior to delivery	plasma	electrochemiluminescent immunoassays (MesoScale Diagnostics (MSD, Rockville, MD))	
Jia et al., 2019 (29)	C3	1120 (1019–1311) µg/mL	43	1438 (1264–1603) µg/mL	30	<0.001	Median (IQR)	Mann Whitney U test	after admission to hospital	serum	immune transmission turbidity method on an automated AU 5800 biochemical analyzer (Beckman Coulter, Brea, CA, USA)	EOPE

(Continued)

TABLE 3 Continued

Reference	Factor	PE		Control		p-value	Test		Time	Sample	Technique	Notes
		Values	n	Values	n							
Jia et al., 2019 (29)	C3	1196 (1012–1340) µg/mL	30	1434 (1271–1602) µg/mL	30	<0.001	Median (IQR)	Mann Whitney U test	after admission to hospital	serum	immune transmission turbidity method on an automated AU 5800 biochemical analyzer (Beckman Coulter, Brea, CA, USA)	LOPE
Sarween et al., 2018 (32)	C3	1.73 ± 0.34 g/L	88	1.74 ± 0.31 g/L	107	0.838	Mean ± SD	t-test	n.a.	serum	Hitachi Cobas 6000 Turbidimeter (Roche diagnostics, West Sussex, UK)	
Derzsy et al., 2010 (33)	C3	1.56 (1.45–1.78) g/L	60	1.79 (1.60–1.91) g/L	60	<0.05	Median (IQR)	Mann Whitney U test	n.a.	serum	radial immunodiffusion	
Ari et al., 2009 (46)	C3	1.45 ± 0.26 g/L	21	1.46 ± 0.18 g/L	24	n.s.	Mean ± SD	One-way ANOVA	n.a.	serum	automated Dade Behring Nephelometer II (Dade Behring Marburg GmbH, Marburg, Germany)	
Mellembakken et al., 2001 (34)	C3	1.31 (1.01–1.60) g/L	15	1.20 (1.03–1.36) g/L	19	n.s.	Median (IQR)	Mann Whitney U test	before labor	plasma	nephelometric technique	
Buyon et al., 1986 (35)	C3	162 ± 4 mg/dL	17	165 ± 4 mg/dL	24	n.s.	Mean ± SD	t-test	during labor	serum	radial immunodiffusion	
Massobrio et al., 1985 (36)	C3	118 ± 4.3 mg/dL	38	116 ± 4mg/dL	30	n.s.	Mean ± SD	t-test	before labor	serum	radial immunodiffusion	
Chen et al., 2021 (27)	C3a	1889 (148.5–23,335) ng/mL	32	34.6 (9.86–308.1) ng/mL	48	<0.01	Median (IQR)	Mann Whitney U test	n.a.	plasma	in-house ELISA	SPE ¹
He et al., 2020 (28)	C3a	257.7 (214.7–398.1) ng/mL	21	383.1 (321.3–448.2) ng/mL	63	0.16	Median (IQR)	Mann Whitney U test	~36 weeks	plasma	ELISA (Quidel Corporation, San Diego, CA, USA)	
Wiles et al., 2018 (47)	C3a	43.7 (29.6–60.2) ng/mL	18	34.2 (30.2–50.3) ng/mL	20	0.48	Median (IQR)	Mann Whitney U test	at routine outpatient attendance	plasma	ELISA (Hycult Biotech HK354)	
Ma et al., 2018 (48)	C3a	30.1 ± 3.3 µg/mL	24	25. 9 ± 2.1 µg/mL	32	0.28	Mean ± SEM	t-test	n.a.	serum	ELISA (BD Biosciences)	
He et al., 2016 (30, 49)	C3a	679.73 (14.32–26,821.68) ng/mL	30	38.63 (14.43–308.15) ng/mL	30	<0.001	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel Corporation, San Diego, CA, USA),	EOPE
He et al., 2016 (30, 49)	C3a	4,018.80 (67.64–26,515.6) ng/mL	30	34.80 (9.86–162.26) ng/mL	30	<0.001	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel Corporation, San Diego, CA, USA),	LOPE
Ye et al., 2015 (50)	C3a	701.39 ± 263.55 ng/mL	52	29.8 ± 5.3 ng/mL	60	<0.05	Mean ± SD	t-test	prior to delivery	serum	ELISA (USCN Life Science Inc., Wuhan, China)	SPE ¹

(Continued)

TABLE 3 Continued

Reference	Factor	PE		Control		p-value	Test		Time	Sample	Technique	Notes
		Values	n	Values	n							
Burwick et al., 2013 (51)	C3a	3373 ± 916 ng/mL	25	3219 ± 548 ng/mL	25	n.s.	Mean ± SD	t-test	day of enrollment	plasma	ELISA (BD Biosciences)	SPE ²
Denny et al., 2013 (52)	C3a	63.8 ± 4.37 ng/mL	72	54.9 ± 1.62	43	n.s.	Mean ± SD	t-test	prior to delivery	plasma	ELISA (USCN Life Science Inc., Wuhan, China)	
Halmos et al., 2012 (39) & Derzsy et al., 2010 (33)	C3a	1358 (854.8–2142) ng/mL	60	751.6 (194.6–1660) ng/mL	60	<0.05	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel)	
Soto et al., 2010 (38)	C3a	2127.4 (698.6–15,820) ng/mL	54	2364.7 (557.9–6642.7) ng/mL	134	n.s.	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Assay Designs, Inc., Ann Arbor, MI, USA)	
He et al., 2020 (28)	C3c	1503.0 ± 332.1 mg/L	21	1653 ± 343.4 mg/L	63	0.288	Mean ± SD	t-test	~36 weeks	plasma	immunoturbidimetric assay	
Griffin, 1983 (53)	C3c	72 ± 33.5 mg/dL	15	63.8 ± 17.2 mg/dL	18	n.s.	Mean ± SD	t-test	before labor	plasma	radial immunodiffusion	
Massobrio et al., 1985 (36)	C3d	6 ± 0.6 mg/dL	38	4 ± 0.8 mg/dL	30	n.s.	Mean ± SD	t-test	before labor	plasma	radial immunodiffusion	
Wang et al., 2021 (26)	Factor B	353.5 (328.5–369.3) mg/L	49	333.0 (308.0–365.0) mg/L	147	0.036	Median (IQR)	Mann Whitney U test	upon inclusion	serum	fully automated ARCHITECT ci16200 Integrated System Chemistry/Immunology Analyzer	
He et al., 2020 (28)	Factor B	548.2 ± 83.8 mg/L	21	595.0 ± 118.8 mg/L	63	0.311	Mean ± SD	t-test	~36 weeks	plasma	immunoturbidimetric assay	
Assaf et al., 2024 (54)	Factor D	34.32 ± 7.28 ng/mL	30	15.93 ± 2.36 ng/mL	29	<0.001	Mean ± SD	t-test	upon inclusion	serum	ELISA (#ELK1128; ELK Biotechnology, Co., Ltd., Wuhan)	
Assaf et al., 2024 (54)	Factor D	44.75 ± 2.81 ng/mL	30	15.93 ± 2.36 ng/mL	29	<0.001	Mean ± SD	t-test	upon inclusion	serum	ELISA (#ELK1128; ELK Biotechnology, Co., Ltd., Wuhan)	SPE ³
David et al., 2021 (55)	Factor D	11,972 pg/mL (3240)	19	3607 pg/mL (5947)	19	<0.05	Median (IQR)	Mann Whitney U test	n.a.	serum	MILLIPLEX MAP™ Human Complement Panel	
Liu et al., 2021 (56)	Factor D	3161 ± 214.7 ng/mL	33	2153 ± 201.4 ng/mL	32	<0.01	Mean ± SD	t-test	before delivery	plasma	ELISA (RayBiotech Inc, Norcross, GA)	
Poveda et al., 2015 (57)	Factor D	5932.1 ± 2036.2 ng/mL	18	4314.5 ± 665 ng/mL	54	<0.01	Mean ± SD	t-test	~34 weeks	serum	ELISA (Abcam, USA)	
Blakey et al., 2023 (31)	iC3b	489 ± 153 ng/mL	33	606 ± 157 ng/mL	33	0.003	Mean ± SD	t-test	within seven days prior to delivery	plasma	electrochemiluminescent immunoassays [MesoScale Diagnostics (MSD, Rockville, MD)]	

EOPE, early-onset pre-eclampsia; IQR, interquartile range; LOPE, late-onset pre-eclampsia; n.a., not available; SD, standard deviation; SEM, standard error mean; SPE, severe pre-eclampsia. ¹SPE defined according to the 2013 American College of Obstetricians and Gynecologists (ACOG) hypertension in pregnancy guidelines (40). ²SPE defined according to the 2002 ACOG hypertension in pregnancy guidelines (58). ³SPE defined according to the 2019 ACOG hypertension in pregnancy guidelines (59).

TABLE 4 Terminal pathway.

Reference	Factor	PE		Control		<i>p</i> -value	Test		Time	Sample	Technique	Notes
		Values	n	Values	n							
Chen et al., 2021 (27)	C5a	44.41 (2.91–7935.5) ng/mL	32	13.5 (2.08–278.7) ng/mL	48	<0.01	Median (IQR)	Mann Whitney U test	n.a.	plasma	home-made ELISA	SPE ¹
He et al., 2020 (28)	C5a	21.9 ± 6.8 ng/mL	21	28.9 ± 7.5 ng/mL	63	0.025	Mean ± SD	t-test	~36 weeks	plasma	ELISA (Quidel Corporation, San Diego, CA, USA)	
Burwick et al., 2019 (60)	C5a	41.0 (26.9–51.0) ng/mL	16	34.2 (28.9–38.6) ng/mL	16	n.s.	Median (IQR)	Mann Whitney U test	at delivery	plasma	ELISA (BD Biosciences, San Jose, CA)	
Wiles et al., 2018 (47)	C5a	1.25 (1.25–2.17) ng/mL	18	1.45 (1.25–3.37) ng/mL	20	0.19	Median (IQR)	Mann Whitney U test	at routine outpatient attendance	plasma	ELISA (Hycult Biotech HK349)	
Ma et al., 2018 (48)	C5a	100.4 ± 5.9 ng/mL	24	76.4 ± 3.0 ng/mL	32	0.001	Mean ± SEM	t-test	n.a.	serum	ELISA (BD Biosciences)	
He et al., 2016 (30, 49)	C5a	35.16 (1.76–7935.46) ng/mL	30	12.87 (2.31–31.70) ng/mL	30	0.003	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel Corporation, San Diego, CA, USA),	EOPE
He et al., 2016 (30, 49)	C5a	84.82 (3.18–3845.73) ng/mL	30	14.33 (1.91–933.20) ng/mL	30	0.001	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel Corporation, San Diego, CA, USA),	LOPE
Ye et al., 2015 (50)	C5a	10.37 ± 2.73 ng/mL	52	8.54 ± 2.74 ng/mL	60	<0.05	Mean ± SD	t-test	prior to delivery	serum	ELISA (USCN Life Science Inc., Wuhan, China)	SPE ¹
Burwick et al., 2013 (51)	C5a	8.2 ± 1.30 ng/mL	25	4.5 ± 0.50 ng/mL	25	<0.01	Mean ± SD	t-test	day of enrollment	plasma	ELISA (BD Biosciences)	SPE ²
Denny et al., 2013 (52)	C5a	63.8 ± 4.37 ng/mL	72	54.9 ± 1.62	43	n.s.	Mean ± SD	t-test	prior to delivery	plasma	ELISA (USCN Life Science Inc., Wuhan, China)	
Soto et al., 2010 (38)	C5a	19.7 (4.3–94.1) ng/mL	54	12.4 (1.2–87.1) ng/mL	134	<0.05	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (American Laboratory Products Company, Windham, NH)	
Haeger et al., 1993 (61)	C5a	5.0 ± 0.8 ng/mL	8	4.8 ± 0.8 ng/mL	8	n.s.	Mean ± SD	t-test	time of delivery	plasma	radioimmunoassay	
Assaf et al., 2024 (54)	C5b-9	11.41 ± 1.93 ng/mL	30	7.88 ± 1.11 ng/mL	29	0.01	Mean ± SD	t-test	upon inclusion	serum	ELISA (#ELK3025; ELK Biotechnology, Co., Ltd., Wuhan)	
Assaf et al., 2024 (54)	C5b-9	22.59 ± 2.22 ng/mL	30	7.88 ± 1.11 ng/mL	29	<0.001	Mean ± SD	t-test	upon inclusion	serum	ELISA (#ELK3025; ELK Biotechnology, Co., Ltd., Wuhan)	SPE ³
Blakey et al., 2023 (31)	C5b-9	237 (198–335) ng/mL	33	237 (185–334) ng/mL	33	0.753	Median (IQR)	Mann Whitney U test	within seven days prior to delivery	plasma	electrochemiluminescent immunoassays (MesoScale Diagnostics (MSD, Rockville, MD))	

(Continued)

TABLE 4 Continued

Reference	Factor	PE		Control		<i>p</i> -value	Test		Time	Sample	Technique	Notes
		Values	n	Values	n							
Chen et al., 2021 (27)	C5b-9	290.2 (112.3– 616.2) ng/mL	32	110.4 (8.59–323.5) ng/mL	48	<0.01	Median (IQR)	Mann Whitney U test	n.a.	plasma	home-made ELISA	SPE ¹
He et al., 2020 (28)	C5b-9	463.4 (419.0–542.6) ng/mL	21	427.7 (302.3–632.0) ng/mL	63	0.6	Median (IQR)	t-test	~36 weeks	plasma	ELISA (Quidel Corporation, San Diego, CA, USA)	
Burwick et al., 2019 (60)	C5b-9	187 (134–234) ng/mL	16	131 (95–158) ng/mL	16	0.03	Median (IQR)	Mann Whitney U test	at delivery	plasma	ELISA (BD Biosciences, San Jose, CA)	
Wiles et al., 2018 (47)	C5b-9	294 (225–429) ng/mL	18	238 (193–360) ng/mL	20	0.30	Median (IQR)	Mann Whitney U test	at routine outpatient attendance	plasma	ELISA (Hycult Biotech HK342)	
Burwick et al., 2018 (62)	C5b-9	2900 (1396–4558) ng/mL	57	1374 (1064–2332) ng/mL	54	<0.001	Median (IQR)	Mann Whitney U test	day of enrollment	plasma	ELISA	PE
Burwick et al., 2018 (62)	C5b-9	2778 (1633–4230) ng/mL	104	1374 (1064–2332) ng/mL	54	<0.001	Median (IQR)	Mann Whitney U test	day of enrollment	plasma	ELISA	SPE ¹
Burwick et al., 2018 (62)	C5b-9	2906 (1740–5848) ng/mL	15	1378 (1096–2440) ng/mL	19	n.s.	Median (IQR)	Mann Whitney U test	day of enrollment	plasma	ELISA	EOPE
Burwick et al., 2018 (62)	C5b-9	2966 (1578–4256) ng/mL	53	1378 (1096–2440) ng/mL	19	0.003	Median (IQR)	Mann Whitney U test	day of enrollment	plasma	ELISA	EOSPE
He et al., 2016 (30, 49)	C5b-9	328.71 (112.27–1871.26) ng/mL	30	116.54 (22.00–323.54) ng/mL	30	<0.001	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel Corporation, San Diego, CA, USA),	EOPE
He et al., 2016 (30, 49)	C5b-9	306.56 (105.17–554.33) ng/mL	30	139.71 (8.59–317.55) ng/mL	30	<0.001	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel Corporation, San Diego, CA, USA),	LOPE
Burwick et al., 2013 (51)	C5b-9	444 ± 171 ng/mL	25	348 ± 172 ng/mL	25	0.04	Mean ± SD	t-test	day of enrollment	plasma	ELISA (BD Biosciences)	SPE ²
Halmos et al., 2012 (39) & Derzsy et al., 2010 (33)	C5b-9	75.9 (50.8–116.3) ng/mL	60	59.9 (42.1–86.6) ng/mL	60	<0.05	Median (IQR)	Mann Whitney U test	n.a.	plasma	ELISA (Quidel)	
Haeger et al., 1993 (61)	C5b-9	5.0 ± 1.6 AU/mL	8	1.4 ± 0.2 AU/mL	8	<0.05	Mean ± SD	t-test	time of delivery	plasma	ELISA	

EOPE, early-onset pre-eclampsia; EOSPE, early-onset sever pre-eclampsia; IQR, interquartile range; LOPE, late-onset pre-eclampsia; n.a., not available; SD, standard deviation; SEM, standard error mean; SPE, severe pre-eclampsia. ¹SPE defined according to the 2013 American College of Obstetricians and Gynecologists (ACOG) hypertension in pregnancy guidelines (40). ²SPE defined according to the 2002 ACOG hypertension in pregnancy guidelines (58). ³SPE defined according to the 2019 ACOG hypertension in pregnancy guidelines (59).

TABLE 5 Complement regulators.

Reference	Factor	PE		Control		<i>p</i> -value	Test		Time	Sample	Technique	Notes
		Values	n	Values	n							
Godtfredsen et al., 2022 (63)	C1-inhibitor	0.17 (0.15–0.19) g/L	117	0.17 (0.16–0.19) g/L	117	0.14	Median (IQR)	Wilcoxon matched-pairs signed rank test	upon inclusion	plasma	BN II analyser (Siemens Healthcare Diagnostics, Marburg, Germany)	
Derzsy et al., 2010 (33)	C1-inhibitor	0.19 (0.17–0.22) g/L	60	0.18 (0.16–0.20) g/L	60	n.s.	Median (IQR)	Mann Whitney U test	n.a.	serum	radial immunodiffusion	
Mellembakken et al., 2001 (34)	C1-inhibitor	0.23 (0.15– 0.55) g/L	15	0.21 (0.17–0.24) g/L	19	n.s.	Median (IQR)	Mann Whitney U test	before labor	plasma	single radial immunodiffusion (NOR-Partigen; Behringwerke A/G, Marburg, Germany)	
Hsieh and Cauchi, 1983 (37)	C1-inhibitor	11.4 ± 2.3 mg/dL	15	13.1 ± 1.5 mg/dL	32	<0.001	Mean ± SD	t-test	n.a.	serum	radial immunodiffusion	
Derzsy et al., 2010 (33)	C4b-binding protein	790.1 (614.5–980.8) µg/mL	60	823.9 (610.2–1004) µg/mL	60	n.s.	Median (IQR)	Mann Whitney U test	n.a.	serum	home-made ELISA	
Velásquez et al., 2020 (64)	CD59	32.0 (27–39) ng/mL	104	23.7 (20–31) ng/mL	54	<0.001	Median (IQR)	Mann Whitney U test	day of enrollment	plasma	ELISA (MyBioSource, San Diego, CA)	SPE ¹
Feinberg et al., 2005 (65)	CR1 /CD35	42.1 ± 4.0 ng/mL	29	37.7 ± 2.9 ng/mL	29	n.s.	Mean ± SD	t-test	just prior to labor	plasma	ELISA (T Cell Sciences, Cambridge, MA)	
Yasmin et al., 2024 (66)	Factor H	324.21 ± 131.04 µg/mL	26	556.95 ± 191.52 µg/mL	31	<0.0001	Mean ± SD	t-test	third trimester	serum	ELISA (#DY4779; R&D Systems, Inc., Minneapolis, Canada)	
Assaf et al., 2024 (54)	Factor H	33.93 ± 2.33 ng/mL	30	27.6 ± 2.41 ng/mL	29	0.02	Mean ± SD	t-test	upon inclusion	serum	ELISA (#ELK1720; ELK Biotechnology, Co., Ltd., Wuhan)	
Assaf et al., 2024 (54)	Factor H	59.02 ± 11.87 ng/mL	30	27.6 ± 2.41 ng/mL	29	<0.001	Mean ± SD	t-test	upon inclusion	serum	ELISA (#ELK1720; ELK Biotechnology, Co., Ltd., Wuhan)	SPE ³
Wang et al., 2021 (26)	Factor H	397.5 (358.0–436.8) mg/L	49	404.0 (372.0–429.0) mg/L	147	0.664	Median (IQR)	Mann Whitney U test	upon inclusion	serum	fully automated ARCHITECT ci16200 Integrated System Chemistry/ Immunology Analyzer	
He et al., 2020 (28)	Factor H	473.4 ± 70.2 mg/L	21	465.2 ± 50.8 mg/L	63	0.72	Mean ± SD	t-test	~36 weeks	plasma	immunoturbidimetric assay	

(Continued)

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TABLE 5 Continued

Reference	Factor	PE		Control		<i>p</i> -value	Test		Time	Sample	Technique	Notes
		Values	n	Values	n							
Jia et al., 2019 (29)	Factor H	351 (319–374) µg/mL	43	370 (339–398) µg/mL	30	0.009	Median (IQR)	Mann Whitney U test	after admission to hospital	serum	immune transmission turbidity method on an automated AU 5800 biochemical analyzer (Beckman Coulter, Brea, CA, USA)	EOPE
Jia et al., 2019 (29)	Factor H	354 (321–372) µg/mL	30	366 (348–408) µg/mL	30	0.031	Median (IQR)	Mann Whitney U test	after admission to hospital	serum	immune transmission turbidity method on an automated AU 5800 biochemical analyzer (Beckman Coulter, Brea, CA, USA)	LOPE
Wiles et al., 2018 (47)	Factor H	672 (567–805) µg/mL	18	1116 (1006–1171) µg/mL	20	<0.0001	Median (IQR)	Mann Whitney U test	at routine outpatient attendance	plasma	ELISA (Microvue SC5–9 Plus EIA)	
Ari et al., 2009 (46)	Factor H	933 ± 298 mg/L	21	1004 ± 342 mg/L	24	n.s.	Mean ± SD	one-way ANOVA	n.a.	serum	radial immunodiffusion, Binding Site (Human factor H NanoRID Kit, Birmingham, UK)	
Mei et al., 2017 (67)	Factor I	37.65 µg/mL	38	73.11 µg/mL	44	<0.05	Mean	t-test	n.a.	plasma	ELISA (CusAb Company, Wuhan, China)	
Blakey et al., 2023 (31)	Properdin	4828 ± 806 ng/mL	33	6877 ± 1421 ng/mL	33	<0.001	Mean ± SD	t-test	within seven days prior to delivery	plasma	electrochemiluminescent immunoassays (MesoScale Diagnostics (MSD, Rockville, MD))	
Blakey et al., 2023 (31)	Properdin	5282 ± 1467 ng/mL	35	7021 ± 1317 ng/mL	35	<0.001	Mean ± SD	t-test	within seven days prior to delivery	plasma	electrochemiluminescent immunoassays (MesoScale Diagnostics (MSD, Rockville, MD))	
Kolialexi et al., 2016 (68)	Vitronectin	44.04 (24.91–64.51) ng/mL	10	193.30 (118.92–508.73) ng/mL	40	<0.001	Median (IQR)	Mann Whitney U test	first trimester prenatal screening	plasma	ELISA (Mabtech, Sweden)	EOPE

EOPE, early-onset pre-eclampsia; IQR, interquartile range; LOPE, late-onset pre-eclampsia; n.a., not available; SD, standard deviation; SPE, severe pre-eclampsia. ¹SPE defined according to the 2013 American College of Obstetricians and Gynecologists (ACOG) hypertension in pregnancy guidelines (40). ³SPE defined according to the 2019 ACOG hypertension in pregnancy guidelines (59).

associated with CP activation induced by the deposition of pathogenic immunoglobulins in the placenta (79).

The AP is also consistently activated in PE, as demonstrated by the reduction in circulating C3 levels and the increased levels of Ba, Bb, and C3a fragments, being markers of AP activation. Similar results are also consistent with the higher levels of circulating FD in PE (54–57), which is responsible for excessive C activation. Interestingly, the increase in Bb levels is consistent with increased FD levels in PE, considering that FD serves as a serine protease acting on FB to yield the active fragment Bb (57). It is also noteworthy to consider that FD levels may increase in parallel to decreased kidney function and be responsible for enhanced AP activation, particularly in the circulation (80). Elevated circulating levels of C3a in the first trimester of pregnancy were demonstrated as an independent predictive factor for PE (45). Moreover, C3a levels were particularly increased in LOPE (30). Accordingly, local C3 deposition in the placentae of PE patients is significantly higher compared to the control placentae (81).

Evidence about terminal pathway components in PE is rather coherent among studies, reporting increased C5a and C5b-9 levels in the maternal circulation, both in EOPE and LOPE (30, 49). Interestingly, a significant correlation was found between C5b-9 levels and anti-angiogenic factors (*i.e.*, soluble endoglin and sFlt-1). We should keep in mind that the increase in C5a and C5b-9 may also be directly influenced by additional factors, such as coagulation and fibrinolysis. Thus, urinary excretion of C5b-9 has been proposed as a more valuable marker of SPE than plasma evaluation (51).

Taken together, existing data show that C regulation is also impaired in PE. During normal pregnancy, FH levels usually increase throughout trimesters of healthy pregnancies (28), suggesting that a balanced regulatory response is crucial to avoid excessive systemic AP activation, despite allowing local C3-dependent mechanisms required to support a normal pregnancy. Interestingly, three studies consistently reported that circulating levels of FH were significantly reduced in PE (29, 47, 66). Yasmin et al. identified lower FH levels in sera collected in the first trimester of pregnancies with PE, proposing FH as a potential predictive biomarker of PE (66). The relative abundance of FH in the third trimester of pregnancy, and particularly the increased FH/C3 ratio, could prevent AP activation, while a reduction in FH may participate in the C dysregulation observed in PE. This scenario is also consistent with an overall reduction of other C regulators, *i.e.*, properdin (31) and FI (67). Reduced concentrations of properdin in maternal plasma allowed to distinguish cases of PE from healthy pregnancies with excellent diagnostic accuracy (31). Excessive AP activity may determine reduced circulating levels of properdin via consumption from tissue deposition.

The overall emerging scenario is an abnormal C activation and regulation in PE. Decreased circulating levels of C1q are frequently observed in combination with lower levels of C4, which may be due to increased consumption of C4 via aberrant activation of the CP or LP. Reduced concentrations of early AP components (*i.e.*, C3, FB, or FH) are also consistent with an extensive depletion of the cascade components due to excessive activation or poor inhibition, while C activation products (*e.g.*, Bb, C3a, C5a, or C5b-9) are increased as a result of the extensive cleavage of C components. A pivotal role in the dysregulation of C activation seems to be carried out by the

reduction in FH levels, which could be caused by deficiency or consumption of FH. Interestingly, a recent study by Lokki et al. identified FH variants that predispose to PE onset, confirming the multifactorial nature of the pathophysiological mechanisms underlying PE (82).

Jia and colleagues demonstrated that the best predictive indicator of PE was the combination of five C factors (C1q, Bb, FH, C3, and C4), both in EOPE and LOPE, displaying a potential as diagnostic markers for severe PE (29).

4.2 Pitfalls of complement testing in pre-eclampsia

An accurate analysis of a wide range of C components in PE is of utmost importance for prediction and diagnosis, although the reliability of these measurements is often challenging. The main concern of C assessment for diagnosing disease and monitoring therapy is that measured concentrations of C components may widely vary among different laboratories due to a lack of protocol uniformity for pre-analytical sample handling (*e.g.*, collection, processing, and storage), use of different calibrators and techniques (*e.g.*, nephelometry, turbidimetry, and ELISA), or antibodies targeting different epitopes. These pitfalls unveil an urgent need for standardization, in concert with adequate pre-analytical sample handling and storage. However, up to now, performing ELISA with well-defined antibodies has proven to be the most reproducible method for reflecting the actual state of C activation *via* quantification of C-derived split products next to the native proteins, while nephelometry and turbidimetry do not allow to distinguish between the native non-activated C proteins and their activated split products (83).

Furthermore, laboratories do not always analyze the proper sample type for C analysis. The use of plasma or serum can lead to different results when certain C factors are considered. In particular, plasma is preferentially chosen for assessing C byproducts to avoid *in vitro* C activation that occurs during serum preparation due to coagulation and fibrinolysis activation. EDTA-plasma (at least a final concentration of 10 mM), any activation of C is minimized (83). For other factors, the binding of C components to fibrinogen and fibrin in plasma should be taken into account (*e.g.*, C1q) (84). Inconsistencies may also be due to sample storage, thawing conditions, and repeated freezing-thawing cycles, which could be crucial variables for specific C components, particularly activation products (85).

Non-uniformity in the timing of sample collection is another issue that needs to be discussed when examining PE, and a serious limitation when comparing the studies collected in this systematic review. Although it may be assumed that the closer the disease onset, the more the potential biomarkers are expected to vary, a different matter is the potential predictive value of the C-components in PE. It is noteworthy that the vast majority of studies gathered in the current review measured C-component expression at the time of PE diagnosis (*i.e.*, in the third trimester of pregnancy). Only a few studies also measured C-components during the first trimester, with limited but encouraging results. He et al. observed significant fluctuations in circulating levels of FB, FH, C1q, C3c, and C4 in PE pregnancies from the first trimester

onwards (28). Altered FD and C5a levels in women with PE in the first trimester were also reported (56), as well as FH (66). These preliminary findings emphasize the urgent need to investigate variations in the C components during pregnancy to identify potential predictive markers at early stages. As PE can have multiple causes, it is unlikely that a single timing strategy for biomarker testing can be used to predict all cases of PE (86). Despite requiring a massive effort from clinicians and researchers in terms of number of enrolled women, it is crucial that we embark on prospective longitudinal studies involving blood sample collection at various stages of pregnancy. These studies may allow us to explore the actual time threshold of biomarkers with predictive significance and ensure proper matching for gestational age between healthy and pregnancies with PE.

Diverging results among studies may also be biased by the cohort selection due to different ethnicity, sample size, symptom severity (mild/severe), onset (preterm/term), and presence of comorbidities. For instance, women with African ancestry are at greater risk of PE, likely due to the involvement of specific factors, among which Bb fragment has been accounted (43), while factor B activation has not been observed in specific Caucasian cohorts (33). This evidence suggests that some C factors may serve as predictors of PE only in specific sub-groups of patients, offering a promising avenue for further investigation and potentially explaining the variations among the cohorts analyzed in this review.

Several lines of evidence support the concept that PE should not be considered a single disorder, and the guidelines no longer support the classification into “mild” and “severe” PE. Therefore, the definition of PE severity may not be consistent across studies, based on different updates of the American College of Obstetricians and Gynecologists (ACOG) hypertension in pregnancy guidelines (40, 58, 59), and may lead to bias in the cohort selection. It is also worth considering that EOPE and LOPE may be explored as separate entities reflecting underlying differences in etiology. The complexity of defining and classifying PE was further highlighted by Than *et al.*, who described four molecular clusters of PE (i.e., canonical/placental, metabolic, immunological, and maternal PE) by proteomic analysis that exhibit distinct clinical phenotypes (87), in line with maternal hemodynamic characteristics of different hypertensive disorders of pregnancy phenotypes (88).

The presence of comorbidities is a crucial factor that must be taken into consideration. Interestingly, several studies that were specifically excluded from the current systematic search report the usefulness of C-component assessment in specific subgroups of patients. For example, predictive value was found for FB and FH serum levels in PE associated with gestational diabetes mellitus (89).

4.3 Strengths and limitations of the systematic literature review

To our knowledge, this is the first systematic literature review focusing on currently available research about the assessment of C

components as circulating biomarkers of PE. The main strength of the current systematic search includes the comprehensive coverage of available measurements of circulating C components in PE, which were summarized as ready-to-use information.

Nevertheless, we can recognize some methodological limitations. First, not all scientific information may be included in bibliographic databases being reported as unpublished conference papers or congress proceedings. In addition, we included only research papers in which numerical values for the measurement of the C-component were reported, thus possibly neglecting other seminal studies in which numerical values were not explicitly reported. Lastly, only publications in English were included, which may lead to language and country-specific biases, potentially limiting the generalizability of our findings.

5 Conclusion and perspectives

Since C activation can occur in several scenarios, the specificity of a single C-component test is low, so a panel of tests should be preferred (90). C multiplex ELISA assays, such as panels for simultaneous testing of C activation fragments and intact components or regulators (91), may be particularly advantageous from a technical and reproducibility standpoint. Thus, multiplex assays can help to address the need for a thorough analysis of the status of C activation and regulation and ensure a comprehensive overview of the C cascade. Here, we propose that multiplex C testing should be performed throughout pregnancy to raise awareness of the importance of planning prospective longitudinal studies to identify early predictive markers for PE.

With the mounting evidence of C activation, the potential of C-targeted therapy for the treatment of PE is a promising avenue. The emergence of new drugs specifically targeting C underscores the growing necessity for soluble biomarkers as companion tests to select and monitor patients who would benefit from these innovative treatments, instilling hope for improved outcomes in the management of PE.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Author contributions

AB: Conceptualization, Data curation, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing. CA: Conceptualization, Data curation, Funding acquisition, Writing – original draft. AM: Data curation, Methodology, Writing – original draft. GZ: Writing – review &

editing. TS: Writing – review & editing. GR: Funding acquisition, Supervision, Writing – review & editing. RB: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1419540/full#supplementary-material>

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Proangiogenic properties of complement protein C1q can contribute to endometriosis

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Endometriosis (EM) is defined as the engraftment and proliferation of functional endometrial-like tissue outside the uterine cavity, leading to a chronic inflammatory condition. While the precise etiology of EM remains elusive, recent studies have highlighted the crucial involvement of a dysregulated immune system. The complement system is one of the predominantly altered immune pathways in EM. Owing to its involvement in the process of angiogenesis, here, we have examined the possible role of the first recognition molecule of the complement classical pathway, C1q. C1q plays seminal roles in several physiological and pathological processes independent of complement activation, including tumor growth, placentation, wound healing, and angiogenesis. Gene expression analysis using the publicly available data revealed that C1q is expressed at higher levels in EM lesions compared to their healthy counterparts. Immunohistochemical analysis confirmed the presence of C1q protein, being localized around the blood vessels in the EM lesions. CD68⁺ macrophages are the likely producer of C1q in the EM lesions since cultured EM cells did not produce C1q *in vitro*. To explore the underlying reasons for increased C1q expression in EM, we focused on its established pro-angiogenic role. Employing various angiogenesis assays on primary endothelial endometriotic cells, such as migration, proliferation, and tube formation assays, we observed a robust proangiogenic effect induced by C1q on endothelial cells in the context of EM. C1q promoted angiogenesis in endothelial cells isolated from EM lesions (as well as healthy ovary that is also rich in C1q). Interestingly, endothelial cells from EM lesions seem to overexpress the receptor for the globular heads of C1q (gC1qR), a putative C1q receptor. Experiments with siRNA to silence gC1qR resulted in diminished capacity of C1q to perform its angiogenic functions, suggesting that C1q is likely to engage gC1qR in the pathophysiology of EM. gC1qR can be a potential therapeutic target in EM patients that will disrupt C1q-mediated proangiogenic activities in EM.

KEYWORDS

C1q, complement system, endometriosis, angiogenesis, gC1qR, ovary, endothelial cells

Introduction

Endometriosis (EM) is a chronic disease characterized by the presence of functional endometrium outside the uterine cavity (1). It is a very frequent gynecological disorder that affects around 10–15% of reproductive-aged women, with an overall prevalence of about 18% (2, 3). EM is commonly associated with pelvic pain, dyschezia, dyspareunia, dysmenorrhea, and infertility (4). The ectopic tissue forms lesions, characterized by a unique microenvironment involving inflammation and angiogenesis. According to pathophysiology and localization, the endometriotic lesions consisting of endometrial glands and stroma can be divided into three different types: superficial peritoneal EM, ovarian endometrioma (OMA), and deep infiltrating EM (5).

EM often presents symptoms that mimic other conditions, thus, delaying its early diagnosis. The time between symptom onset and EM diagnosis is usually long and the negative impact of this delay (between 6 to 10 years worldwide, irrespective of the healthcare system) can seriously affect patient's lifestyle and clinical outcomes (6, 7). A careful history of menstrual symptoms and chronic pelvic pain provides a basis for suspecting EM. No screening tools or tests are currently validated for EM prediction.

Treatment for EM varies, based on symptom severity and a desire to conceive. No current treatment permanently cures the disease. Medical therapy is commonly initiated for pain control without surgical confirmation of the disease. Such treatment is intended to reduce pain through various mechanisms, including minimizing inflammation, interrupting or suppressing cyclic ovarian hormone production, inhibiting the action and synthesis of estradiol, and reducing or eliminating menses. Surgical approaches to relieve EM-related pain can be used as the first-line therapy, or initiated after failed medical therapies (8).

Retrograde menstruation is the most widely accepted pathological mechanism of the onset of EM (9). However, not all women affected by retrograde menstruation develop EM, suggesting a multifactorial cause, including defective immune surveillance (10). Several studies have suggested that EM is due to aberrant immune activation (e.g., immune cell recruitment, cell adhesion and exaggerated inflammatory processes), facilitating the implantation and survival of endometriotic lesions (11, 12).

The complement system is a crucial component of the humoral immunity that acts as a bridge between innate and adaptive immunity (13, 14). Complement is involved in host defense against infectious agents and altered self, playing a pivotal role in the activation of inflammatory processes. It has been shown that the complement system is one of the most impaired pathways in EM, although the mechanisms underlying its involvement in the pathogenesis of EM have not been fully elucidated (15).

Beyond the canonical functions carried out by complement, alternative roles of several complement components have emerged, particularly with regard to C1q (16, 17). C1q is the first recognition subcomponent of the complement classical pathway. It is a complex protein composed by 18 chains organized in a tulip bouquet-like structure comprising a triple-helical collagen-like region and C-terminal globular heads (18). An emerging complement

activation-independent function of C1q concerns its capability to promote angiogenesis. It has been reported that C1q on its own can act as a proangiogenic factor in placental development, wound healing, post-stroke *restitutio ad integrum*, and tumor progression (19). In animal models, C1q deficiency is correlated with pre-eclampsia-like symptoms, a gestational syndrome characterized by angiogenic dysfunction; thus, C1q seems crucial during normal development of the placental tissue (20). Moreover, Bossi et al. demonstrated a pro-angiogenic role of C1q in the wound healing process, being localized in granulation tissue, independent of complement activation, based on permeability, proliferation, and migration of endothelial cells (ECs) (21). C1q is also involved in the angiogenic process during post-stroke ischemia recovery. On the other hand, few studies demonstrated that C1q could also drive tumour angiogenesis (22, 23).

It is widely recognized that blood supply is essential for the survival of endometriotic implants and further development of EM, since it can provide nutrients and growth factors, and promote recruitment of inflammatory cells to the endometriotic lesions (24). Microvascular ECs are also active participants in inflammatory processes via secretion of pro-inflammatory mediators and modulation of adhesion and migration of leukocytes through the expression of adhesion molecules and chemokines (25), in addition to being crucial players in angiogenic processes. As demonstrated by Agostinis et al., ECs may respond very differently to pro-inflammatory and pro-angiogenic stimuli depending on their tissue of origin (25).

In this study, we aimed to investigate the presence of C1q in endometriotic lesions and its potential proangiogenic role in EM. Thus, we evaluated its effects in classical angiogenic processes, such as cell motility, proliferation, and tube formation in ECs isolated from the EM lesions as compared to ECs isolated from healthy ovaries, the most frequent site of EM engraftment.

Methods

Reagents and antibodies

The following antibodies were used: mouse mAb anti-human CD34 (#MA5-15331), Alexa Fluor 647 donkey anti-mouse IgG (H+L) (#A-31571), Alexa Fluor 594 donkey anti-goat IgG (H+L) (#A-11058), and Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (#A-21206) were purchased from ThermoFisher Scientific (Massachusetts, USA); rabbit mAb anti-human CD31/PECAM1 (#ZRB1216) from Sigma Aldrich (Missouri, USA); mouse mAb anti-human vWF (#M0616), rabbit pAb anti-human vWF (#IS527), rabbit mAb anti-human CK8/18 (#M3652), mouse mAb anti-human CD31/PECAM1 (#M0823), mouse anti-human CD68 (#14-0688-80), and goat anti-mouse FITC-conjugated F(ab)' (#F0479) from Dako (Milan, Italy); mouse mAb anti-human VE-cad was kindly provided by E. Dejana (Mario Negri Institute, Milan, Italy); FITC-conjugated goat anti-rabbit (#11-4839-81) was purchased from Jackson ImmunoResearch (Milan, Italy); mouse anti-human CD31 FITC (#557508) was purchased from BD

Bioscience (New Jersey, USA). Other chemicals were purchased from Sigma Aldrich.

Gene expression profiling analysis

The analysis of C1q gene expression levels via its three chains (*CIQA*, *CIQB*, and *CIQC*) in control endometrium and different EM lesions (peritoneal, deep, and OMA) was conducted using data obtained from the Gene Expression Omnibus of the National Centre for Biotechnology Information (NCBI) with the accession number GSE141549. Microarray analysis on samples obtained from 43 endometrium biopsies of healthy woman, 101 endometrium biopsies of EM patients and 190 EM lesions, and data normalization, have been described by Gabriel and colleagues (26). Stages were defined following the rASRM classification (27).

Patient enrollment and specimen collection

Women included in this study were recruited from the Endometriosis Clinic at the I.R.C.C.S. 'Burlo Garofolo' Hospital (Trieste, Italy). All patients underwent surgical removal of ovarian endometriotic cysts through laparoscopy and subsequently received histological confirmation of the diagnosis. Patients positive for human immunodeficiency virus-1, hepatitis B virus, hepatitis C virus, or other sexually transmitted diseases, and patients with comorbidities, including peritoneal neoplasms, teratomas, endometrial polyps or any other proliferative diseases, were excluded from the study. During laparoscopy, endometrial biopsies were obtained using VABRA aspirator. The severity of EM was assessed using the revised American Society for Reproductive Medicine (rASRM) classification. All participants signed an informed consent form, following the approval of the Ethics Committee of the Friuli-Venezia Giulia region (Italy) (Protocol N 0023422/P/GEN/ARCS 2021). Upon enrollment, detailed information regarding medical history (e.g., age of menarche, EM diagnosis, family history of EM, number of pregnancies, and infertility diagnosis) and EM symptoms (e.g., ovulation, pre-menstrual and post-menstrual pain, dysmenorrhea, dyspareunia, dyschezia, and dysuria) were collected.

Tissue samples collected from EM lesions or endometrial biopsies were fixed in 10% buffered formalin and embedded in paraffin for immunohistochemical (IHC) analysis. A section of the lesion (about 1 cm²) was minced and preserved in Trizol (Invitrogen) for total RNA isolation, and a section was sliced for cell isolation. Prior to surgery, whole blood sample was collected in an EDTA-containing vacutainer tube for a group of patients and immediately processed for immunophenotyping.

Healthy ovarian samples were obtained from patients undergoing ovariectomy/annexectomy during sex reassignment surgery, or patients suffering from gynecological disorders unrelated to ovaries (e.g., leiomyomas). Patients were enrolled at the Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Trieste, Italy. All participants signed an informed consent form,

following the approval of the Regional Ethical Committee of FVG (CEUR), Udine, Italy (Prot. 0010143/P/GEN/ARCS 2019).

Immunophenotyping by flow cytometry

Prior to surgery for ovarian endometriotic cyst removal, whole blood samples were collected in an EDTA-containing vacutainer and processed within 2h. Antibodies against human-CD45 FITC, human-CD3-PerCP-Cy5.5, and human-CD56-PE (1:50) were added to 100 µl of sample and incubated for 30 min at room temperature (RT) in the dark. Red blood cells were then lysed with High-Yield Lyse Fixative-Free Lysing Solution (Life Technologies) for 15 min. Labeled samples were acquired using Attune NxT Flow Cytometer (ThermoFisher) equipped with a 488 nm laser, and analyzed with Attune Cytometric Software v5.3.0. Gating strategy used to identify and characterize CD56⁺CD3⁺ Natural Killer (NK) cells is shown in [Supplementary Figure 1](#).

Cell isolation and culture

Human umbilical vein ECs (HUVECs) were isolated and cultured, as described by Tedesco et al. (28). ECs were isolated from both endometriotic ovarian cysts (EECs) and healthy ovarian biopsy (OVECs), following the procedure described by Agostinis et al. (29). The protocol was slightly modified for the isolation of OVECs. Briefly, the tissue was minced and digested with 0.5% trypsin (Sigma-Aldrich) (0.1% for OVECs) and 50 µg/ml DNase I (Roche, Milano, Italy) overnight at 4°C, and then with collagenase type 1 (3 mg/ml) (Worthington Biochemical Corporation, DBA, Milano, Italy) for 30 min - (20 min for OVECs) at 37°C. After assessing cell viability with Trypan blue, ECs were positively selected with a mix of Dynabeads[®] CD31 (4x10⁸ beads/mL, Invitrogen; Thermo Fisher Scientific-11155D) and Dynabeads[™] M-450 (Life Technologies, Milan, Italy) coated with Ulex europaeus 1 lectin (Sigma-Aldrich), and then seeded on a fibronectin-gelatin coated T12.5 cm² flask. Cells were maintained in Human Endothelial Serum Free Medium (HESFM, Gibco, Carlsbad, CA) supplemented with 1% Penicillin-Streptomycin (PS), 20 ng/mL basic Fibroblast Growth Factor (bFGF), 10 ng/mL Epidermal Growth Factor (EGF), 10% v/v fetal bovine serum (FBS, Life Technologies), and 10% v/v normal human serum (NHS; Sigma-Aldrich). For OVECs, hydrocortisone (1:100; Sigma Aldrich) was added. For the endometriotic lesions, the CD31⁺ epithelial/stromal cells (EMCs) were also kept and seeded on a gelatin-coated flask with HESFM + 10% FBS. Cells were maintained at 37°C in a humidified atmosphere in a 5% v/v CO₂ incubator, and the culture medium was changed every 2–3 days depending on the growth of the cells.

Flow cytometry analysis and immunofluorescence for primary cell characterization

ECs (5x10⁵) were fixed in 3% v/v paraformaldehyde (PFA) in dark for 15 min. Next, primary antibodies: anti-human CD31/

PECAM1-FITC (1:25), anti-human vWF (1:50), and anti-human CK8/18 (1:50), were incubated for 45 min at 4°C, diluted in Saponin A (Farmitalia Carlo Erba, Milan, Italy) when permeabilization was needed, or in PBS + 1% (w/v) BSA for cell membrane staining. Incubation with FITC-conjugated anti-mouse and anti-rabbit secondary antibodies (1:300 in Saponin A) was performed for 30 min on ice in the dark. Cells were resuspended and fixed in 1% PFA. Fluorescence was acquired using Attune NxT Flow Cytometer (ThermoFisher) equipped with a 488 nm laser, and analyzed with the Attune Cytometric Software v5.3.0.

For immunofluorescence characterization, cells were seeded on round glass coverslips of 13 mm diameter and allowed to reach 70% of confluency. Cells were then fixed with 3% PFA for 15 min at RT in the dark and washed twice with PBS+ 0.1% Tween 20. For blocking, permeabilization and quenching simultaneously, cells were incubated with a buffer containing PBS + 1% w/v BSA + 0.1% Triton X-100 + 50 mM glycine for 30 min at RT. After washing primary antibodies were added in PBS + 2% w/v BSA for 1h at RT. Cells were washed again twice and incubated with Cy3-conjugated secondary antibodies (1:300) for 30 min in the dark. During the last 5 min of incubation, DAPI was added (1:1000). Coverslips were then washed twice and mounted on glass slides with a drop of Fluorescence Mounting Medium. Images were acquired with Leica DM3000 microscope and Leica DFC320 camera.

Immunohistochemistry

Tissue samples were fixed in 10% buffered formalin and paraffin embedded. 4–5µm tissue sections were deparaffinized with xylene and rehydrated with decreasing percentage of ethanol (100%, 95%, 70%) and H₂O. Antigen retrieval was performed for 20 min at 98.5°C in Tris-HCl/EDTA buffer, pH 9.0, for C1q, and in Citrate buffer, pH 6.0, for C4d and CD133 staining. Neutralization of the endogenous peroxidases was performed by adding H₂O₂ for 5 min; the blocking of nonspecific binding was achieved via incubation with PBS+2% w/v bovine serum albumin (BSA) for 30 min. Sections were incubated overnight at 4°C with primary antibodies, rabbit anti-human C1q (1:500), rabbit anti-human C4d (1:100), or rabbit anti-human CD133 (1:50) diluted in PBS, followed by incubation with anti-rabbit horseradish peroxidase (HRP)-conjugate (1:500) for 30 min at RT. Staining was performed by 3-amino-9-ethylcarbazole (AEC) chromogen substrate (Vector Laboratories). Sections were counterstained with Mayer haematoxylin (DiaPath) and examined under a Leica DM 2000 optical microscope. Images were acquired using Leica DFC 7000 T digital camera (Leica Microsystems, Wetzlar, Germany).

Double-staining immunofluorescence of paraffined section

Paraffined tissue sections of endometriotic ovarian lesions were deparaffinized with xylene and rehydrated with decreasing ethanol

percentage (100%, 95%, 70%) and H₂O. Antigen retrieval was performed in Tris-HCl/EDTA buffer, pH 9.0, for 20 min at 98.5°C. Slides were incubated with PBS + 1% w/v BSA + 0.01% Triton X-100 for 1h to prevent nonspecific binding. Primary antibodies diluted in the blocking buffer were added in the following combination: rabbit anti-human C1q (1:300) and mouse anti-human CD68 (1:100), rabbit anti-human C1q and mouse anti-human vWF (1:50), and rabbit anti-human C1q and mouse anti-human CD34 (1:50). After overnight incubation at 4°C, the anti-rabbit-Cy3 (1:300) and anti-mouse-Alexa Fluor 488 (1:750) secondary antibodies were added for 2h at RT. After 10 min of incubation with DAPI (1:500), slides were mounted using a Fluorescence Mounting Medium (Dako, Golstrup, Denmark), and images were acquired with a Leica DM 2000 (Leica, Wetzlar, Germany) fluorescence microscope using a Leica DFC 7000 T digital camera.

Gene expression analysis

Tissue samples from endometriotic lesions were minced and lysed using Trizol reagents (1 mL for 50–100 mg of tissue). Total RNA extraction was carried out using the PureLink™ RNA Mini Kit (Invitrogen, ThermoFisher). For isolated primary cells, RNA extraction was performed by lysing the cells with RNA Lysis Buffer and using the Total RNA Purification kit (Norgen Biotek Corp., Thorold, Canada), following the manufacturer's protocol. Isolated RNA was then quantified using NanoDrop™ 2000/2000c spectrophotometer (ThermoFischer Scientific, Massachusetts, USA) and reverse transcribed in to cDNA using SensiFAST™ cDNA Synthesis kit (Meridian Life Science, Memphis, USA). For RT-qPCR, Power SYBR™ Green Master Mix (Applied Biosystems, Life Technology, USA) was used and the reaction was performed using Corbett Rotor-Gene™ 6000 (Qiagen, Hilden, Germany). Expression levels of the human C1qA, C1qB, and C1qC genes were assessed through a comparative quantification analysis, considering reaction efficiency and normalization against the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1).

Surface biotinylation assay

OVECs and EECs (1x10⁶/well) were seeded on to a 6-well plate. The following day, cells were washed with ice-cold PBS + 1 mM MgCl₂ + 0.1 mM CaCl₂ and then incubated with EZ-Link™ Sulfo-NHS-Biotin (1 mg/mL) for 30 min on ice on an orbital shaker. To remove the excess of biotin, quenching was performed with 0.1 M glycine for 5 min at RT with shaking. Cells were collected with a scraper and washed thrice with ice-cold PBS. Cell pellets were resuspended into RIPA buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl pH. 7.4, 0.1% protease inhibitors), kept on ice for 20 min, and then centrifuged at 14,000 x g for 10 min at 4°C. From each lysate, an input (1:10) was taken and resuspended into 2X Laemmli buffer. The remaining lysates were incubated with High Capacity

TABLE 1 List of primers used for Real-Time quantitative PCR.

Gene	Melting Temperature (C°)	Forward sequence Reverse sequence	Accession number
<i>C1QA</i>	60	TGGAGTTGACAACAGGAGGC CGATATGGCCAGCACACAGA	NM_001347465.2
<i>C1QB</i>	60	ACCCAGGGGATAAAAGGAGAG GGCAGAGAAGGCGATTTTCTG	NM_001371184.3
<i>C1QC</i>	60	AGGATGGGTACGACGGACTG CTTCTGCCCTTGGGTCCTC	NM_001347619.2
<i>GAPDH</i>	60	CCAGGTGGTCTCCTCTGACTT GTTGCTGTAGCCAAATTCGTT	NM_001357943.2

Streptavidin Agarose Resin (50% slurry, 0.02% sodium azide; Thermo Scientific) on a rotary shaker for 2h at 4°C. Streptavidin resin was washed three times in RIPA buffer, followed by centrifugation at 5,000 x g for 5 seconds. After the last washing step, the supernatant was carefully removed and 20 µL of 2 x Laemmli buffer was added to the resin to elute the biotinylated cell surface proteins. Samples were either stored at -80°C, or used immediately for Western Blot analysis.

Western blot analysis

Cell lysate proteins were separated on a 10% SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane using the semi-dry transfer apparatus Trans-Blot Turbo System (BIO-RAD). After 1h of incubation with 5% skimmed milk in PBS + 0.1% Tween 20, the membrane was probed with anti-gC1qR (2 µg/mL; a kind gift from Prof Berhane Ghebrehiwet, State University New York, Stony Brook, NY, USA) and anti-actin antibodies overnight at 4°C. Membrane was washed three times, every time for 5 minutes, and then incubated with LI-COR IRDye secondary antibodies (1:20,000 dilutions of anti-rabbit IgG IRDye® 800CW and anti-mouse IgG IRDye® 680RD) for 1h at RT. After three washing steps, the fluorescence intensity was measured by the Odyssey® CLx near-infrared scanner (LI-COR Biosciences, Lincoln, NE, USA). Image acquisition, processing and data analysis were performed using Image Studio 5.2 (LI-COR Biosciences).

Angiogenesis assays

Migration assay was performed in a trans-well system using EECs, OVECs or HUVECs. Cells were stained with the fluorescent dye FAST DiI™ and seeded in FluoroBlok™ Inserts (1.5x10⁵ cells/insert). The lower chamber of the trans-well was loaded with VEGF (20 ng/mL), as a positive control, or C1q (10 µg/mL). After 18h, the fluorescence was read with INFINITE 200 Fluorescence Plate Reader, applying a bottom reader protocol. The percentage of migration was compared against a calibration curve established with an increasing number of labeled cells.

Wound healing assay with primary ECs was performed after seeding 5x10⁴ cells/well in a 24-well plate. After reaching a confluence of 60–70%, the endothelial monolayer was perturbed at the center of the well with a tip. Then, cells were stimulated with VEGF (20 ng/mL) as a positive control, or C1q (10 µg/mL), and maintained at 37° in a 5% v/v CO₂ incubator. Images of the wound fields were captured at different time points (0 and 8h) and the percentage of wound closure was compared among the different conditions.

Tube formation assay with primary ECs was performed by seeding 5x10⁴ cells in a CultureSlides onto a Matrigel® drop and stimulated with VEGF (20 ng/mL), as a positive control, or C1q (10 µg/mL). After 18 h, using TiEsseLab BDS 600 microscope, the capillary-like structures formed by ECs in the Matrigel® matrix were counted, comparing the different conditions.

Proliferation assay with primary ECs was performed seeding 7x10³ cells/well in a 96-well plate and then stimulating them with VEGF (20 ng/mL), as a positive control, or C1q (10 µg/mL). After 24h, MTS was added to each well and cell proliferation was measured using PowerWave Select X Microplate Reader (450 nm).

Binding of C1q to endothelial cells

ECs were grown to confluence in 96-well tissue culture plates (Corning) and incubated with C1q (10 µg/mL), for 5, 15, 30, 60, or 120 min at RT. Bound C1q was probed by polyclonal rabbit anti-human C1q (1:300; Dako) and alkaline phosphatase-conjugated secondary antibody (1:20,000; Sigma–Aldrich). The enzymatic reaction was developed using p-nitrophenyl phosphate (Sigma–Aldrich; 1 mg/mL) as a substrate and read kinetically at 405 nm using a Titertek Multiskan ELISA reader (Flow Labs).

Statistical analysis

Data were analyzed by GraphPad Prism software 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Unpaired two-tailed Mann-Whitney test was used for the analysis of C1q gene expression and for GEP analysis; paired t test was applied for angiogenesis experiments. Results were expressed as mean ± standard error mean

(SEM) of three independent experiments performed in duplicate. P-values <0.05 were considered statistically significant.

Results

Gene expression analyses reveal higher levels of C1q expression in EM lesions compared to patient-derived uterine endometrium

To gauge C1q expression in EM, we interrogated the publicly available database EndometDB developed by the Turku University (Finland), which collected gene expression data from 190 EM patients and 43 healthy controls (26). Gene expression profiling (GEP) analysis revealed that all the three genes of C1q (*C1QA*, *C1QB*, and *C1QC*) were up-regulated in all histotypes of EM lesions (peritoneal, deep, and OMA) compared to healthy control endometrium (CE). As shown in Figure 1A, significantly lower C1q expression levels were detected in CE or in patient-derived endometrium (PE) as compared to EM lesions, especially when analyzing peritoneal EM and OMA. In these last two histotypes, an association between C1q expression level and disease severity (stage I-IV) was also observed, although it was not statistically significant (Figure 1B).

Demographic data and clinical features and immunophenotype of EM patients

A cohort of EM patients ($n = 20$), aged between 30 and 57 years, was enrolled at the I.R.C.C.S. “Burlo Garofolo” Hospital (Trieste, Italy). The participating patients’ demographic data and clinical features are listed in Table 2. Of note, most of the patients (15/20; 75%) were diagnosed with stage III–IV, according to the standard classification of the revised American Society for Reproductive Medicine (rASRM) (27). Furthermore, 60% of the EM patients had full-term pregnancies, while 25% received a diagnosis of infertility. At the time of enrollment, 50% of patients were undergoing hormonal therapies (*i.e.*, progestins, combined oestrogen–progestins, or hormone-releasing intrauterine devices). To immunophenotype circulating leukocytes in EM patients, we focused on natural killer (NK) cells. It is widely regarded that the uterine environment is partly regulated by uterine NK (uNK) cells, which are characterized by lower cytotoxicity ($CD16^{low}$, $CD56^{bright}$); they play a key role in establishing a suitable milieu for embryo implantation. The uNK cells in the peritoneal fluid as well as uterine endometrium of women affected by EM showed a lower cytotoxic activity. In the EM lesions, it revealed a lower capability to induce apoptosis in endometrial cells (30); this reduction seemed associated with the severity of the disease (31). Based on CD56 expression, NK cells were classified in $CD56^{++/bright}$

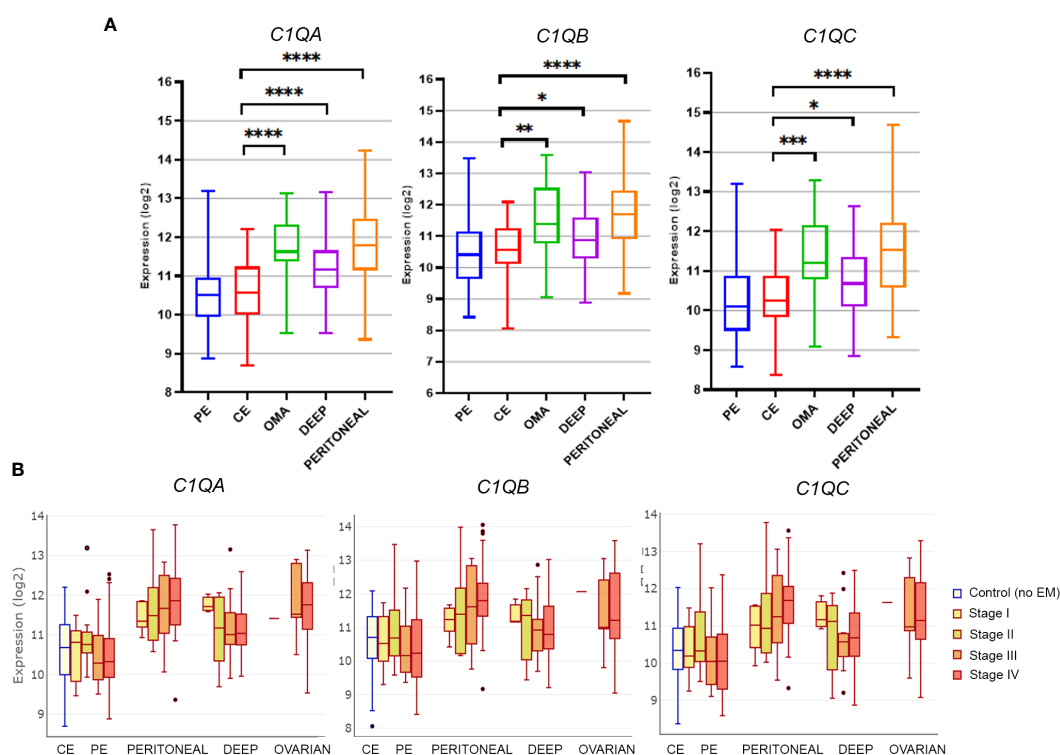


FIGURE 1

Gene expression analysis of C1q in EM lesions based on EndometDB. (A) Histograms representing *C1QA*, *C1QB*, and *C1QC* mRNA expression in control endometrium (CE), patient endometrium (PE), and in different EM lesions (peritoneal; deep; and ovarian, OMA). Gene expression profiling (GEP) analysis, based on data extracted from GEO (GSE141549), revealed a significantly higher expression of all three C1q genes in EM lesions as compared to CE. (B) Analysis of C1q gene expression in EM patients clustered into different disease stages (stage I–IV). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Mann–Whitney U Test).

TABLE 2 Demographic characteristics of the enrolled EM patients.

EM Patients (n = 20)		
Age, years (SD)	32.5 (7.3)	
Age at menarche, years (SD)	12.5 (1.6)	
Body-mass index, kg/m ² (SD)	22.1 (2.8)	
Fertility status	n	%
Pregnancy	12	60%
Infertility	5	25%
n.a.	3	15%
rASRM classification	n	%
Stage I	2	10%
Stage II	3	15%
Stage III-IV	15	75%
Adenomyosis	n	%
Yes	4	20%
No	16	80%
Ongoing medical therapy	n	%
Yes	10	50%
No	10	50%

Stages were defined following the revised American Society for Reproductive Medicine (rASRM) classification (27). n, number; n.a., not available; SD, standard deviation.

and CD56^{+/dim} subsets, since the CD56^{++/bright} CD16⁻ NK subset represents pre-terminally differentiated NK cells, capable of proliferation and cytokine/proangiogenic factor production with low cytotoxic properties (32). Interestingly, a significantly higher percentage of circulating CD56^{++/bright} NK cells was observed in EM patients compared to healthy women (Supplementary Figure 1). Conversely, no significant differences in CD45⁺⁺, CD3⁺, or total NK percentage were observed between EM patients and healthy controls (Supplementary Table 1).

Distribution of C1q in endometriotic lesions, eutopic endometrium, and healthy ovary

We investigated the local distribution of C1q in ectopic and eutopic endometrium via immunohistochemistry (IHC) using EM tissue samples. C1q displayed an intense positivity around the vessels in endometriotic lesions (Figure 2A; red arrows). C1q staining was particularly evident in some isolated cells scattered in the stroma (Figure 2B; yellow arrows). However, eutopic endometrium of the EM patients (Figure 2C) (and from healthy women) (Supplementary Figure 2) was almost negative for C1q staining. We also analyzed C1q expression in the ovary, which is the most frequent site of EM engraftment. Surprisingly, we observed C1q expression in healthy ovary tissue (Figure 2D), even though the

staining was considerably weaker compared to endometriotic lesions (Figure 2E). To understand whether C1q presence could be associated with the classical pathway activation, we investigated also C4d expression. We noticed a weak positivity for C4d; however, its intensity was not comparable to C1q staining (Figures 2F, G). Unlike most complement components that are synthesized by hepatocytes, C1q is known to be primarily produced by macrophages (and adherent monocytes); however, it seems that under certain circumstances, ECs are capable of expressing C1q or binding it (19). To discern the cells capable of synthesizing C1q within the microenvironment of endometriotic lesions, we conducted double immunofluorescence (IF) analysis for C1q and von Willebrand Factor (vWF) as an EC marker (Figure 3A), CD68 as a macrophage marker (Figure 3B), or CD34 as a marker of newly formed vessels or endothelial progenitor cells (EPCs) (Figure 3C). As shown in Figure 3, several CD68⁺ and CD34⁺ cells exhibited a positive staining for C1q. Furthermore, co-labelling with vWF revealed that the C1q staining was associated with mature endometriotic endothelium (Figures 3D, E). To exclude the expression of C1q by CD34⁺ EPCs, we analyzed also the presence of CD133⁺ cells in the EM tissue. As shown in Supplementary Figure 3, we failed to detect the presence of CD133⁺ cells in EM lesions.

C1q transcripts are locally expressed in endometriotic lesions but not in isolated primary cells

To dissect further the role of C1q in EM, we isolated primary cells from patient-derived endometriotic lesions. After enzymatic digestion of the tissue, ECs were purified using magnetic beads coated with anti-CD31 (Figure 4A). As revealed by the IF (Figure 4B), the CD31⁻ cells consisted of a mixture of epithelial and stromal cells, called as endometriotic epithelial/stromal cells (EMCs), which positively stained for mucin-1/epithelial membrane antigen and vimentin, whereas the CD31⁺ endometriotic ECs (EECs) were absolutely positive for endothelial markers (i.e., CD31, VE-cadherin, vWF, and CD34; Supplementary Figure 4). Following the same protocol, we also isolated and characterized ECs from healthy ovary tissue (OVECs) as a control of acceptor tissue (Supplementary Figure 4). Gene expression analysis using Real-Time quantitative PCR (RT-qPCR) revealed local expression of the three C1q genes (C1QA, C1QB, and C1QC) in all analyzed EM tissues, albeit with a notable degree of variability among samples. Conversely, neither EMCs nor EECs showed expression of C1q transcripts (Figure 4C). Peripheral blood mononuclear cells (PBMCs) were used as positive control and calibrator. Interestingly, clustering EM patients based on disease severity (i.e., I-III stages and IV stage) revealed a significant difference in C1q expression between the two groups (I-III stages vs IV stage), with a higher level of C1q transcripts in the most severe group (Figure 4D). Moreover, significantly higher levels of C1q transcripts were found in patients presenting adenomyosis concomitant with EM (Figure 4E).

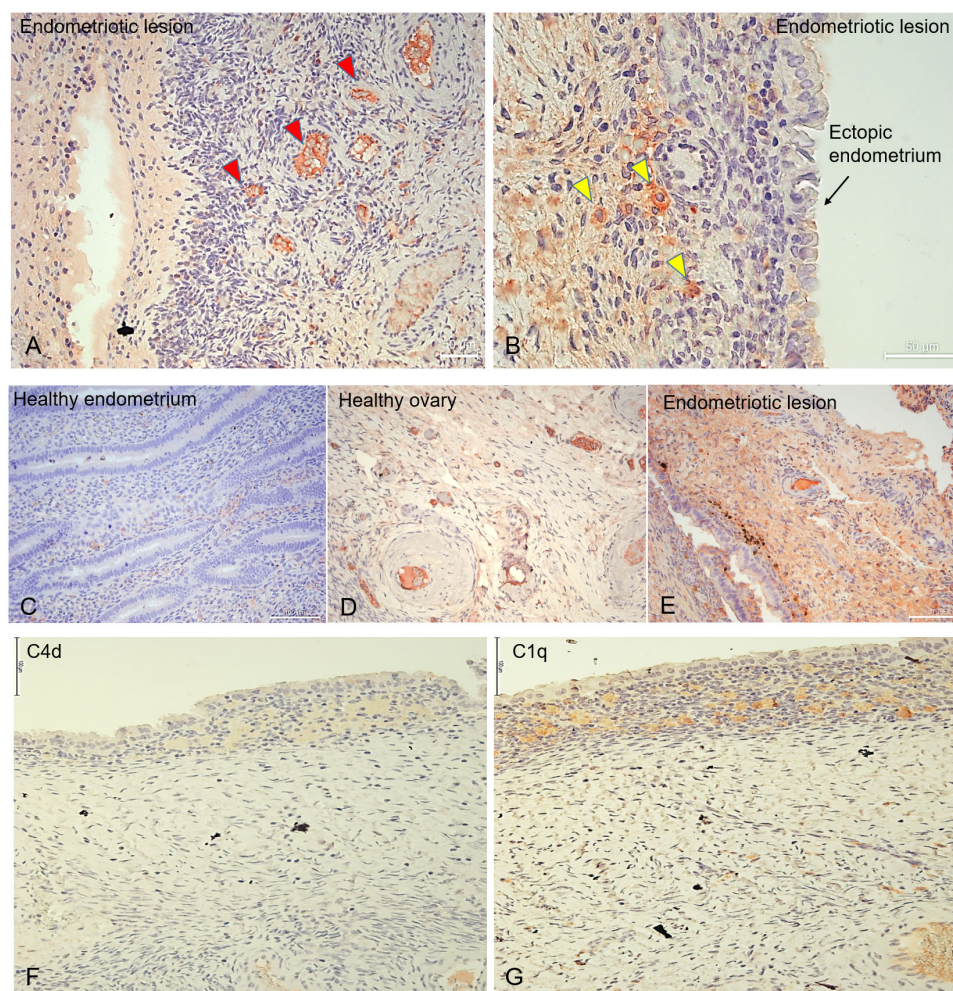


FIGURE 2

C1q is abundantly present in endometriotic lesions and in healthy ovary. Representative microphotographs showing the presence of C1q in ovarian endometriotic lesions (A, B, E), patients' eutopic endometrium (C), and healthy ovary (D). AEC (red) chromogen was used to visualize the binding of rabbit anti-human C1q antibody. Red arrows indicate vessels (A), while yellow arrows indicate isolated cells scattered in EM stroma which resulted due to positive staining for C1q (B). (F, G) Representative microphotographs showing the presence of C4d (F) or C1q (G) in serial sections of endometriotic lesions. C1q is present in the endometriotic lesion; however, the classical pathway is feebly activated. AEC (red) chromogen was used to visualize the binding of secondary antibodies. Nuclei were counterstained in blue with Harris Hematoxylin. Magnification, 10x (A, C-E); 20x (B). Scale bars, 50 µm (A, B); 100 µm (C-G).

C1q promotes angiogenesis in endothelial cells isolated from both endometriotic lesions and healthy ovary

To investigate the potential proangiogenic activity of C1q, we first assessed the ability of ECs to bind to C1q. We analyzed the behavior of EECs in comparison to OVECs, as a control of normal tissue, and to HUVECs, as a widely accepted model of ECs. The binding of C1q to ECs was evaluated by whole cell-ELISA assay, incubating live cells with C1q (10 µg/mL) for different time points (0, 15, 30, 60, or 120 min). As shown in Figure 5A, both EECs and OVECs reached the plateau after 10 min of incubation, whereas HUVECs did after 60 min. Furthermore, both these microvascular ECs were able to bind a higher amount of C1q compared to HUVECs.

Next, to investigate a proangiogenic role of C1q in EM, we performed a series of functional assays using EECs or OVECs, involving migration, scratch, tube formation, and proliferation (Figure 5B). HUVECs were used as positive control. We also carried out the above-mentioned experiments in parallel using VEGF, a well-known proangiogenic factor. Migration and scratch assays revealed that C1q was able to significantly promote the motility of both EECs and OVECs, in a VEGF-comparable manner (Figures 6A, B). The results obtained with OVECs suggested that C1q could play a pivotal role also in physiological angiogenesis in the healthy ovary. These observations were further corroborated by tube formation assay (Figure 6C), where ECs were loaded onto a 3D matrix of Matrigel® in the presence or absence (REST; resting cells) of the stimuli (C1q or VEGF). The capillary-like structures forming closed tubes were counted after 18h; EECs were found to form a

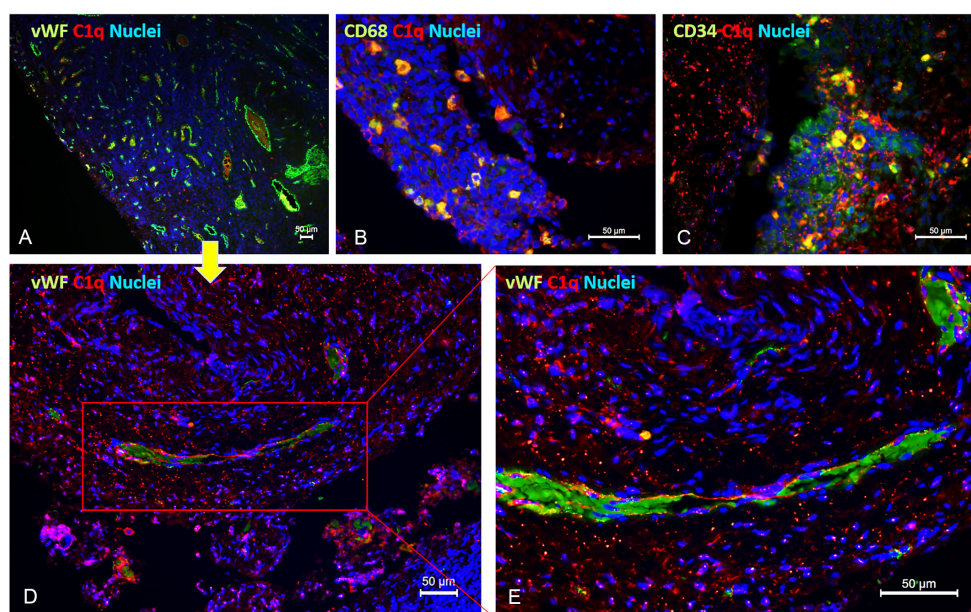


FIGURE 3

Double immunofluorescence microscopy for C1q in EM lesions. Representative images showing double staining for C1q (red) and vWF (A, D, E), CD68 (B), or CD34 (C) (green) in endometriotic lesions. After deparaffinization, tissue sections were incubated overnight with anti-human C1q and anti-human vWF, CD68, or CD34 primary antibodies, followed by incubation with anti-rabbit Cy3 and anti-mouse Alexa Fluor™ 488 secondary antibodies. Cell nuclei were stained with DAPI. Scale bars, 50 μm.

higher number of tubes in all the experimental conditions compared to OVECs. Moreover, we analyzed the ability of C1q to enhance the proliferation using an MTS assay, which revealed that C1q indeed enhanced the cell proliferation rate (Figure 6D).

C1q can induce endothelial cell motility via gC1qR

Given the effect of C1q on EECs, we aimed to elucidate the mechanisms underlying the proangiogenic capabilities of C1q by investigating the potential involvement of a putative C1q receptor. To this end, we characterized the expression of the receptor for the globular head of C1q (gC1qR), also known as hyaluronic acid-binding protein 1 (HABP1) or p32, in EECs and OVECs. First, IF was employed to detect gC1qR expression in both permeabilized and non-permeabilized ECs, as shown in Figures 7A, B. Remarkably, gC1qR exhibited elevated expression in permeabilized EECs and OVECs. Specifically, we observed a ubiquitous cytoplasmatic expression with a granular pattern, indicating potential co-localization with mitochondria. Interestingly, results from non-permeabilized cells also suggested the presence of gC1qR also at the cell surface, implicating its likely localization for C1q binding. To characterize the cell surface fraction of gC1qR, we carried out a cell surface biotinylation assay followed by SDS-PAGE and Western blot analysis (Figure 7C). The surface labeling with a biotinylated derivative confirmed gC1qR presence on the cell membrane of EECs as well as

OVECs. Notably, we observed a higher amount of gC1qR in EECs considering both total protein and cell membrane fraction, compared to OVECs (Figure 7D).

To assess the functional involvement of gC1qR in C1q-dependent proangiogenic effects, we conducted short interfering RNA (siRNA) experiments in HUVECs as a model of EC behavior. Following 48h of silencing, HUVECs transfected with siC1QBP (gC1qR gene) or siCTRL were tested in wound healing assay using C1q or VEGF as stimulus. Silencing of gC1qR resulted in a significantly reduced response of HUVECs to C1q, as evidenced by a lower percentage of wound closure after scratching (Figure 7E). The findings confirm that proangiogenic effects of C1q is likely to be mediated through gC1qR. Conversely, no significant differences were observed after VEGF stimulation or under resting conditions.

Discussion

Although an ever increasing body of scientific research has emerged on EM over recent years, the precise mechanisms governing its pathogenesis remain poorly understood, necessitating further investigation for the development of novel therapeutic strategies (34). The local and systemic immunological dysfunction is a well-established component in EM pathophysiology (35). In the current study, we enrolled a cohort of EM patients ($n = 20$), and we collected endometrial biopsies, a fragment of the ovarian cyst, and the peripheral blood. The latter was used to perform flow cytometric immunophenotyping, to analyze in particular the NK cell subset. In

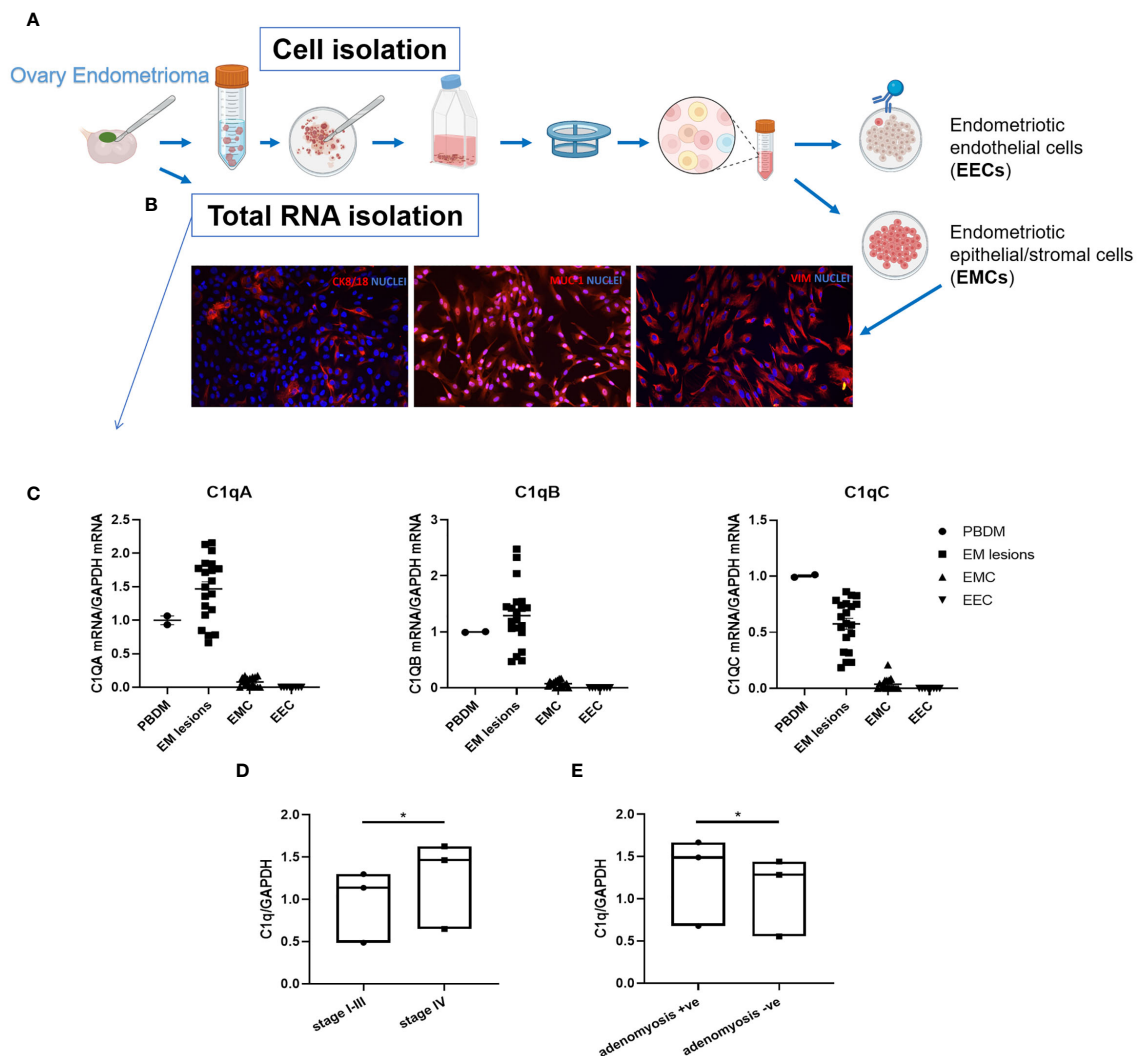


FIGURE 4

C1QA, *C1QB*, and *C1QC* gene expression in endometriotic lesions and primary isolated endometriotic cells. **(A)** Graphical representation of primary EM cell isolation procedure. **(B)** Characterization of endometriotic cells (EMCs) isolated from EM ovary cysts by immunofluorescence. Around 10% of EMCs resulted positively stained for cytokeratin (CK)8/18, and 100% were positive for mucin-1 (MUC-1) and vimentin. Cell nuclei were stained with DAPI. **(C)** After total RNA extraction and retro-transcription, C1q gene expression was analyzed by performing RT-qPCR. Peripheral blood mononuclear cells (PBMCs) were used as positive control. GAPDH was used as the housekeeping gene. Scatter plots were generated with the software GraphPad Prism 8.4.3. **(D, E)** The clustering of EM patients in stages (I-III or IV) or for adenomyosis presence respectively highlighted a significant difference in terms of C1q expression, with higher C1q levels in the most severe group **(D)**, and in adenomyosis-positive EM patients **(E)**. C1q expression was evaluated by examining the collective mean of individual *C1QA*, *C1QB*, and *C1QC* genes. Data are expressed as box-plots (median, interquartile range). * $p < 0.05$ (unpaired two-tailed t-test).

normal endometrium, CD56^{bright} uNK cells represent the major lymphocyte population (36). In EM, a decreased activity of peritoneal blood and peritoneal NK cells has consistently been reported, displaying reduced cytotoxicity and chemotaxis (37–41). It has been proposed that the abrogated NK cell function may be partially due to changes in the frequency of circulating CD56⁺ and/or CD16⁺ NK cells in EM patients, although a real consensus is yet to emerge (42). Interestingly, we observed a significantly higher percentage of circulating CD56^{++/bright} NK cells in EM patients compared to healthy women. This is consistent with an overall scenario of reduced NK cytotoxicity and defective immunosurveillance towards autologous cells.

A significantly impaired complement pathway in EM is widely acknowledged (15). In this study, we focused on the first recognition subcomponent of complement classical pathway, namely C1q, due to its involvement in angiogenic processes, which are fundamental for the maintenance of endometriotic implants (43). Sikora and colleagues have reported significantly higher levels of C1q, mannan-binding lectin (MBL), and C1-inhibitor in the peritoneal fluids of EM patients compared to control group (44). Recent multi-omics analysis also revealed an upregulation of *C1QA* expression in EM (45). Thus, we first examined the role of C1q in EM by gene expression analyses based on EndometDB. As previously observed for the complement component C3 (46), a higher expression of C1q

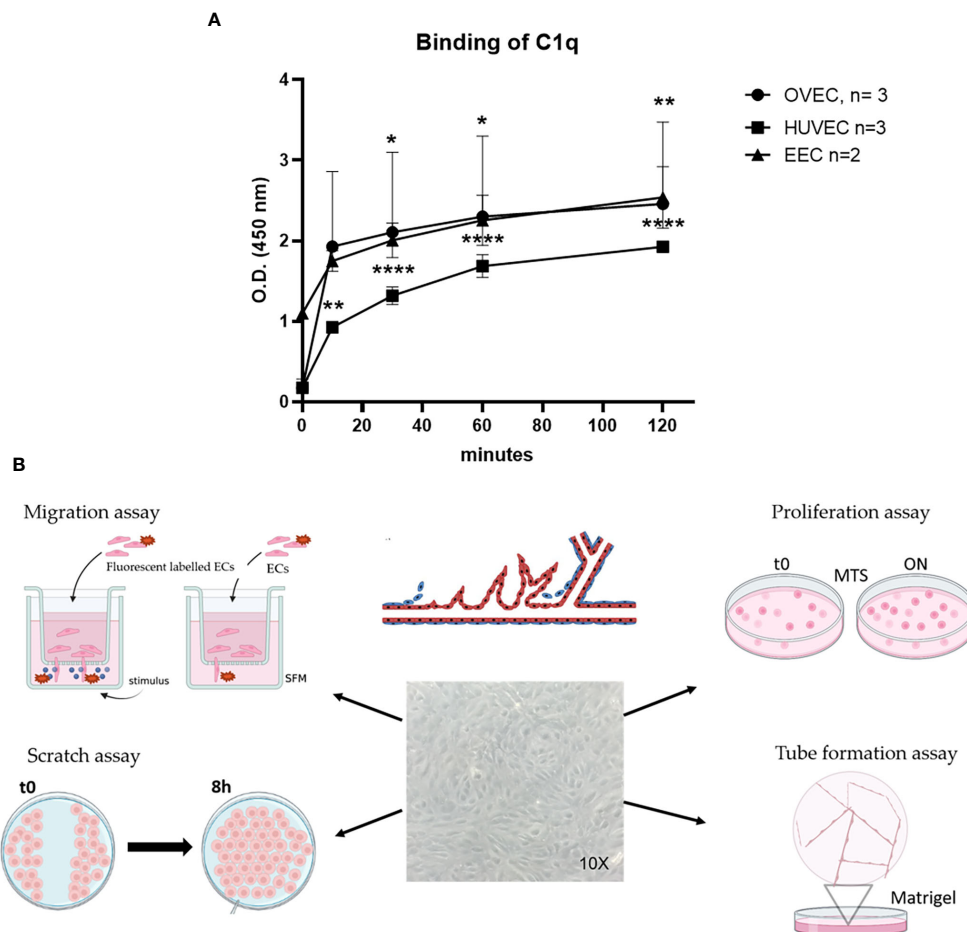


FIGURE 5

Binding of C1q to EECs, OVECs, or HUVECs. (A) Different endothelial cells [ECs; i.e., ECs isolated from healthy ovary (OVECs), $n = 3$; human umbilical vein ECs (HUVECs), $n = 3$; and endometriotic ECs (EECs)] grown to confluence on 96-well tissue culture plates were incubated with 10 $\mu\text{g/mL}$ of purified C1q at different time points (0, 15, 30, 60, or 120 minutes) at room temperature. The binding of C1q was revealed by whole-cell ELISA assay. The data are presented as mean $\pm$ SD of three separate experiments. (B) Schematic representation of functional assays for the evaluation of C1q proangiogenic properties by migration, scratch, proliferation, and tube formation assays. Image created with [BioRender.com](#), as an adaptation from Laschke et al. (33). * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

genes was observed in EM tissues compared to uterine endometrium, in particular in OMA and peritoneal lesions. Interestingly, OMA manifested a correlation with the severity of the disease, suggesting an association between C1q expression and lesion development. Furthermore, we found a significantly increased expression of C1q in EM patients with concomitant adenomyosis. The main histologic feature of adenomyosis is the presence of endometrial glands and stroma within the myometrium (47), suggesting the key role played by angiogenesis in the pathogenesis of adenomyosis (48). The observation of higher levels of C1q transcripts in patients with more severe EM (IV stage) and those with concomitant adenomyosis is clinically relevant, and suggests a potential association between C1q expression and disease prognosis. However, due to the small sample size, this conclusion needs validation using larger cohorts.

Based on bioinformatics dataset, we further investigated C1q expression also at the protein level focusing exclusively on OMA. We observed the presence and the local synthesis of C1q in endometriotic lesions. IHC revealed a substantial presence of C1q

in EM tissues, suggesting a likely activation of the complement classical pathway, only to be deterred by the very low intensity of C4d staining, as a byproduct of cascade activation. This observation, together with our previous findings concerning the role of C3 in EM (46), appears that the to suggest that complement activation in EM lesions mostly takes place via the alternative pathway. This is consistent with the involvement of the coagulation pathway in EM, which can directly activate C3 (i.e. by activating plasmin) (49). Complement activation at the EM lesion site can consistently contribute to local inflammation, chemotactic leukocyte infiltration, and damage to colonized healthy tissue.

We compared the C1q expression in EM lesions with the patients' eutopic endometrium, as the likely tissue of origin of EM, and with healthy ovary, being the main engraftment site for ectopic endometrium. Surprisingly, eutopic endometrium was almost negative for C1q staining, while healthy ovary tissues showed a moderate positivity, indicating a potential role of C1q in physiological processes involving ovary. Based on the strong structural analogy between C1q and adipokines (belonging to the

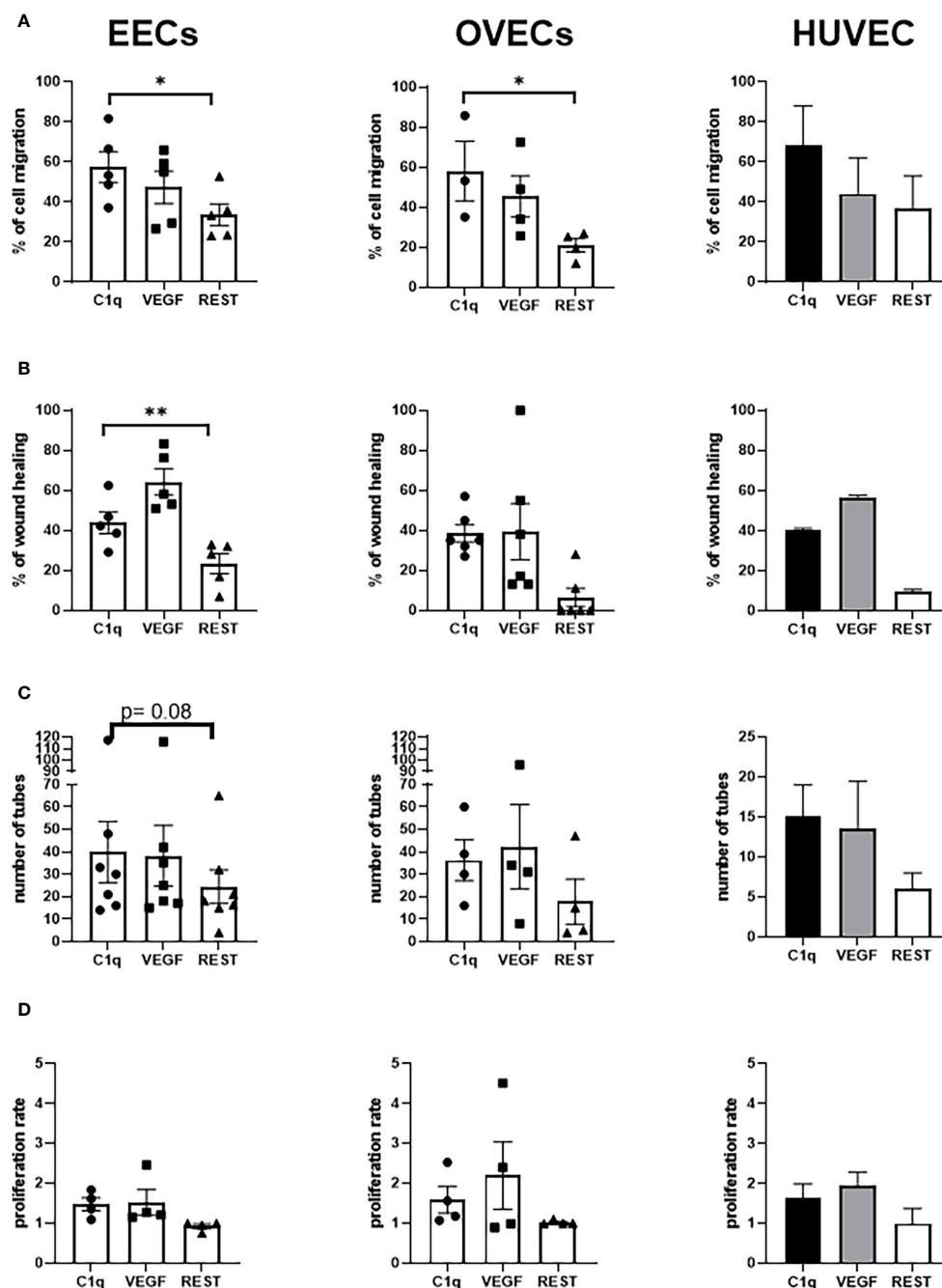


FIGURE 6

C1q promotes angiogenesis in EECs and OVECs. **(A)** Migration assays were performed in a trans-well system using endothelial cells (ECs) isolated from endometriotic cysts (EECs), healthy ovary (OVECs), or human umbilical vein (HUVECs). Cells were stained with FAST DiI™, seeded in FluoroBlok™ Inserts (1.5×10^5 cells/insert), and the lower chamber was loaded with C1q (10 $\mu\text{g/mL}$) or VEGF (20 ng/mL), as chemoattractant stimuli. After 24h, fluorescence was read via INFINITE 200 Fluorescence Plate Reader. Data are expressed as mean $\pm$ standard deviation (SD). * $p < 0.05$; ** $p < 0.01$. **(B)** Wound healing assays were performed using EECs, OVECs, and HUVECs. Cells (5×10^4 /well) were grown until 60–70% of confluence in a 24-well plate. After scratching the middle of endothelial monolayer, cells were stimulated with C1q (10 $\mu\text{g/mL}$) or VEGF (20 ng/mL). Images of the wound fields were captured after 18h, allowing calculation of percentage wound closure. Data are expressed as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$. **(C)** Tube formation assays were performed in EECs, OVECs, and HUVECs. Cells (5×10^4) were seeded onto Matrigel® in CultureSlides, and stimulated with C1q (10 $\mu\text{g/mL}$) or VEGF (20 ng/mL). After 18h, using TiEsseLab BDS 600 microscope, the capillary-like structures formed by ECs were manually counted, comparing the different conditions. Data are expressed as mean $\pm$ SD. * $p < 0.05$. **(D)** Proliferation assays were performed using EECs, OVECs, and HUVECs. Cells (7×10^3 /well) were seeded in a 96-well plate, and stimulated with C1q (10 $\mu\text{g/mL}$) or VEGF (20 ng/mL) for 24h. MTS was then added in each well, and cell proliferation was measured using PowerWave Select X Microplate Reader. Data are expressed as mean $\pm$ SD. REST, resting cells.

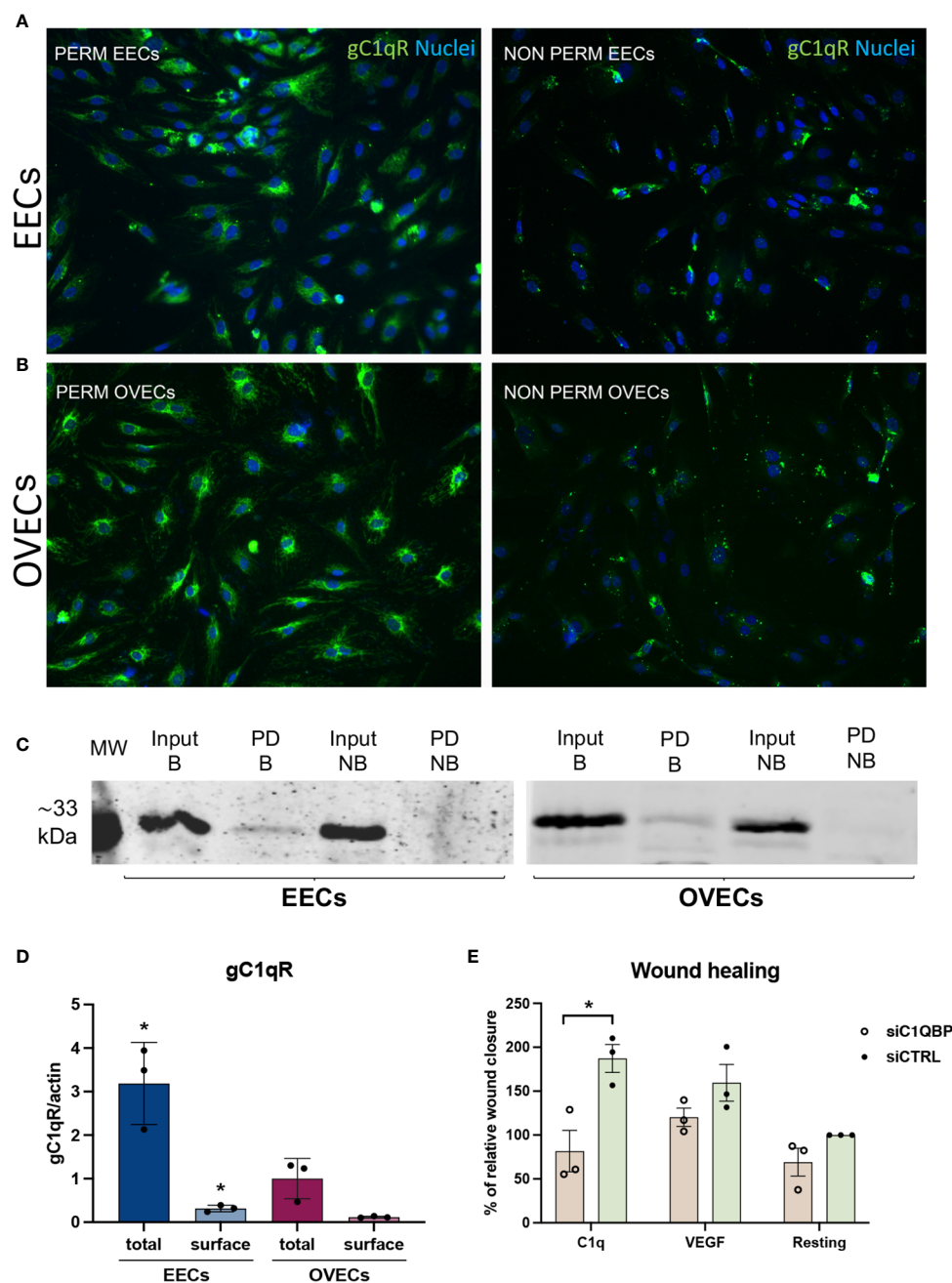


FIGURE 7

gC1qR is expressed in EECs and OVECs and can modulate C1q-induced proangiogenic behaviour. (A, B) Representative images displaying gC1qR positive staining (green) in EECs and OVECs, comparing permeabilized and not permeabilized cells. Cell nuclei were stained with DAPI (blue). (C, D) Surface biotinylation assay for the detection of gC1qR fraction present on the cell surface of EECs ($n = 3$) and OVECs ($n = 3$). Cells were treated with Sulfo-NHS-biotin reagent, biotinylated cell surface proteins were isolated upon binding to a Streptavidin-coated resin, and separated on a 10% SDS-PAGE. Membrane was then probed with anti-gC1qR antibody and anti-rabbit IRDye 800CW secondary antibody by Western blot analysis. Signal intensity was detected using an Odyssey CLx near-infrared scanner (LI-COR Biosciences, Lincoln, NE, USA). Image acquisition, processing, and data analysis were performed with Image Studio 5.2 (LI-COR Biosciences). β -actin was used to normalize the results. Data are expressed as mean $\pm$ standard deviation (SD). EECs displayed a higher amount of gC1qR compared to OVECs, considering both total protein and cell surface fraction; $*p < 0.05$. B, biotinylated; MW, molecular weights; NB, not biotinylated; PD, pull down. (E) Wound healing assay was performed using HUVECs ($n = 3$) after transfection with siC1QBP (gC1qR gene) or siCTRL for 48h. After scratching the middle of endothelial monolayer, cells were stimulated with C1q (10 μ g/mL) or VEGF (20 ng/mL). Images of the wound fields were captured at 0 and 8h, percentage wound closure was calculated. The percentage of wound healing was considered as relative to siCTRL in resting conditions (100% of wound healing). Data are expressed as mean $\pm$ SD; $*p < 0.05$.

C1q/TNF superfamily) (18) and their close involvement in the regulation of sex hormone synthesis (50), it is possible that C1q may play a role in ovarian steroidogenesis as well as follicle maturation. Furthermore, Sang Wook Yoo and colleagues reported the presence of a range of complement components (i.e., C1qA, C1qB, C1qC, C1r, C1s, C2, C3, C4BP, C4A, C4B, C7, C8 α , C8 β , C9, Factor D, Factor H, FH-1, FH-5, Factor I, properdin, and MASP-1) in human follicular fluids, whereas C5 and C6 were notably absent (51). This evidence suggests that C1q could play a role in granulosa cell function during the follicular process and in oocyte maturation.

We tried to determine the local cellular origin of C1q in the EM lesions. As expected, double IF results highlighted a high grade of co-labeling with macrophages (CD68⁺ cells), but also unexpectedly revealed that ECs in their early stage of maturation (CD34⁺ cells) were able to synthesize C1q. Of note, since EM microenvironment is characterized by inflammation, leukocyte infiltration, and activation of coagulation, we cannot totally exclude a partial deposition of C1q derived from the blood circulation as well, in addition to local synthesis. Plasma is a possible source of C1q, interacting with the membrane of ECs. However, we think that the involvement of circulating C1q could be marginal because binding of plasma C1q to target cells usually leads to complement activation through the classical pathway, which apparently does not occur in EM, as evident from the low level of C4d deposition.

Accumulating evidence support the involvement of C1q in several physiological and pathological processes related to angiogenesis (16, 21, 23). Studies have highlighted the proangiogenic role of C1q in wound healing process (21, 52). Notably, C1q is localized in granulation tissue and, independently from complement activation, can stimulate permeability, proliferation, and migration of ECs. Additionally, C1q is involved in the angiogenic process during the post-stroke ischemia recovery (53). Thus, C1q could function as a proangiogenic factor, offering novel therapeutic possibilities (19).

Considering the previously established heterogeneity in ECs behavior across different tissue districts (25), we isolated primary ECs from endometriotic cysts in patient (EECs), and compared their response to C1q stimulation together with HUVECs and OVECs, the ECs isolated from healthy ovary. C1q promoted EC proliferation, migration, and tube formation.

Hayuningtyas et al. demonstrated that the collagen-like region of C1q induced the phosphorylation of DDR2 (Discoidin Domain Receptor 2), p38 kinase, and ERK (Extracellularly Regulated Kinase) 1/2 in a fibroblast cell line. Through the binding to DDR2, C1q enhanced fibroblast migration; DDR2-specific siRNA reduced C1q-mediated cell migration for wound healing (52). On the contrary, Bossi and colleagues showed that the main contribution to EC activation is attributable to the globular (gC1q) domain of C1q (21). In addition to achieving the same pro-angiogenic effects using only the globular portion of C1q, they demonstrated the neutralizing effect of a specific antibody against gC1qR, whereas an antibody against cC1qR (a putative receptor for the collagen region of C1q i.e. calreticulin) did not bring about a statistically significant inhibition. Therefore, we investigated the role of gC1qR in mediating the effect induced by C1q in the current

study. We also identified gC1qR as a putative receptor involved in C1q-mediated angiogenic processes, being present on the cell surface of both EECs and OVECs. Interestingly, antibody neutralization of cell-surface gC1qR has been shown to inhibit angiogenesis, preventing cell migration and tube formation in HUVECs (54). Silencing of gC1qR gene via RNA interference could impair the ability of C1q to promote EC migration. The expression of cell-surface gC1qR at comparable levels may also justify the slight differences in terms of C1q binding or angiogenic response between EECs and OVECs. Moreover, the presence of C1q in the healthy ovary is likely attributable to its inherent proangiogenic role, which is a fundamental process in follicle maturation (55).

Conclusions

C1q is abundantly present within endometriotic lesions and is expressed in normal ovary tissue as well, suggesting its potential role in physiological ovary angiogenesis. C1q in EM lesion microenvironment is expressed mainly by resident macrophages, although the ability to express C1q by ECs in an early phase of their differentiation still remains to be clarified. Our data demonstrate that C1q promotes angiogenesis of endometriotic and ovary ECs. C1q, supporting angiogenesis, appears as a central factor for the maintenance and growth of EM engraftment. Thus, this study opens up a new opportunity for identifying novel therapeutic targets for treating EM, possibly targeting C1q-gC1qR interaction that mediates angiogenesis in EM lesions.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human subjects were approved by Regional Ethical Committee of FVG (CEUR), Udine, Italy (Prot. 0010143/P/GEN/ARCS 2019). The studies were conducted in accordance with the local legislation and institutional requirements. The patients provided their written informed consent to participate in this study.

Author contributions

CA: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. MT: Data curation, Methodology, Software, Writing – original draft. GZ: Investigation, Methodology, Software, Writing – review & editing. AB: Data curation, Formal analysis, Investigation, Methodology,

Software, Writing – original draft. SP: Methodology, Software, Validation, Writing – review & editing. MS: Methodology, Software, Writing – review & editing. LP: Investigation, Resources, Software, Validation, Writing – review & editing. FR: Methodology, Project administration, Software, Writing – review & editing. GL: Data curation, Software, Validation, Writing – review & editing. AM: Data curation, Formal analysis, Software, Writing – review & editing. AS: Data curation, Methodology, Validation, Writing – review & editing. BS: Data curation, Methodology, Software, Visualization, Writing – review & editing. EV: Data curation, Methodology, Resources, Writing – review & editing. GG: Data curation, Investigation, Methodology, Resources, Writing – review & editing. GR: Project administration, Resources, Supervision, Writing – review & editing. UK: Conceptualization, Formal analysis, Project administration, Resources, Writing – review & editing. RB: Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1405597/full#supplementary-material>.

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Role of factor H-related protein 3 in *Pseudomonas aeruginosa* bloodstream infections

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Pseudomonas aeruginosa is a leading cause of nosocomial bloodstream infections. The outcome of these infections depends on the virulence of the microorganism as well as host-related conditions and factors. The complement system plays a crucial role in defense against bloodstream infections. *P. aeruginosa* counteracts complement attack by recruiting Factor H (FH) that inhibits complement amplification on the bacterial surface. Factor H-related proteins (FHRs) are a group of plasma proteins evolutionarily related to FH that have been postulated to interfere this bacterial evasion mechanism. In this study, we demonstrate that FHR-3 competes with purified FH for binding to *P. aeruginosa* and identify EF-Tu as a common bacterial target for both complement regulator factors. Importantly, elevated levels of FHR-3 in human serum promote complement activation, leading to increased opsonization and killing of *P. aeruginosa*. Conversely, physiological concentrations of FHR-3 have no significant effect. Our findings suggest that FHR-3 may serve as a protective host factor against *P. aeruginosa* infections.

KEYWORDS

FHR-3, factor H, complement system, *P. aeruginosa*, bloodstream infection

Introduction

Pseudomonas aeruginosa is one of the most common Gram-negative organisms causing nosocomial bloodstream infections with a high-rate mortality ranging from 30% to about 50% (1–4). The reasons for the poor outcome of these infections are multifactorial and include the frequent emergence of multidrug-resistant strains, the intrinsic virulence of the pathogen, and host related-factors (5, 6). A remarkable feature of *P. aeruginosa*

bloodstream infections is their rapid progression. Many deaths occur shortly after infection (3, 4), suggesting that an altered host's early innate immune system response predisposes to this type of infection.

The complement system is a crucial early innate immune effector and the main defensive mechanism against *P. aeruginosa* in blood (7). Complement is activated by three different pathways: classical, lectin and alternative pathway. The result of the activation of any of the three pathways is the formation of C3 convertases which cleave C3 to generate C3b molecules that are deposited on the bacterial surface. Surface-bound C3b organizes additional C3 convertases, which amplify complement activation and opsonization of the bacteria. Clustering of C3b molecules around the surface-bound C3-convertase generates the C5 convertases that cleave C5 into C5a and C5b. C5b initiates the formation of the membrane attack complex that may lead to bacterial lysis, while C5a facilitates the recruitment of leukocytes to the site of the infection. C3b in the bacterial surface is inactivated to iC3b and C3dg. iC3b is a major ligand for the complement receptors CR3 and CR4 that mediate the phagocytosis of the microorganism by macrophages and other phagocytic leukocytes.

Complement activation is strictly controlled by regulatory proteins that maintain the homeostasis of the system and prevent damage to self-tissues. Factor H (FH), a 155-kDa circulating plasma glycoprotein that consists of 20 short consensus repeats (SCR) domains, is the master complement regulator (8). FH binds to C3b by its N- and C- terminal domains, both in the fluid phase and deposited on surfaces, and acts as a cofactor for Factor I to proteolyze C3b into inactive C3b (iC3b) or accelerates the decay of the C3 convertase (9). Not surprisingly, binding FH from the host plasma is a common strategy exploited by many bacterial pathogens to elude the complement system attack (10, 11).

In humans, the *CFH* gene is located in chromosome 1 adjacent to the genes encoding the complement FH-related proteins 1 to 5 (*CFHR-1-5*). Although the FHRs proteins have evolved from gene duplications of the *CFH* gene, they lack the FH regulatory domains and cannot regulate the complement system. In contrast, the FHRs have conserved the domains that FH uses to bind ligands in host cells and pathogens and therefore can compete with FH for binding to these ligands (11). Ligand competition between FH and the FHRs is referred to as “FH-deregulation” and has been postulated as a mechanism to interfere the complement evasion mechanisms mediated by the incorporation of FH to the bacterial surface (9). However, whilst the biological role of FH binding to *P. aeruginosa* has been well determined (12), the biological consequences of the interaction of the FHRs with the bacterial surface are still poorly understood.

In this study, we focused on FHR-3 because it shows the highest degree of amino acid identity to the microbial binding domain present in the SCR6 and 7 of FH among FHRs proteins (Supplementary Figure 1). FHR3 is a plasma protein, which is expressed in four glycosylated forms with molecular masses ranging from 45 to 56 kDa (8). Initial estimations indicated that plasma concentration of FHR-3 was 50–100 µg/ml (13, 14). However, more recent studies using specific FHR-3 monoclonal antibodies have

demonstrated that the concentration of FHR-3 in human plasma is remarkably as low as 0.14–4 µg/ml (15, 16).

In this manuscript, we investigated the effect of FHR-3 on the complement activation induced by *P. aeruginosa* and whether this FH related protein influences host defense against *P. aeruginosa* infections.

Materials and methods

Bacterial strains

PAO1 and its isogenic *wzz2*-deficient mutant (PAO1Δ*wzz2*) were previously described (17). *wzz2* gene encodes a lipopolysaccharide (LPS) O antigen chain length regulator. Interruption of *wzz2* gene results in production of LPS devoid of the very long O antigen, conferring a serum-sensitive phenotype to the strain.

Two *P. aeruginosa* clinical strains, B205 and B75, collected from patients with bloodstream infection were also used in this study. Bacterial cells were grown in Luria Bertani (LB) broth at 37°C with shaking or in LB solidified with 1.5% agar.

Human serum and complement proteins

Blood samples were collected from two donors with an FHR-1/FHR-3 deficiency. Human samples were taken after informed consent of each participant. The study was aligned with the Helsinki Declaration and was approved by the Institutional Review Board and the Regional Ethics Committee. Blood was coagulated at 37°C for 30 min and then centrifuged at 4,500 x g for 20 min at 4°C to separate the serum. Equal volumes of serum from the two donors with FHR-1/FHR-3 deficiency were mixed to get a pool of FHR-3-deficient human sera (ΔFHR-3). Human serum was aliquoted and stored at –80°C until its use. In some experiments, sera were heat inactivated (HI) at 56°C for 30 min prior to use.

Factor H and recombinant human FHR-3 proteins were supplied by HycultBiotech and Elabscience, respectively.

Purification of EF-Tu

P. aeruginosa EF-Tu was purified from strain PAO1 harboring the plasmid pUCP18ApGw(*tufB*) which encodes His-tagged EF-Tu (18) using nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Briefly, bacterial cells were suspended in lysis buffer consisting of 50 mM sodium phosphate (pH 8), 300 mM NaCl, 10 mM imidazole, and lysozyme (1 mg/ml) (Sigma- Aldrich, St. Louis, MO). The cells were disrupted by sonication (10 sets of 30-s pulses), and the resulting homogenate was centrifuged at 12,000 X g to remove cellular debris. Ni-NTA (5 ml in a 50% slurry) was added to the resulting supernatant, and the suspension was swirled at 4°C at 100 rpm for 1 h on a rotary shaker. The Ni-NTA resin was washed

three times with 20 ml of wash buffer consisting of 50 mM sodium phosphate buffer (pH 8.0), 300 mM NaCl, and 20 mM imidazole. The His-tagged protein was eluted with 1.5 ml of 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 250 mM imidazole. Purity (>97%) was confirmed by SDS-PAGE analysis.

FH and FHR-3 binding assays

Binding of FH to bacterial cells was determined by whole-cell ELISA. Briefly, 96-well round-bottom polystyrene microtiter plates were coated overnight at 37°C with 1×10^8 bacterial cells resuspended in phosphate-buffered saline (PBS). Next, wells were blocked with PBS containing 1% bovine serum albumin (PBS-BSA) and incubated with PBS as negative control, FH (20 µg/ml), recombinant human FHR-3 protein at different concentrations depending on the experiment or FHR-3-deficient human sera (10%) diluted in PBS. FH was detected with the specific mouse mAb antibody OX-24 (Abcam). Finally, wells were incubated with an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Sigma), and developed with p-nitrophenyl phosphate (Sigma) in 50mM carbonate-bicarbonate buffer, pH 9.6, 5mM MgCl₂. Absorbance was measured at 415 nm. Negative controls values were < 0.1 optical density units and were subtracted from the experimental values. Washing steps with PBS were included between incubations that were performed for 1 h at 37°C.

Binding of FHR-3 to EF-Tu was also determined by ELISA using the protocol described above using microtiter plate wells that were coated overnight at 4°C with 1 µg of purified EF-Tu dissolved in 100 µl of PBS. FHR-3 was detected with the mouse mAb antibody MBI-6 (19).

To identify the binding region of FHR-3 and FH in EF-Tu, we generated a library of 25 synthetic N-terminal biotinylated 20-mer overlapping peptides spanning the entire *P. aeruginosa* EF-Tu molecule. Peptides dissolved in PBS-Tween 0.05% at 10 µg/ml were captured on streptavidin-coated microtiter plates overnight at 25°C and incubated for 1 h at 37°C with recombinant human FHR-3 (2 µg/ml) or purified human FH (10 µg/ml) diluted in PBS. After three washing steps, FHR-3 and FH were detected using the specific mouse mAb antibodies MBI-6 or OX-24, respectively. Primary antibodies were detected as described above.

C3 deposition assays

Deposition of C3 on the bacterial cells was also determined by whole-cell ELISA. Microtiter plates were coated and blocked as described above and incubated for 15 min at 37°C with FHR-3-deficient human sera (25%) diluted in PBS supplemented with FHR-3 (2 µg/ml or 100 µg/ml of serum) or not. HI-serum was used as control. Next, microtiter plate wells were incubated sequentially with a mouse mAb anti-human C3 that recognizes an epitope in the β-chain (C3-12.17) (20), an alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (Sigma), and developed with p-nitrophenyl phosphate (Sigma) in 50 mM carbonate-bicarbonate buffer (pH 9.6) plus 5 mM MgCl₂.

C3a and C5a quantification

Bacterial cells (1×10^9 CFU) of *P. aeruginosa* were incubated for 15 min at 37°C in FHR-3-deficient human sera (25%) diluted in PBS supplemented with FHR-3 (500 ng/ml or at 25 µg/ml) or not. FHR-3-deficient human sera incubated with FHR-3 without bacteria was used as negative control. After incubation, bacterial suspension was centrifuged and the amount of C3a and C5a present in the supernatant was quantified using the Human C3a/Complement C3a ELISA (Invitrogen) or the Human C5a/Complement C5a ELISA Kit (Invitrogen) following the manufacturer's instructions.

Serum resistance assays

Serum resistance assays were performed as previously described (21, 22). Briefly, a total of 1×10^6 CFU of *P. aeruginosa* grown exponentially at 37°C in LB were incubated at 37°C in FHR-3-deficient human sera diluted in PBS supplemented or not with recombinant FHR-3. Optimal serum concentrations were determined empirically for each strain in the current setting. HI-FHR-3-deficient human sera was used as control. At different time points, survival was determined by counting colonies on LB agar plates.

Statistical analysis

Comparisons among experimental groups were assessed using one-way ANOVA with multiple comparisons. All results are reported as the mean with standard deviation. Differences were considered statistically significant at $P < 0.05$.

Results

FHR-3 competes with FH for the binding to *P. aeruginosa*

The competition for ligands between FH and the FHRs is termed “FH-deregulation” and has been proposed as a mechanism to disrupt the complement evasion strategies facilitated by the binding of FH to the bacterial surface (9). We evaluated the impact of FHR-3 on the binding of FH to *P. aeruginosa* using purified proteins. Bacterial cells were incubated with FH, and decreasing amounts of recombinant FHR-3, and the binding of FH to the bacterial surface was determined using a FH specific monoclonal antibody. FHR-3 reduced the amount of FH bound to *P. aeruginosa* PAO1 in a dose-dependent manner (Figure 1A). When the amount of recombinant FHR-3 and FH were at approximately equimolar ratios to those determined in normal human serum by Pouw et al. (15, 16) (FH: FHR-3 of 1:0.0066), FHR-3 had no effect on the binding of FH to the bacteria (Figure 1B). By contrast, at FH: FHR-3 ratios to those estimated in normal human serum by Fritsche et al. (13) (FH: FHR-3 of 1:0.27), FH bound on the bacterial surface of PAO1 was

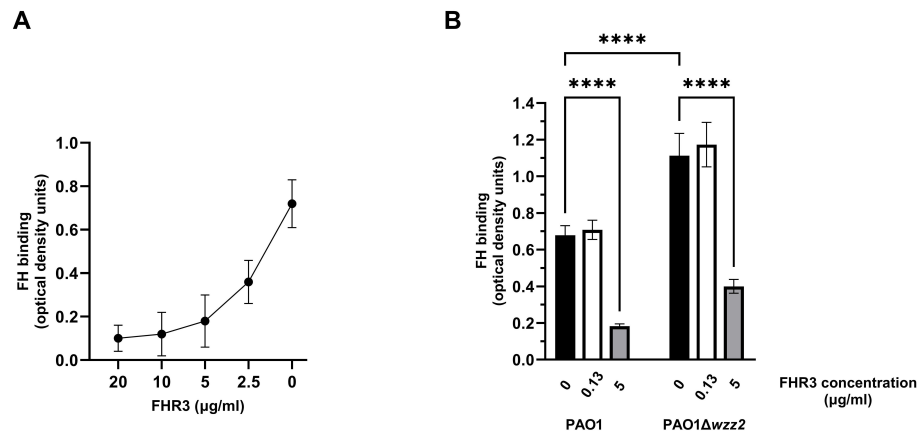


FIGURE 1

FHR-3 competes with FH for the binding to *P. aeruginosa*. ELISA binding assays of purified FH to the reference strain PAO1 and the serum-sensitive isogenic LPS-deficient strain PAO1Δwzz2 in the presence of FHR-3. In (A) the strain PAO1 was incubated with purified FH (20 μg/ml) in the presence of decreasing amounts of recombinant FHR-3 (x-axis on a logarithmic scale). In (B) the strains were incubated with purified FH (20 μg/ml) in the absence (black columns) or presence of FHR-3 at 0.13 μg/ml (white columns) or at 5 μg/ml (grey columns). Bound FH was detected with the mAb OX-24. Bars represent the mean of at least three independent experiments done in duplicate, and SD is indicated by error bars. Statistical analyses were performed using one-way ANOVA with multiple comparisons; *P* values are indicated on the bars with asterisks. *****P* < 0.0001.

approximately 30% that seen in the absence of FHR-3 (Figure 1B). A similar pattern was observed when we determined the impact of FHR-3 on the binding of FH to the serum-sensitive strain PAO1Δwzz2 (Figure 1B). These results demonstrate antagonism between FH and FHR-3 for binding to the bacterial surface and suggest that FH and FHR-3 share a common binding site on *P. aeruginosa*.

It is known that EF-Tu is a surface-exposed protein that mediates the binding of FH to *P. aeruginosa* (12). FH has two binding domains for EF-Tu; one is located in the SCRs 6-7 and the other in the SCRs 18-20 (12). Given the high homology between FH SCRs 6-7 and FHR-3 SCRs 1-2 (Supplementary Figure 1), we hypothesized that EF-Tu might be an FHR-3-binding protein of *P. aeruginosa*. To test this hypothesis, we purified recombinant *P. aeruginosa* EF-Tu and conducted FHR-3 binding experiments. ELISA assays of recombinant EF-Tu incubated with recombinant human FHR-3 demonstrated that EF-Tu binds FHR-3 (Figure 2A). Furthermore, this binding was dose-dependent, as shown for the different concentrations of FHR-3.

To localize the binding region of FHR-3 and FH in EF-Tu, we used a library of synthetic N-terminal biotinylated peptides spanning the entire *P. aeruginosa* EF-Tu molecule (Figure 2B). Peptide mapping was performed using recombinant FHR-3, or purified human FH. Quantitative ELISA analysis revealed that the highest reactivity of both proteins was against peptide ID 6 (Figure 2C). FHR-3 reacted almost exclusively with peptide ID 6, while FH was also bound to peptide ID 23.

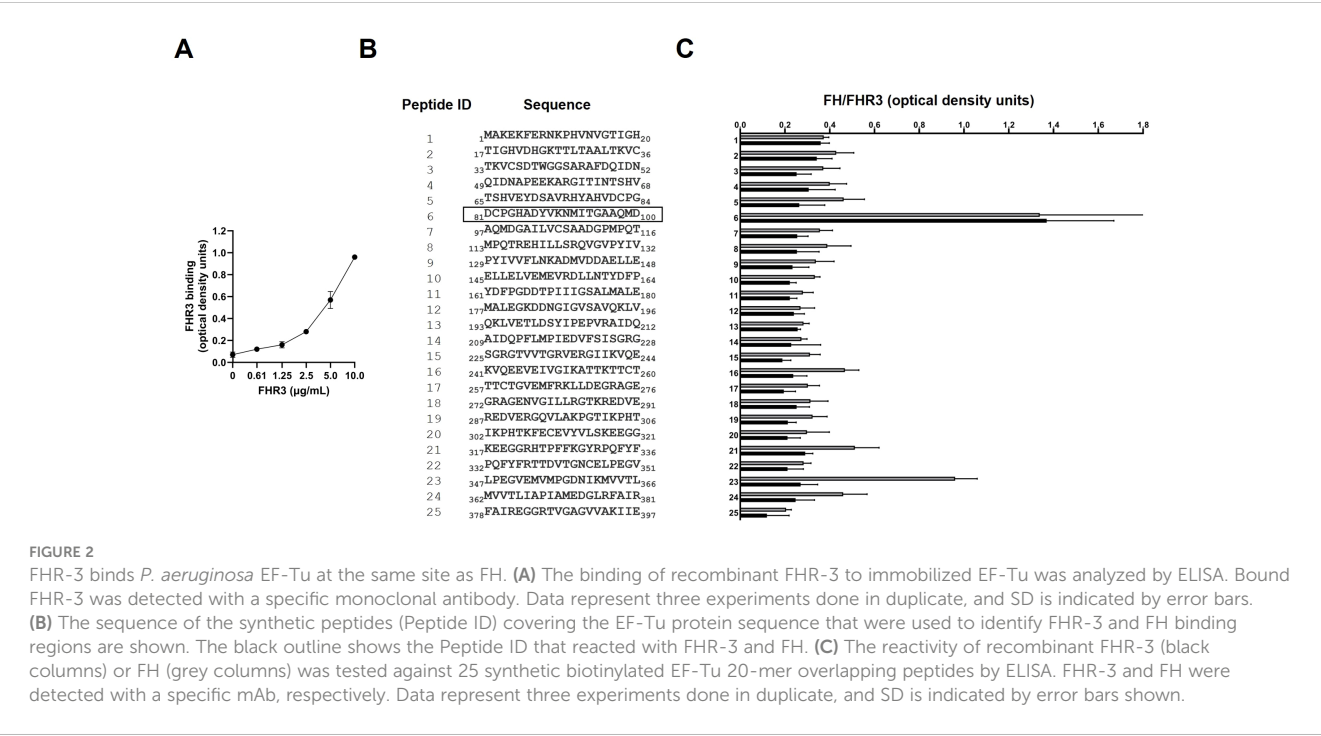
FHR-3 increases the binding of FH to *P. aeruginosa* in human serum

We next assessed the influence of FHR-3 on the binding of FH to *P. aeruginosa* using FHR-3-deficient human sera (ΔFHR-3) at

10% supplemented with FHR-3 at 100 μg/ml or 2 μg/ml of serum, according to Fritsche et al. (13) or Pouw et al. (15), respectively. Conversely to the results obtained with purified FH, *P. aeruginosa* incubated with human ΔFHR-3, supplemented with FHR-3 at 100 μg/ml of serum, acquired higher levels of FH than those without exogenous FHR-3 (Figure 3A). Furthermore, the effect of FHR-3 on FH binding to *P. aeruginosa* was more remarkable on the serum-sensitive strain PAO1Δwzz2 than on the serum-resistant strain PAO1, with increment rates of approximately 100% and 20%, respectively. FHR-3 at 2 μg/ml of serum did not affect the binding of FH to *P. aeruginosa*. Interestingly, FHR-3 had no impact on the binding of FH to the bacteria when human serum was heat-inactivated (HI-ΔFHR-3) (Figure 3B), suggesting that the influence of FHR-3 on the binding of FH to the bacteria requires a functional complement system and may be attributed to the formation of C3b, the natural ligand of both proteins. These results lead us to hypothesize that FHR-3 bound to C3b facilitates the assembly of C3 convertase on *P. aeruginosa*, resulting in a much higher deposition of C3b that recruits FH rather than competing with FH for the binding to C3b.

To test this hypothesis, we determined the effect of FHR-3 on the deposition of C3 on the bacteria (Figure 4). FHR-3 deficient serum (25%) supplemented with FHR-3 at 100 μg/ml of serum increased the amount of C3 fragments deposited on the serum-sensitive strain PAO1Δwzz2, but had no effect on the serum-resistant strain PAO1 (Figure 4). Low concentrations of FHR-3 (2 μg/ml of serum) had no effect on the deposition of C3 fragments on *P. aeruginosa*.

We also quantified the amount of generated C3a to directly assess the FHR-3-induced C3 convertase activity (Figure 5A). The addition of FHR-3 at 100 μg/ml of serum to the FHR-3 deficient serum increased the production of C3a induced by the *P. aeruginosa* independently of the strain tested. As anticipated, the serum lacking FHR-3 and supplemented with FHR-3 at 2 μg/ml of serum showed a production of C3a comparable to that observed without supplementation.

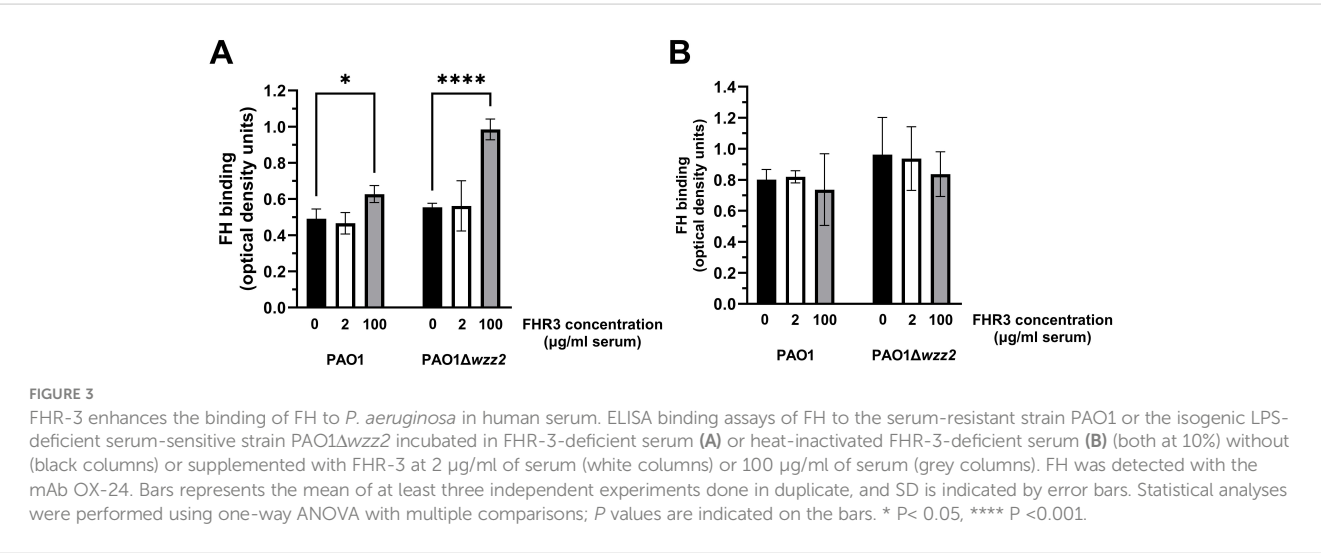


To further confirm that FHR-3 enhanced complement activation, we went one step further. We evaluated the formation of C5a in the human sera incubated with the bacteria in the presence or not of exogenous FHR-3 (Figure 5B). Consistent with the previous results, both strains produced higher levels of C5a when incubated in sera supplemented with FHR-3 at 100 µg/ml demonstrating that, at this concentration, this protein promotes the *P. aeruginosa*-induced activation of the complement system. However, low concentrations of FHR-3 (at 2 µg/ml of serum) had no effect on the production of C5a.

Overall, these results indicate that FHR-3, at the serum concentrations estimated by Fritsche et al. (13), enhances the activation and deposition of C3 on *P. aeruginosa*. In contrast, we did not observe any effect at the serum concentrations reported by Pouw et al. (15).

FHR-3 promotes the killing of *P. aeruginosa*

Cleavage of C5 into C5a and C5b initiates the formation of the membrane attack complex that may result in the lysis of the microorganism. To investigate whether FHR-3-enhanced complement activation impacts on bacterial survival, we evaluated the viability of *P. aeruginosa* incubated in strain-optimized concentrations of ΔFHR-3 serum supplemented or not with recombinant FHR-3 (Figure 6). The addition of FHR-3 at any concentration did not impair the survival rate of the highly serum-resistant strain PAO1, which was able to grow in the human sera at 45%. On the other hand, the serum-sensitive strain PAO1Δwzz2 was efficiently cleared by the human sera (2.5%). Interestingly, the



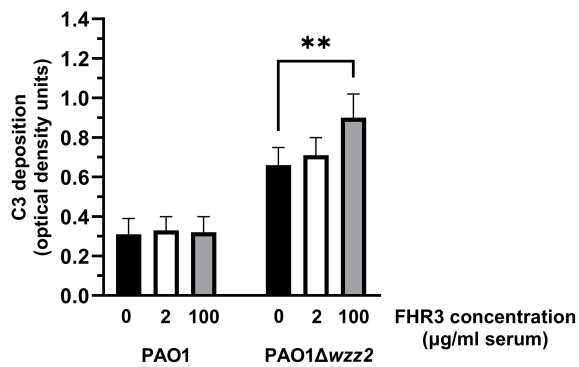


FIGURE 4

FHR-3 increases the deposition of C3 on *P. aeruginosa*. FHR-3 deficient serum (25%) without (black columns) or supplemented with FHR-3 at 2 μg/ml of serum (white columns) or at 100 μg/ml of serum (grey columns) were incubated for 15 min at 37°C with PAO1 or the isogenic serum-sensitive strain PAO1Δwzz2. Deposition of C3 on the bacterial surface was determined by ELISA. Bars represent the mean of at least three independent experiments done in duplicate, and SD is indicated by error bars. Statistical analyses were performed using one-way ANOVA with multiple comparisons; *P* values are indicated on the bars. ** *P* < 0.01.

addition of FHR-3 at (100 μg/ml of serum) slightly increased the killing of this strain, but the addition of a low concentration of FHR-3 (2 μg/ml) did not enhance the clearance of this strain. This observation was further confirmed with two additional *P. aeruginosa* clinical strains isolated from bloodstream infections, B75 and B205. FHR-3 deficient human sera killed *P. aeruginosa* B75 and B205 more efficiently with the addition of FHR-3 at the concentration estimated by Fritsche et al. (13) (100 μg/ml of serum), than without it. The presence of FHR3 at the concentration reported by Pouw et al. (15) (2 μg/ml of serum) did not result in improved clearance of these isolates.

Discussion

In this work, we have characterized a previously unrecognized interaction between the FHR-3, a member of the FH/FHRs protein family, and *P. aeruginosa*. It has been already reported that this microorganism binds different members of the FH protein family, including FH, FHL-1, and FHR-1 (12, 23, 24). *P. aeruginosa* utilizes FH and FHL-1 to inactivate C3b and to prevent formation of the C3 convertase to evade the host complement system (12, 23). However, the biological significance of the binding of the FHR proteins remained poorly investigated.

It has been suggested that FHRs proteins act as decoys molecules that displace FH from the surface of the pathogen due to their overlapping ligand spectrum with this complement inhibitor (9). Our competition studies using purified FH and FHR-3 support this idea. The most likely explanation for this result is that, given the high homology between FH SCRs 6-7 and FHR-3 SCRs 1-2 (Supplementary Figure 1), they share the same bacterial target. In this regard, it is noteworthy that most FH-binding microbial proteins also bind FHR proteins through those FH domains that are conserved among the FHR proteins (9, 25). *P. aeruginosa* is not an exception, therefore it is logical that the FH binding protein EF-Tu is also the ligand of FHR-3. A linear EF-Tu binding motif for both FH and FHR-3 was identified within domain 1 of this protein suggesting that this region of EF-Tu is surface exposed and thus accessible to ligand binding. In fact, comparisons of *P. aeruginosa* EF-Tu structure to EF-Tu structures from other microorganisms and three-dimensional modeling indicate that FH and FHR-3 binding region identified in this study is located on the surface exposed portion of the protein in the domain 1 distant from the typical consensus sequence of the GTP/GDP binding domain (26). Since EF-Tu is a well-conserved protein which is surface exposed in many different microorganisms, it is reasonable to hypothesize that this region may be a common FH microbial

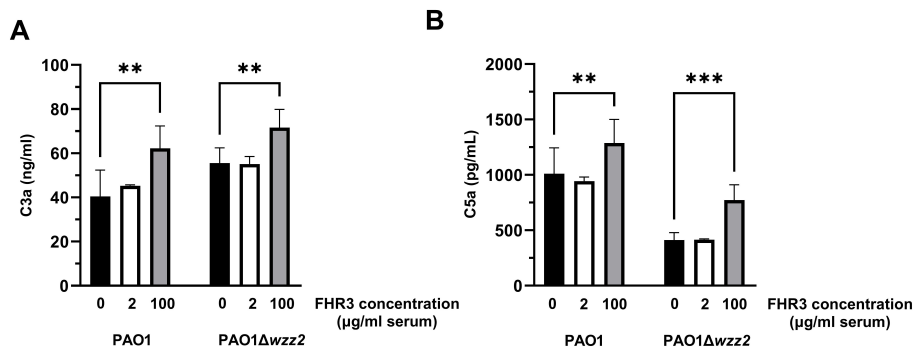


FIGURE 5

P. aeruginosa-induced generation of C3a and C5a is enhanced by FHR-3. FHR-3 deficient serum (25%) without (black columns) or supplemented with FHR-3 at 2 μg/ml of serum (white columns) or at 100 μg/ml of serum (grey columns) were incubated for 15 min at 37°C with the serum-resistant strain PAO1 or the isogenic serum-sensitive strain PAO1Δwzz2. Levels of C3a (A) and C5a (B) were determined by ELISA. Bars represent the mean of at least three independent experiments done in duplicate, and SD is indicated by error bars. Statistical analyses were performed using one-way ANOVA with multiple comparisons; *P* values are indicated on the bars. ** *P* < 0.01, *** *P* < 0.001.

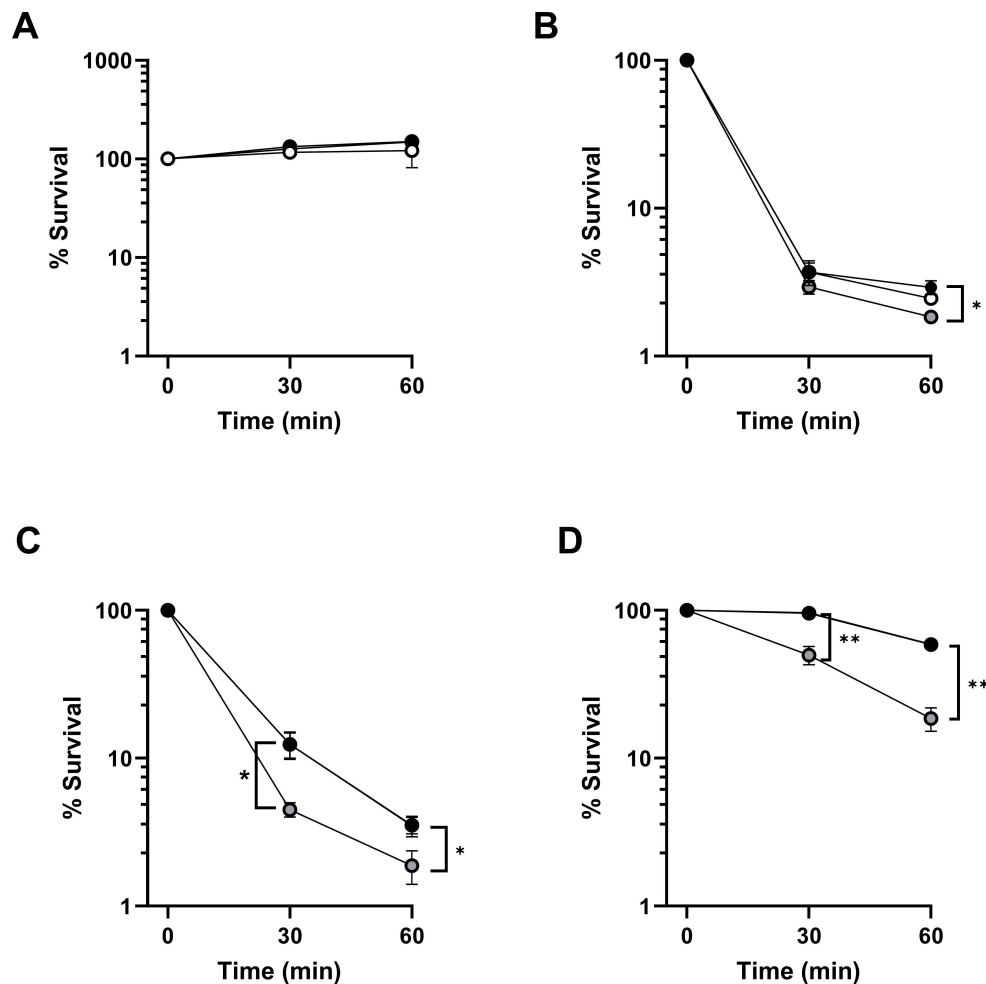


FIGURE 6

FHR-3 increases *P. aeruginosa* complement-mediated killing. Survival of strains PAO1 (A), PAO1Δwzz2 (B), and the clinical isolates from bloodstream infections B75 (C) and B205 (D) at different time points after incubation in strain-optimized concentrations of FHR-3 deficient serum (PAO1, 45%; PAO1Δwzz2, 2.5%; B75, 2.5% and B205, 45%) not supplemented with FHR-3 (black circles) or supplemented with FHR-3 at 2 μg/ml of serum (white circles), or 100 μg/ml of serum (grey circles). Black and grey circles are overlaid in panel A, while black and white circles are overlaid in panels C and (D) Percentage survival at each time point was calculated in respect to the number of viable bacteria at time 0. Dots represent the mean of at least three independent experiments done in duplicate, and SD is indicated by error bars. Statistical analyses were performed using one-way ANOVA with multiple comparisons. *P* values; * *P* < 0.05, ** *P* < 0.01.

binding motif. Thus, the identification of the FH binding domain on EF-Tu may facilitate the generation of protective monoclonal antibodies to block the binding of FH to the pathogens avoiding complement inhibition.

When the competition experiments were performed using active human FHR-3-deficient sera supplemented with FHR-3, the results obtained were the opposite to those obtained with purified components. FHR-3 enhanced the binding of FH to the bacteria by a mechanism that is dependent of the complement activation, because in the experiments conducted with heat-inactivated serum, FHR-3 had no influence on the binding of FH to the bacteria. To explain this apparent contradiction, we postulate that surface-bound FHR-3 activates complement through the alternative pathway by promoting the formation of an active C3bBb convertase and the enhanced binding of FH to the bacteria was a consequence of the increased amount of C3b deposited on its surface, the natural ligand of FH.

The results of our experiments measuring the FHR-3-induced deposition of C3 and generation of C3a and C5a mediated by activation of the complement on *P. aeruginosa* supports this idea.

Our observations are in line with several lines of evidence supporting that the FHRs proteins promotes complement activation independently of FH (27–30). Further experiments will be required to characterize the underlying mechanisms that govern FHR-3-induced activation of the complement alternative pathway.

To our knowledge, only one study has provided direct evidence of the role of FHR-3 in bacterial infection. Caesar et al. demonstrated that FHR-3 competes with FH for binding to *Neisseria meningitidis* factor H binding protein (fHbp), promoting complement activation (31). Interestingly, these observations may explain the results of a genetic association study that linked a variant in the *CFHR-3* gene, associated with increased expression of the FHR-3 protein, with protection from *N. meningitidis* infection (32).

In our study, we show that FHR-3 enhances complement activation in the bacterial surface, which will result in increased amount of C3 deposition and killing of the bacteria. We believe that this effect of bacterial-bound FHR-3 increasing complement activation and opsonization may be a general strategy used by the innate immune system against different pathogens.

Our functional studies indicate that FHR-3 promotes the complement-mediated killing of *P. aeruginosa*. The effect of FHR-3 on the clearance of two bloodstream infection isolates was clear. However, FHR-3 had no impact on the viability of the highly serum-resistant strain PAO1 (7) or a poor effect on its isogenic highly susceptible mutant PAO1Δwzz2. This observation leads us to hypothesize that FHR-3 fine-tunes complement activation. Thus, its effect on bacterial clearance may rely on the specific phenotype of each isolate and the complement concentration at the site of infection. This observation is consistent with the high variable survival rates for different *P. aeruginosa* strains in blood (7).

It is noteworthy that the effect of FHR-3 on complement deposition and the killing of *P. aeruginosa* is only observed when we use levels of FHR-3 similar to those employed by Caesar et al. in their experiments with *N. meningitidis* (31). These levels are significantly higher than the physiological concentration of FHR-3 in human serum as subsequently reported by Pouw et al. (15). Indeed, in our experiments using FHR-3 concentrations within the range reported by Pouw et al., FHR-3 had no effect on complement activation on *P. aeruginosa*.

In light of these results, it is unlikely that FHR-3 levels in the blood significantly influence the outcome of *P. aeruginosa* infections. Nonetheless, our data does not rule out the possibility that the demonstrated effect of FHR-3 on complement deposition and killing of *P. aeruginosa* *in vitro* could occur *in vivo* in some infected tissues. Although a preliminary study suggests that FHR-3 does not function as an early major acute phase protein (15), other research has demonstrated that FHR-3 is more abundant in certain specific tissues when they are infected than in serum (33). Thus, *in vitro* studies are a limitation to investigate the effect of FHR-3 on bacterial infections and future studies should focus on the use of *P. aeruginosa* *Cfhr-3* knock-out mice model of infection to evaluate the role of this complement regulator factor *in vivo*.

Interestingly, *CFHR3* copy numbers impact on the FHR-3 serum levels and usually differ between individuals (15). Moreover, the deletion of *CFHR3* is a frequent polymorphism in humans that originated long time ago by a non-homologous recombination event. Its allelic frequency ranges from 0.5% in sub-Saharan populations to almost non-existence in Asiatic. In European populations it is around 2%, which translates into 4% homozygotes and 32% heterozygotes for the *CFHR3* deletion (15, 34). Future studies should be focused to know whether *CFHR3* deletion or low copy numbers of this gene is associated with an increased risk for the development of *P. aeruginosa* infections.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Institutional Review Board and the Regional Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AA-G: Investigation, Writing – original draft, Writing – review & editing, Data curation, Methodology. HM-M: Data curation, Investigation, Methodology, Writing – review & editing. MM-B: Investigation, Writing – review & editing. MV: Investigation, Writing – review & editing. AD-S: Investigation, Writing – review & editing, Formal analysis, Supervision. JG: Supervision, Writing – review & editing, Formal analysis. SR: Supervision, Writing – review & editing, Conceptualization, Data curation, Formal analysis. SA: Conceptualization, Data curation, Supervision, Writing – review & editing, Formal analysis, Funding acquisition, Investigation, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1449003/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Schematic representation of the human FH, FHL-1 and FHR-3 Functional sites in FH are indicated in brackets on FH. Vertical alignment and colors show Short

Consensus Repeats (SCR) homologous to SCRs 6-8 and 19-20 of FH. The degree of amino acid sequence identity (in percentage) between FH and FHR-3 is indicated below FHR-3 SCRs. FH-like protein 1 (FHL-1) is a splice variant of FH.

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Role of complement factor D in cardiovascular and metabolic diseases

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In the genesis and progression of cardiovascular and metabolic diseases (CVMDs), adipose tissue plays a pivotal and dual role. Complement factor D (CFD, also known as adipsin), which is mainly produced by adipocytes, is the rate-limiting enzyme of the alternative pathway. Abnormalities in CFD generation or function lead to aberrant immune responses and energy metabolism. A large number of studies have revealed that CFD is associated with CVMDs. Herein, we will review the current studies on the function and mechanism of CFD in CVMDs such as hypertension, coronary heart disease, ischemia/reperfusion injury, heart failure, arrhythmia, aortic aneurysm, obesity, insulin resistance, and diabetic cardiomyopathy.

KEYWORDS

CFD, adipsin, inflammation, cardiovascular disease, metabolic diseases

1 Introduction

Cardiovascular and metabolic diseases (CVMDs) constitute the principal causes of death globally. Cardiovascular diseases (CVDs) constitute more than two-thirds of the deaths associated with a high body mass index (BMI) (1). The pathogenesis of these diseases remains incompletely clarified. Although researchers have made great progress in the prevention and treatment of CVMDs, the morbidity, disability and mortality rates remain high.

The development and prognosis of CVMDs are closely related to adipose tissue. For each 5 kg/m² increase in BMI, the risks of CVDs will increase varied from 10% to 49% (2). Adipose tissue is an energy storage organ as well as an active and inflammatory organ capable of releasing adipokines (3). Adipokines modulate lipid metabolism, glucose metabolism, inflammation, and blood pressure. CFD is a serine protease that is produced mainly by adipose tissue and macrophages. CFD is an essential rate-limiting enzyme in complement alternative pathway (AP). As an adipokine, CFD regulates

metabolism by increasing adipocyte differentiation and lipid accumulation, protecting beta-cells, and promoting insulin secretion. As a complement factor, CFD amplifies complement cascade activation and protects cells from infection, while it induces low-grade inflammation (LGI) in CVDs.

Mounting evidence suggests that CFD plays a significant role in CVMDS by regulating immune system homeostasis and metabolic balance. CFD levels are implicated in the etiology of several CVMDS. In a large population-based cohort, participants with higher CFD have a greater adverse cardiovascular profiles (4). CFD is positively associated with CVDs incidence. For each 1 standard deviation increase in the plasma concentration of CFD, the incidence of CVDs is 15% greater (5). Increasing CFD levels are correlated with obesity, hypertension, LGI, and endothelial dysfunction (6). However, CFD often acts as a protective factor in diabetes and diabetic cardiomyopathy.

Given the controversial role of CFD in different contexts of cardiovascular system immunity and metabolism (Figure 1), summarizing the specific effects of CFD on CVMDS is crucial for further research. In this review, we summarize the progress of CFD in CVMDS in recent years.

2 Introduction of CFD

2.1 The structure and maturation of CFD

CFD is a differentiation-dependent serine protease with a molecular weight of 24.4 kDa. Two variants of immature human CFD exist: variant 1, which is composed of 251 amino acids with an 18-amino acid leader sequence, and variant 2, which is composed of 260 amino acids with a 25-amino acid leader sequence (7). Apart from the pro-peptide, the two variants have the same polypeptide chain and form the same mature pro-CFD. As an inactive zymogen, pro-CFD consists of 235 amino acids and matures after the cleavage of a 6-amino acid peptide. Activated human CFD consists of a single serine protease domain of 228 amino acids with a catalytic triad (7). This process seems to occur during or immediately after secretion (8). Over 99% of plasma CFD exists in the form of CFD instead of the pro-CFD form (9).

Human CFD is not glycosylated. In contrast, mouse CFD is highly glycosylated, possessing an molecular mass of approximately 42 and 45 kDa and being more stable in circulation (7, 10).

CFD circulates in a self-inhibited form and possesses relatively low proteolytic activity in blood (11). CFD has a narrow substrate specificity. CFD only binds to an open complement factor B (CFB) conformation in its Mg^{2+} -dependent complex with C3b, triggering reversible conformational changes in the catalytic center of CFD (12). After the cleavage of CFB, CFD returns to an inactive resting-state conformation in circulation (13).

2.2 The production and metabolism of CFD

CFD is primarily and constitutively secreted by adipocytes, is also produced by macrophages and monocytes, and is synthesized by a small number of brain astrocytes (14). Among all the complement components, CFD has the lowest level in blood, ranging from 1 to 2 $\mu\text{g}/\text{mL}$, with no difference between healthy men and women (15). The CFD level in heart tissue depends on its serum level, whereas heart tissue expresses CFD mRNA at relatively low levels. Encircling the heart and blood vessels, epicardial adipose tissue (EAT) and pericardial adipose tissue (PVAT) synthesize CFD and deliver it to the heart via exosomes (16, 17). CFD is filtered through the glomerulus and then reabsorbed and decomposed by the renal tubules (7). It is estimated that the fractional catabolic rate of plasma CFD is 60% per hour, resulting in a lower serum concentration under nonpathological conditions (18). However, serum CFD levels can be influenced by renal dysfunction (19). CFD plasma levels increase approximately tenfold in patients with end-stage renal failure (20). However, CFD levels increase in the urine and decrease in the plasma of patients with Fanconi syndrome because of selective impairments in the renal tubular epithelium (21).

2.3 The physiological role of CFD

The role of CFD in protecting cells from infection has been well recognized. CFD deficiency causes insufficient terminal C5b-9

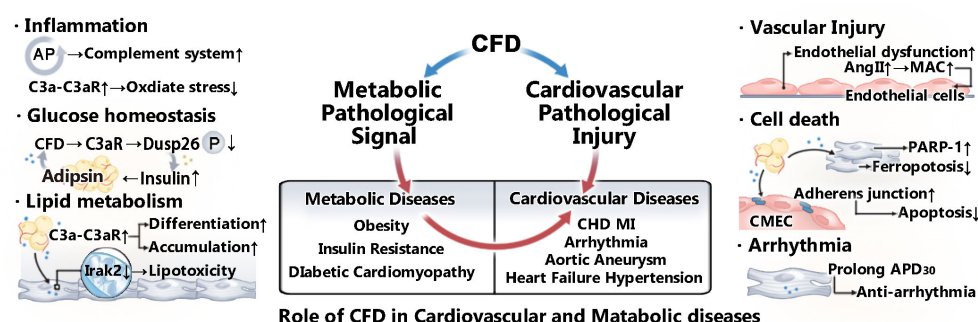


FIGURE 1

Role of CFD in CVMDS. CFD plays a role in various cardiovascular and metabolic diseases by regulating metabolic pathological signals and mediating cardiovascular pathological injury. CFD is closely related to the activation of the complement system. In addition, as an adipokine, it plays a vital role in lipid metabolism and glucose homeostasis. Moreover, studies have shown that CFD is associated with vascular injury, apoptosis, and arrhythmia.

complement complex (TCC) generation, which ultimately increases the risk of meningococcal infection approximately 6000-fold (22). In families with CFD deficiency, family members show low AP activity and have meningitis and pneumonia (22, 23). CFD is also involved in other physiological processes. In previous studies, evidence indicating a central role of CFD in energy homeostasis and systemic metabolism has been put forward. CFD promotes the differentiation of adipocytes and the accumulation of lipids by activating the C3a-C3aR pathways in adipocytes and preadipocytes (24–26). In addition, CFD promotes the generation of acylation-stimulating protein (ASP) by catalyzing the conversion of C3 to C3a. As a C3 cleavage product of exopeptidase activity, ASP regulates lipid storage by increasing the activity of diacylglycerol O-acyltransferase 2 (27). CFD, which is upregulated by proliferator-activated receptor gamma (PPAR γ) acetylation, inhibits Wnt/ β -catenin signaling and thus primes bone marrow mesenchymal stem cell adipogenic differentiation in mice (28). As a diurnal factor, CFD plays an adipose-autonomous role in metabolic rhythms. In mouse adipose tissue, the expression of CFD exhibits a rhythm pattern, with a peak at zeitgeber time (zt) 12 and a trough at zt0 (29). No fluctuations in CFD are observed in the plasma (29). PPAR γ

acetylation upregulates CFD transcription, which destabilizes BMAL1 and mediates metabolic rhythms (29). CFD also plays a role in glucose metabolism. Several studies have indicated that CFD promotes insulin secretion and sustains β -cell function via the C3a-C3aR1 pathway (30).

2.4 The pathophysiological role of CFD

CFD contributes to paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), geographic atrophy (GA), and various diseases (Table 1). Patients afflicted with PNH and aHUS exhibit excessive activation of the AP. CFD inhibitors decrease C3 fragment deposition on PNH erythrocytes and abate complement-mediated hemolysis (31). Complement dysregulation is a key component of the pathogenesis of systemic lupus erythematosus (SLE). MBL-associated serine protease (MASP)3 is likely a direct activator of pro-CFD in resting human blood, cleaving inactive pro-CFD into active CFD (32). Compared with healthy individuals, SLE patients exhibit increased MASP-1 and MASP-3 levels. In addition, CFD, CFB and MASP1/3 are deposited along the glomerulus in

TABLE 1 Summary of the main studies investigating the effects of CFD in multiple diseases.

Disease	Species	Characteristic	Treatment	Finding	PMID
CKD	Human	a. 30 healthy subjects b. 30 CKD III & IV participants c. 30 CKD III & IV renal transplant recipients	NA	CFD levels are increased in the microparticles of CKD and transplant patients, which also activates the alternative pathway in serum.	30006493
PNH and aHUS	Human Monkey	a. 3 PNH patients & 4 aHUS participants b. 3 Monkeys	CFD inhibitor ACH-4471	ACH-4471 inhibits complement-mediated hemolysis in patients and blocks the AP activity in monkeys.	27810992
C3G	Human	29 C3G participants	NA	Patients CFD concentrations are inversely correlated with eGFR. AP are systematic activated.	36404708
TMA-associated LN	Human	a. 79 LN-associated TMA participants b. 79 LN participants without TMA	NA	TMA has stronger staining CFD deposited along the glomerulus than LN patients.	36582241
GA	Human	a. 42 GA participants b. 41 GA participants c. 40 GA participants	a. Lampalizumab injection monthly b. every other month injection c. sham control	Lampalizumab treatment reduce lesion area progression.	28637922
GC	Human	4 GC-derived cell lines	NA	All cell lines secrete a large amount of CFD.	12095175
Breast cancer	Human Mouse	CFD-KO mice and mADSCs	NA	In mADSCs, CFD-KO reduces its ability to enhance the proliferation of PDX cells, and addition of CFD can restore this ability.	34439392
IA	Mouse	FF mice	NA	FF mice are resistant to IA and bone destruction because of a lack of CFD.	31167128
NAFLD	Human	908 NAFLD participants	NA	CFD can predict NAFLD remission independently.	34814152

In addition to cardiovascular and metabolic diseases, CFD is also involved in various diseases such as PNH, aHUS, GA, chronic kidney disease, and tumors. Its related pathogenic mechanisms and roles have been widely studied. CKD, chronic kidney disease; TMA, thrombotic microangiopathy; LN, lupus nephritis; GC, gastric cancer; KO, knockout; mADSCs, mammary adipose tissue-derived stem cells; PDX, patient-derived xenograft; IA, inflammatory arthritis; FF, fat-free; NAFLD, nonalcoholic fatty-liver disease.

patients with lupus nephritis (LN), which demonstrates that AP is involved in the pathogenesis of LN (33).

CFD, together with MASP-1, C3, and C5, participates in the hemostatic response. While playing a protective role in preventing bleeding, it also exacerbates thrombotic and inflammatory diseases. While research has shown that platelets secrete CFD and activate AP (34), research has shown that CFD deficiency in mice can prevent thrombosis, and the inhibition of CFD can reduce platelet activation during vascular injury (35). A decrease in CFD in heparinized human whole blood can inhibit platelet activation in a simulated extracorporeal circulation circuit (36).

CFD plays an important role in CVMDs through various pathological mechanisms (Figure 2), which we will discuss in detail later.

3 CFD and the complement system

3.1 General description of the complement system

Complement system activation involves three pathways: the classical pathway (CP), the lectin pathway (LP), and the AP (37). The activation of the CP is driven mainly by antigen-antibody complexes as well as C-reactive protein and other factors (38). LP is triggered by specific microbial polysaccharides. AP precedes CP and LP and is slowly self-activated by hydrolysis (38).

The three pathways mentioned above initiate complement activation, which converges on the common core component C3. C3 is cleaved into C3a and C3b by C3(H₂O)Bb, which initiates

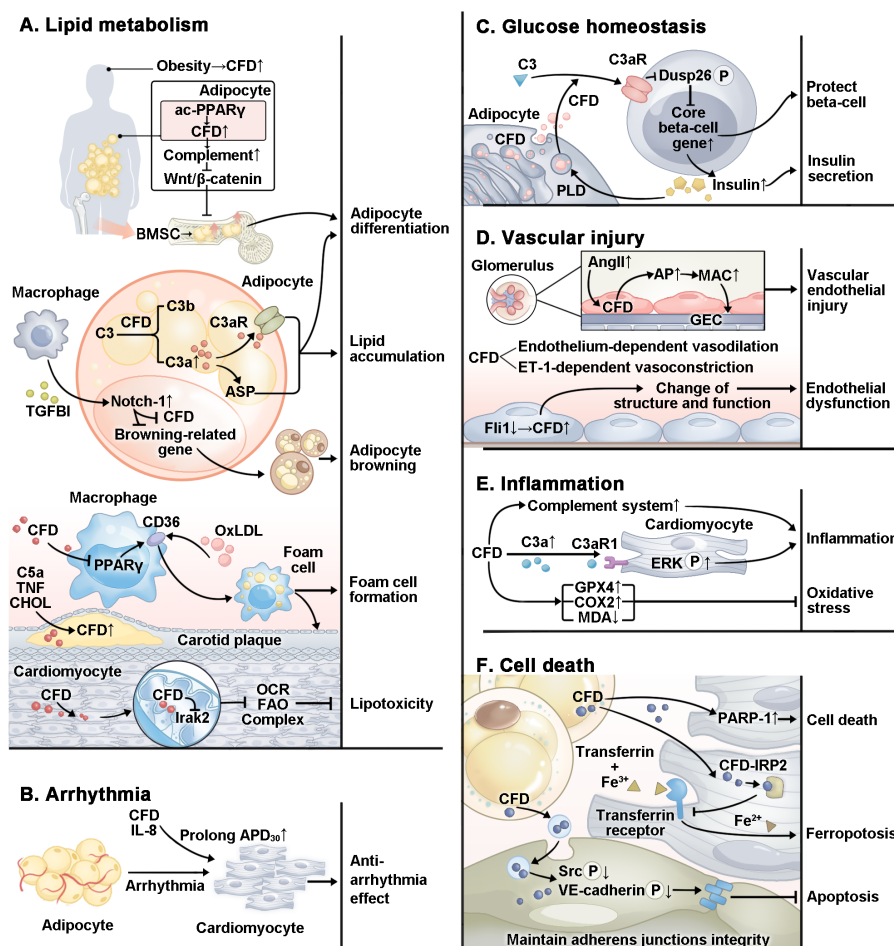


FIGURE 2

Specific mechanism by which CFD plays a role in CVMDs. In CVMDs, CFD plays a complex role. (A) CFD regulates adipocyte differentiation and lipid accumulation through multiple pathways. CFD promotes foam cell formation. However, in cardiomyocytes, CFD inhibits lipotoxicity. (B) CFD plays an antiarrhythmic role by prolonging APD₃₀. (C) CFD protects beta-cells and promotes insulin secretion. (D) CFD is related to vascular endothelial injury and endothelial dysfunction. (E) CFD plays a pro-inflammatory role through the complement system and multiple pathways. In addition, studies have reported that CFD inhibits oxidative stress. (F) CFD protects cardiomyocyte against ferroptosis and maintains the integrity of cardiac microvascular endothelial cell adherens junctions. However, CFD activates PARP-1 and causes cardiomyocytes apoptosis. ac, acetylation; PPARγ, proliferator-activated receptor gamma; TGFβ1, transforming growth factor-beta; OxLDL, oxidized low-density lipoprotein; TNF, tumor necrosis factor; CHOL, cholesterol; Irak2, interleukin-1 receptor-associated kinase 2; OCR, oxygen consumption rate; FAO, fatty acid oxidation; IL-8, interleukin 8; APD, action potential duration; Dusp26, dual specificity phosphatase 26; P, phosphorylation; PLD, phospholipase D; AngII, angiotensin II; GEC, glomerular endothelial cell; Fli1, friend leukemia virus integration 1; ERK, extracellular signal-regulated kinase; GPX4, glutathione peroxidase 4; COX2, cyclooxygenase-2; MDA, malondialdehyde; PARP-1, poly ADP-ribose polymerase-1; IRP2, iron regulatory protein 2; Src, proto-oncogene tyrosine-protein kinase; VE, vascular endothelial.

signal amplification (39). CFD plays a promoting role in the generation of C3(H₂O)Bb. In addition, initial pathways activation results in C5 convertase formation, which causes C5 to be cleaved into C5a and C5b. Moreover, the cleavage of C5 results in a labile conformation of C5b, which then binds successively to C6, C7, C8, and multiple copies of C9 to form the TCC, which is also called as the membrane attack complex (MAC) (37). The MAC can directly create transmembrane pores on cells and bacteria, leading to target swelling and eventual lysis (37).

Since complement cascade activation is a rapidly amplifying proinflammatory response, a variety of proteins that inhibit this reaction tightly regulate complement system activation in multiple ways, such as by modulating convertase activity and controlling the formation of the MAC. Complement factor I and complement factor H can limit the activity of C3 convertase and bind to C3b, preventing novel convertase formation (40).

3.2 The alternative pathway

AP activation starts with the slow spontaneous hydrolysis of C3 to form C3(H₂O). MASP-3 is the exclusive activator of pro-CFD in resting human blood (41), promoting the proteolytic processing of pro-CFD to CFD (42). CFD cleaves C3(H₂O) combined with CFB to the AP liquid phase C3 convertase C3(H₂O)Bb. Through catalysis, C3 is cleaved into C3a and C3b. AP generates the majority of C3b (38). C3b later binds to a surface and recruits CFB to generate the C3bB complex. Subsequently, CFD, which binds to C3bB, becomes transiently active and cleaves CFB into Ba and Bb fragments, generating the AP cell surface C3 convertase C3bBb, which further promotes the generation of C3b. C3b binds to pathogens and acts as an opsonin to enhance phagocytosis. Additionally, when C3bBb accumulates and its local concentration reaches a certain level, C3b may bind to C3bBb, resulting in the generation of the C5 convertase C3bBb3b (43), which catalyzes downstream cascade reactions, resulting in the formation of MAC and lysis of the pathogen. Moreover, properdin combines with C3bBb to form C3bBbproperdin. This process stabilizes C3bBb, increasing its half-life by 5- to 10-fold (44).

3.3 The role of CFD in the amplification loop

In a variety of diseases, complement system activation leads to inflammatory damage, such as PNH and aHUS. Researchers have reported that it regulates the onset and development of ischemia/reperfusion (I/R) injury in recent years. Complement activation occurs early in acute myocardial infarction (AMI) (45). The elevation of C3d and sC5b9 occurs prior to the classical markers of myocardial necrosis in AMI patients (45).

AP has been regarded as a dual system (46). It both takes on a recognition function comparable to that of CP and LP and carries out an amplification function in the complement system. Since CP and LP activation generates C4bC2a, which forms C3b, triggering signal propagation via the amplification loop, AP amplification is a common feature of any kind of initial complement activation (46).

AP amplification is responsible for approximately 80% of CP-induced complement terminal activation (47).

CFD is a pivotal rate-limiting enzyme in AP (Figure 3). The concentration of CFD is closely related to the activity and output of the AP and downstream amplification loop (7). The cleavage of CFB into Ba and Bb fragments by CFD is regarded as the rate-limiting stage for the generation of the C3 convertase C3(H₂O)Bb. An increase in the CFD concentration enhances complement cascade activity. A lack of CFD has been shown to lead to inactive AP (22). More importantly, complement activation initiated by CP and LP can be amplified through the AP. C3b deposited on the cell surface by CP can serve as a site for AP C3 convertase formation (46). Under the catalysis of CFD, more C3bBb is produced and cleaves C3 to form more C3b deposited on the cell surface. Therefore, CFD amplifies the output of all three pathways through a positive feedback mechanism (48).

4 The role of CFD in cardiovascular diseases

The findings summarized above emphasize the role of CFD in the complement system. We will briefly summarize the evidence that suggests possible roles of CFD (Figure 4) in each disease in the following text.

4.1 Hypertension

Many studies have verified the vital role of immune cytokines in hypertension and end-organ damage. Triggered by lifestyle factors, infections and autoimmunity, both chronic initiation and adaptive immune overactivation may result in high blood pressure and tissue damage (49). According to the Maastricht Study (4), participants with higher CFD are characterized by higher blood pressure. As an endogenous vascular elastase, CFD mediates the onset and development of pulmonary hypertension (PAH) (50). Moreover, CFD contributes to an obesity-related decrease in endothelium-dependent vasodilation and endothelin-1-dependent vasoconstriction (51).

As an autoimmune disease, systemic sclerosis (SSc) can lead to varying degrees of involvement in connective tissues, which can cause PAH in patients. The progression of PAH is related to elevated serine elastase activity (52). In patients with systemic sclerosis, CFD is increased in serum and is positively correlated with right ventricular systolic blood pressure and PAH. Patients with elevated CFD levels are at higher risk for PAH-related cardiac dysfunction (53). Importantly, high CFD levels are more specific to PAH than to brain natriuretic peptide levels. Mechanistically, progressive PAH is often associated with abnormalities in the structure and function of vascular and endothelial cells. In SSc, Friend leukemia virus integration 1 deficiency upregulates CFD in human dermal microvascular endothelial cells (19).

Innate immune response activation occurs when the embryo attaches to the uterus. Recent investigations have demonstrated that

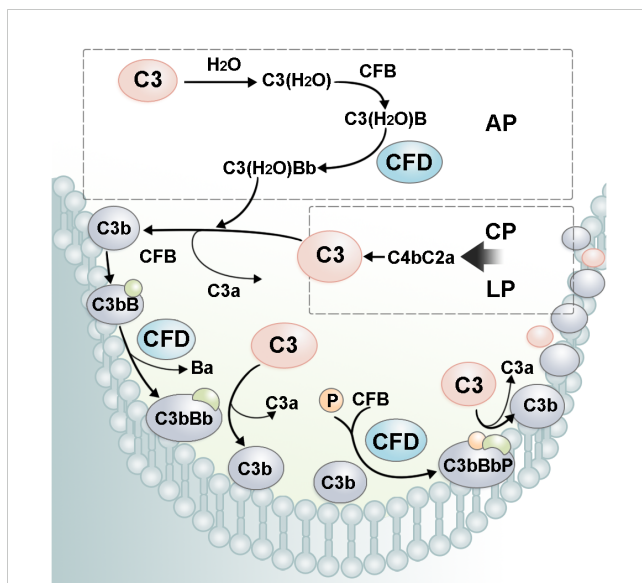


FIGURE 3

The role of CFD in the complement amplification loop. In AP, CFD cleaves $C3(H_2O)B$ into the alternative pathway fluid phase $C3$ convertase, $C3(H_2O)Bb$. $C3(H_2O)Bb$ cleaves $C3$ into $C3a$ and $C3b$. $C3b$ recruits CFB on the cell surface. After cleavage by CFD, alternative pathway cell-surface $C3$ convertase, $C3bBb$, is formed. $C3bBb$ cleaves $C3$ and forms more $C3b$, leading to a rapid amplification of complement cascade activity. P, properdin.

complement system dysregulation mediates the onset of preeclampsia, which is mainly characterized by gestational hypertension. CFD concentrations are reportedly increased in patients with preeclampsia during late gestation (54, 55). The concentration of complement terminal complexes in the placenta of preeclamptic women is higher than that in the placenta of normal women.

4.2 Coronary heart disease

CFD is a risk factor for coronary heart disease (CHD) in postmenopausal women (56). According to a cross-sectional study among 288 women, serum CFD levels are elevated in patients with polycystic ovary syndrome and are influenced by BMI, homeostatic model assessment for insulin resistance and high-sensitivity C-reactive protein (57). Another study showed that CFD can serve as an independent predictor of carotid intima-media thickness (CIMT) (57). Furthermore, CFD may be correlated with the instability of atherosclerotic plaques in patients with CHD (58). A study conducted among 370 patients undergoing diagnostic coronary angiography demonstrated that serum CFD levels have the potential to predict future incidences of AMI and all-cause death in patients with coronary artery diseases (58). CFD is highly expressed in coronary atherosclerotic plaques and adventitial adipocyte tissue (58). This reflects the potential role CFD plays in the development of atherosclerosis.

CFD plays a pathogenic role in CHD through multiple pathways. First, EAT can release proinflammatory factors directly

into the coronary lumen because it is close to myocytes. Previous studies have demonstrated that circulating CFD is exposed to the vascular endothelial layer and is correlated with systemic AP activation, which is correlated with vascular endothelial dysfunction (59). Moreover, pericoronary adipose tissue is bidirectionally linked to endothelial dysfunction (60). In response to angiotensin II stimulation, CFD is overly expressed in glomerular endothelial cells and activates local complement, resulting in the formation and deposition of the MAC complex on endothelial cells and promoting vascular endothelial injury (61). Then, complement induces endothelial cells to release inflammatory cytokines, which may subsequently promote vascular dysfunction (23). An increasing level of CFD is related to low-grade inflammation and endothelial dysfunction (6). Coronary angiography assessments using optical coherence tomography have shown that serum CFD levels can partially predict thin-cap fibroatheromas, which are inflammatory plaques that are prone to cause acute coronary syndrome (62). In addition, atherosclerosis is usually associated with abnormal lipid metabolism, such as increased intracellular cholesterol uptake and decreased excretion. CFD is expressed in the aortic endothelium (63). The expression of CFD, C1q alpha and beta, C2, and C3 is increased in atherosclerotic carotid plaques from acute coronary syndrome patients primed with C5a and tumor necrosis factor followed by cholesterol crystals (64).

Overall, the majority of studies show a promoting effect of CFD on atherosclerosis development. However, some studies have suggested a negative correlation between CFD and arterial injury. In 483 obese adult subjects, circulating CFD concentrations are reduced in individuals with CIMT and asymptomatic carotid atherosclerosis (65). Moreover, the overexpression of CFD inhibits the development of atherosclerosis in mice (66). Mechanistically, CFD downregulates the PPAR γ /CD36 pathway, suppresses oxidized low-density lipoprotein-induced lipid uptake by macrophages, and prevents the formation of foam cells (66). Therefore, more in-depth investigations are still required to determine the specific mechanism of CFD in the onset and progression of CHD.

4.3 Ischemia/reperfusion injury

I/R injury can occur during myocardial infarction (MI) or cardiovascular surgery. AMI can lead to the production and secretion of CFD, and the serum CFD level of patients with AMI is upregulated at admission (17). At the 1-month follow-up, this difference in CFD levels between AMI patients and the control group disappeared (17). Among MI rats, the CFD expression levels in the EAT are markedly greater than those in subcutaneous adipose tissue. Inhibiting CFD activity can alleviate myocardial injury after MI (67). CFD derived from EAT induces cell apoptosis through excessive activation of poly ADP-ribose polymerase-1 in H9c2 cells (67).

However, studies have also suggested that CFD has a cardioprotective effect during MI injury by reducing inflammation, alleviating oxidative stress, and improving adverse

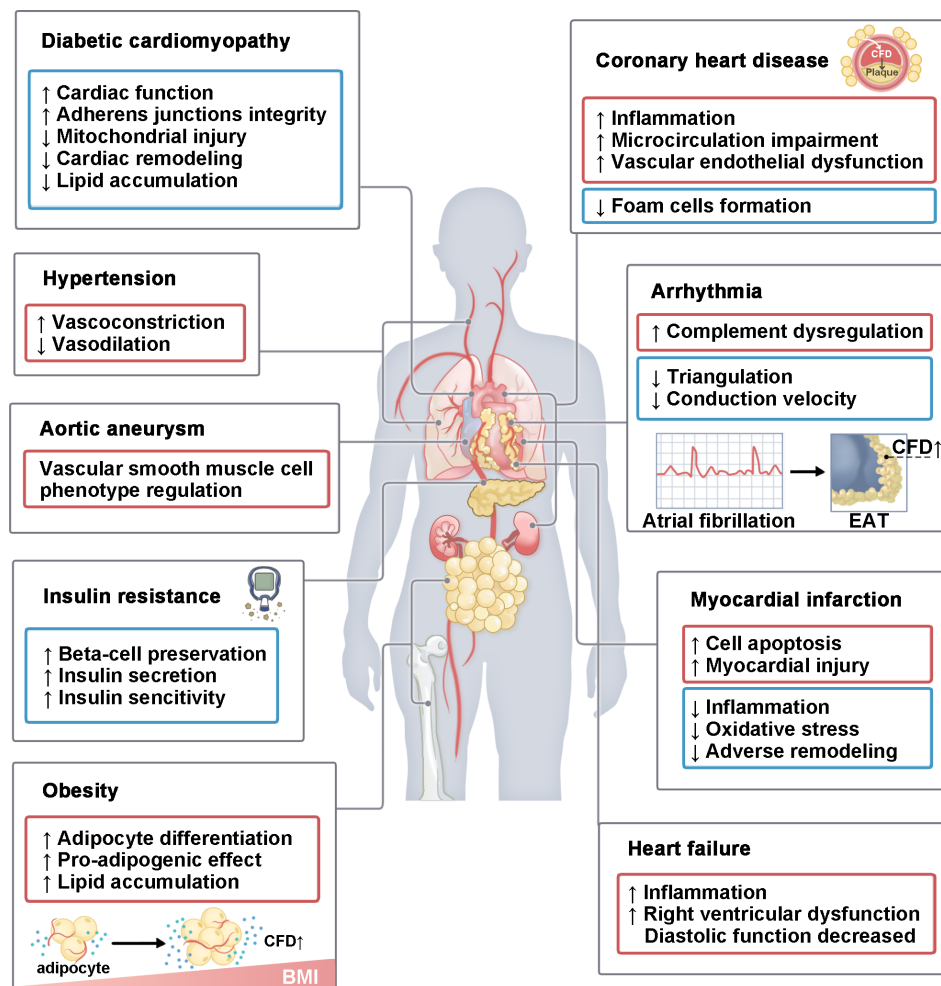


FIGURE 4

The role of CFD in various CVMs. CFD plays a role in various CVMs. It mainly includes diabetic cardiomyopathy, hypertension, aortic aneurysm, insulin resistance, obesity, coronary heart disease, arrhythmia, I/R injury and heart failure. This process involves multiple organs, including the heart, blood vessels, lungs, pancreas, kidneys, bones, and adipose tissue. In some diseases, CFD has both pathogenic and protective effects. BMI, body mass index; EAT, epicardial adipose tissue.

remodeling. Open heart surgery may induce I/R injury. In 32 patients who received elective on-pump coronary artery bypass grafting with cardiopulmonary bypass, plasma CFD declined after surgery and decreased to the lowest level (approximately 30% below the baseline values) at 24 hours after surgery (68). There is an inverse correlation between CFD and IL-6 levels in samples collected at the start of cardiopulmonary bypass and 1 minute after removal of the aortic cross-clamp (68). In one study, CFD overexpression significantly increased the survival rate, preserved the left ventricular ejection fraction, and alleviated myocardial pathological damage in mice after MI surgery (17). CFD alleviates lipid oxidative stress after MI by decreasing cyclooxygenase-2 expression and increasing glutathione peroxidase 4 levels (17). Further observations noted that the CFD treatment of neonatal mouse ventricular myocytes altered the expression of proteins related to ferroptosis and iron metabolism (17). Mechanistically, CFD interacts with IRP2 to regulate iron homeostasis by upregulating the iron storage-related protein ferritin heavy chain

and downregulating the transferrin receptor, thereby protecting cardiomyocytes against ferroptosis and reducing cardiac remodeling after MI (17).

4.4 Heart failure

Experimental and clinical evidence points to inflammatory mediators as a major cause of the development of heart failure (HF), as they can lead to maladaptive processes within the myocardium (39). Uncontrolled complement activation can cause widespread inflammation and tissue damage in HF. C3 and CFD are highly abundant in the right ventricle (69). In HF patients, the plasma concentration of CFD is elevated, especially in those with New York Heart Association functional class III and IV. The concentrations of CFD and terminal complement complexes are associated with the N-terminal pro-B-type natriuretic peptide and C-reactive protein concentrations (70). Increased CFD levels are

also correlated with measures of inflammatory response and deteriorated diastolic function (70). In line with this, increased CFD is closely associated with left ventricular dysfunction ($p < 0.001$), right ventricular dysfunction, and a higher frequency of diastolic dysfunction in patients with systemic sclerosis (53). In conclusion, the concentration of CFD is correlated with the disease severity of patients with HF.

Recently, research has demonstrated that the C3-CFD-C3aR axis plays a crucial role in right ventricular failure. C3a-C3aR1 activated extracellular signal-regulated kinase and upregulated HF- and inflammation-related pathways in rat cardiomyocytes. In mouse models of right ventricular failure, both C3 knockout and CFD knockout attenuate ventricular dysfunction (69).

4.5 Arrhythmia

EAT accumulates and expands in obese and insulin-resistant states. In addition to functioning as an energy reserve system, epicardial fat determines the local inflammatory milieu of the heart via the release of multiple inflammatory and anti-inflammatory adipokines. The expansion of EAT can be proarrhythmic, resulting in hypoxia, inflammatory cytokine release, and the infiltration of fat into the myocardium. Epicardial fat increases the occurrence of atrial arrhythmias (71). Gene expression analysis revealed that the CFD gene was highly abundant in the EAT of patients with atrial fibrillation (AF) (72).

However, another study noted that the expression of CFD is downregulated in patients with AF-related cardiogenic embolism stroke (AF-CE) (73). Although CFD is associated with neutrophils, it is negatively correlated with naive B cells, naive CD4⁺ T cells, and resting NK cells. Bioinformatic analysis and experiments further support that CFD can potentially serve as a diagnostic blood biomarker for AF-CE (73). Thus, the decrease in CFD may imply complement dysregulation, which predicts the risk of AF-CE.

In direct coculture, white fat-like adipocytes (hAdip) increase triangulation, prolong action potential duration, and slow the conduction velocity of human-induced pluripotent stem cells to ventricular cardiomyocytes (74). The presence of MCP-1, IL-6, IL-8, and CFD, which are correlated with the immune response and inflammation, increased in the hAdip-conditioned medium. However, CFD and IL-8 can decrease triangulation and shorten conduction velocity through increasing action potential duration alone (74).

4.6 Aortic aneurysm

Abdominal aortic aneurysm is correlated with vascular smooth muscle cell apoptosis and inflammation, and chronic inflammatory reactions activate the AP and may lead to the progression of atherosclerotic aortic aneurysm aortic wall remodeling. CFD levels are elevated in patients with thoracic aortic aneurysms and abdominal aortic aneurysms (75, 76), suggesting that CFD may promote vascular smooth muscle cell phenotype regulation in the onset of aneurysms.

5 The role of CFD in metabolic diseases

5.1 Obesity and dyslipidemia

The expansion of adipose tissue is often accompanied by the accumulation of immune cells. Emerging evidence shows that obesity is correlated with chronic LFI. There is a positive correlation between C3 and various indicators of body fat (77). Studies have shown that the concentrations of CFD are correlated with the likelihood of obesity (78). In overweight/obese women with a BMI ≥ 25 kg/m², circulating CFD levels are higher than those in subjects with a BMI < 25 kg/m² (57). An increasing number of studies have shown that BMI is positively correlated with CFD (79). Placental macrophages Hofbauer cells secrete CFD. More CFD was released from the placenta of obese mothers than from that of lean mothers (27). Circulating fetal CFD is positively correlated with maternal BMI and maternal insulin resistance (27).

There is still controversy about the type of adiposity associated with CFD. On the one hand, a cross-sectional study suggested that CFD and leptin concentrations are positively associated with BMI and visceral fat mass in patients with cardiometabolic disorders (79). In adults with growth hormone deficiency (AGHD), serum CFD is correlated with total body fat, visceral fat, and insulin resistance. After growth hormone treatment and appropriate lipid-lowering therapy, CFD levels significantly decrease in visceral adipose tissue (80). In contrast, a study has shown that CFD is related to subcutaneous and intrathoracic adipose volumes yet shows no connection with visceral adiposity (78).

In contrast, some studies have found the opposite effect on CFD. Plasma CFD levels are significantly downregulated in high-fat diet (HFD)-fed mice (81) but are slightly increased in isocaloric HFD-fed mice (82). Intriguingly, it has also been reported that CFD levels rise slightly within 48 hours after HFD feeding in mice but then decrease below baseline levels after 6 weeks after initiation of a HFD (83). Plasma proteome analysis has shown that preventative exercise can only partially reverse the decrease in plasma CFD caused by HFD overconsumption (82). Exercise inhibits the CFD-secreted phosphoprotein 1 signaling pathway in diet-induced obese male mice (84). Promoting the browning of white adipose tissue is considered a potential strategy for protecting against obesity. Transforming growth factor-beta, which is derived from macrophages, suppresses CFD mRNA and browning-related gene expression in adipocytes by activating Notch-1 signaling (81).

5.2 Hyperglycemia and insulin resistance

CFD plays an important role in promoting insulin secretion, regulating blood glucose levels and improving insulin sensitivity. In plasma, the level of CFD is decreased in patients with type 2 diabetes mellitus (T2DM) (85). Compared to T2DM patients, T2DM patients with β -cell failure exhibit decreased levels of CFD mRNA in visceral and subcutaneous adipose tissues (30). Animals with genetically absent CFD have glucose intolerance because of

insulinopenia (30). In diabetic mice, CFD preserves beta cells by catalyzing C3a formation and downregulating dual specificity phosphatase 26 expression in β cells (78). Furthermore, CFD activates C3a-C3aR1 to promote insulin secretion (30). In addition, insulin promotes CFD secretion from adipocytes by stimulating phospholipase D activity (86).

5.3 Diabetic cardiomyopathy

Diabetic cardiomyopathy causes fatty acid and lipid accumulation in cardiomyocytes, resulting in lipotoxicity and triggering progressive structural and functional remodeling of the heart. In diabetic cardiomyopathy mice, CFD levels in heart tissue, plasma, white adipose tissue, and brown adipose tissue are significantly decreased (87). Overexpression of CFD relieves cardiac remodeling and improves cardiac function both in mice and rats with diabetic cardiomyopathy (87, 88).

CFD alleviates diabetes-related coronary microvascular complications (87). Specifically, CFD maintains the integrity of cardiac microvascular endothelial cell adherens junctions by downregulating tyrosine-protein kinase Csk and inhibiting VE-cadherin phosphorylation, thereby protecting the microvasculature against hyperpermeability (87). The abnormal processing of mitochondrial calcium and decreased level of free matrix calcium lead to a decrease in cardiomyocyte function. Recent research has revealed that CFD binds to interleukin-1 receptor-associated kinase 2 (Irak2) and inhibits its mitochondrial translocation, downregulating the interaction of Irak2 with prohibition-optic atrophy protein 1 on mitochondria in cardiomyocytes of mice treated with DMC. Inhibition of the Irak2 signaling pathway improves fatty acid oxidation, protects mitochondrial structure and function, and alleviates lipid accumulation (88).

6 Potential therapeutic strategies targeting CFD

The central role of CFD in AP activation and the critical role of CFD in complement cascade amplification make this protein an effective pharmaceutical target for complement-related diseases. As an inhibitor targeting the proximal part of AP, CFD inhibitor selectively block AP activation, impede C3 cleavage, and limit signal amplification of the CP and LP without any effect on the initiation or terminal function of these two complement pathways (8). Since the CP and terminal pathway mediate the bacterial lysis, CFD inhibition potentially reduces the risk of infections compared with C5 blockade or even C3 blockade (31). Previous studies have shown that terminal complement deficiencies patients have increased risk of infection with *Neisseria* species compared to early complement pathway component deficiencies (89). Additionally, C5a participate in the activation of leukocytes to phagocytose pathogens (90). Moreover, inhibiting CFD does not block C3b-mediated opsonization or C3a-mediated inflammation. Thus, CFD inhibitor modulate complement activation while keep “protective” complement activation.

Although CFD inhibitor have the advantage of oral administration, its short half-life requires 2–3 administrations per day (91). In addition, since that low levels of CFD are sufficient for AP activation in serum, complete inhibition of CFD may be vital for therapeutic efficacy (8). Compared to C5 inhibitors targeting downstream pathways, CFD inhibitors cannot fully block complement activation (91). C3 activation by other plasma protease, bypassing factor D, has been described in specific clinical circumstances (89). As a part of the plasma contact activation system, kallikrein cleaves C3 to C3b and C3a (89). These fundings suggest that a combined treatment with C5 inhibitors and CFD inhibitors may achieve better clinical response.

At present, a number of CFD inhibitors have been designed and quite a few of them have entered clinical trials. Benefited by its distinct mechanism, CFD inhibitors may achieve more profound clinical benefits in the future.

Danicopan (ALXN2040) is an oral CFD inhibitor that is mainly used as an add-on therapy in patients with PNH (NCT04469465). Danicopan improved hemoglobin concentrations with no serious adverse events reported in the study (92). Additionally, Danicopan is currently in a phase II clinical trial for patients with GA (NCT05019521). Furthermore, BCX9930 is another oral CFD inhibitor used in phase II clinical trials for the treatment of PNH (NCT04702568) and C3 glomerulopathy (NCT05162066).

There are few anti-CFD antibodies currently in clinical trials. Lanpazumab is a humanized monoclonal antibody targeting the antigen-binding fragment of CFD (93). In a cardiopulmonary bypass simulation, anti-CFD monoclonal antibodies inhibited the formation of inflammatory factors and the activation of neutrophils and platelets (36). Anti-CFD antibodies significantly inhibited the formation of alternative, lectin, and soluble TCCs in SARS-CoV-2-infected monkeys (94). In addition, CFD antibody treatment reduced the levels of proinflammatory cytokines, chemokines, and the plasma endothelial lesion marker plasminogen activator inhibitor-1 and improved coagulation abnormalities (94).

7 Conclusion and perspective

This review has shown the clear function of CFD in a range of CVMDs and identifies the role of CFD as a biomarker in these diseases. Current research shows that it has multiple effects on the cardiovascular system. Although CFD promotes endothelial dysfunction and atherosclerosis development and mediates ventricular diastolic dysfunction, it alleviates oxidative stress and improves adverse remodeling. Furthermore, regarding metabolic diseases, CFD promotes adipose tissue accumulation, controls blood glucose levels, improves insulin sensitivity and relieves diabetic cardiomyopathy. Thus, CFD may mediate interweaving immune responses and energy metabolism.

A large number of studies have confirmed the association between CFD and CVMDs. Many articles have pointed out the predictive value and biomarker role of CFD in CVMDs. However, the role of CFD in each CVMDs remains elusive. More research is required to clarify the effect of CFD on CVMDs and to confirm the specific mechanism by which CFD regulates CVMDs. Understanding the specific mechanism

of CFD in the onset and development of diseases is an important knowledge base for discoveries about the diagnostic value and clinical treatment of CFD in CVMDS. Furthermore, researchers may adopt different methods to assess the safety, toxicology profile, and therapeutic effect of CFD-targeting medicine on CVMDS in animal models and human patients.

Overall, CFD is a prospective biomarker and therapeutic target for CVMDS.

Author contributions

YK: Conceptualization, Investigation, Visualization, Writing – original draft. NW: Conceptualization, Investigation, Writing – review & editing. ZT: Investigation, Writing – review & editing. DW: Investigation, Writing – review & editing. PW: Investigation, Writing – review & editing. QY: Investigation, Writing – review & editing. XY: Investigation, Writing – review & editing. WS: Investigation, Writing – review & editing. ZJ: Investigation, Writing – review & editing. MZ: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Clinical associations of complement-activating collectins, collectin-10, collectin-11 and mannose-binding lectin in preterm neonates

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Introduction: Premature and low-birthweight infants are at especially high risk of perinatal complications, including impaired thermoregulation, infections and respiratory distress. Such adverse effects and the need for invasive procedures are associated with high mortality among preterms. This study focused on the influence of the innate immune system and tested the levels of collectins, collectin-10 (CL-10), collectin-11 (CL-11) and mannose-binding lectin (MBL) in preterm neonates.

Methods: Cord blood was collected from 535 preterms (born at gestational age ≤ 37 weeks). *COLEC10* and *COLEC11* polymorphisms were analyzed by real-time PCR and those of *MBL2* by PCR/PCR-RFLP. The concentrations of collectins in sera from cord blood were determined with ELISA.

Findings: Low concentrations of CL-10 in cord sera (<462 ng/ml corresponding to the 10th percentile) were significantly associated with births at GA ≤ 32 weeks. Median levels of both CL-10 and CL-11 were significantly lower in preterms with very low birthweight (<1500 g), low Apgar 1' score and those who needed prolonged hospitalisation. Lower median CL-10 was also observed in fetal growth restriction cases. An important finding was the decreased concentrations of CL-10, CL-11 and MBL in respiratory distress syndrome (RDS). For CL-10 and CL-11, that relationship was confined to infants born at GA ≥ 33 weeks and/or with body mass at birth ≥ 1500 g. Only CL-10 was found to influence susceptibility to early-onset infections. *COLEC11* heterozygosity for the activity-decreasing polymorphism (rs7567833, +39618 A>G, His219Arg) was

more common in preterm premature rupture of membranes (pPROM) cases, compared with corresponding reference groups. Furthermore, C/T or T/T genotypes at *COLEC11* at rs3820897 (-9570 C>T) as well as MBL deficiency-associated *MBL2* gene variants were more common in preterms diagnosed with RDS than among unaffected newborns.

Conclusion: The complement-activating collectins investigated here could be important for maintaining homeostasis in preterm neonates. Despite similar structure and specificity, MBL, CL-10 and CL-11 manifest a different spectrum of clinical associations.

KEYWORDS

CL-10, CL-11, collectin, complement, mannose-binding lectin (MBL), neonate, prematurity, respiratory distress syndrome (RDS)

1 Introduction

Approximately 11% of babies globally are born preterm (before 37 weeks) (1). In 2022, 7.2% of live births in Poland were premature. Among 305,132 newborns, 2,453 (0.8%) had body mass <1500 g at birth (2). Premature and low-birthweight babies are at especially high risk of perinatal complications, including impaired thermoregulation, infections and respiratory distress. Those complications and the need for invasive procedures are associated with relatively high mortality among preterms: 65.1% of infants who died aged ≤1 year (764/1171) were born prematurely. Furthermore, as many as 40.2% (321/798) of neonatal deaths were associated with birthweight (BW) <1500 g (2). That reflected rather underdevelopment of the respiratory system and other adverse effects of prematurity than low body mass itself. Additionally, perinatal complications may affect the quality of life during childhood and even adulthood (1, 3, 4).

The neonatal immune system is immature, especially in preterm newborns. Furthermore, premature delivery abrogates the transfer of maternal immunoglobulins through the placenta and thus does not allow them to reach a sufficient concentration in the infant's blood. The lack of "immune experience" (exposure to "non-self" antigens) is reflected by a lack of specific T and B lymphocytes and, therefore, impaired cellular and humoral response. Low level or lack of antibodies recognising polysaccharide or glycoconjugate antigens may, at least partially, be compensated by soluble defence lectins, including collectins (5, 6).

Collectins are oligomeric C-type defence lectins. As pattern-recognising molecules (PRMs), they contribute to maintaining homeostasis by elimination of pathogens and altered self-cells by opsonisation or agglutination. Three of these PRMs, mannose-binding lectin (or mannan-binding lectin, MBL), collectin-10 (CL-10; or collectin liver-1, CL-L1) and collectin-11 (CL-11; collectin kidney-1, CL-K1) form complexes with MASPs (MBL-

associated serine proteases), enabling activation of complement via the lectin pathway (LP). Those lectins recognise a variety of carbohydrate residues, including D-mannose, N-acetyl-D-glucosamine and L-fucose, that commonly decorate microbial surface structures [reviewed in (5, 7, 8)]. The ligand specificity of CL-10 and CL-11 may be extended by forming their heterocomplexes called CL-LK (9). Moreover, the interaction of CL-11 with C1q/TNF-related protein 6 (CTRP-6) enables its specific recruitment to molecular conserved motifs, common for a variety of microbes (pathogen-associated molecular patterns, PAMP) or endogenous signals released following host's cell damage or death (danger-associated molecular patterns, DAMP), followed by complement activation (10). As a part of the innate immune system, the mentioned collectins are important players in neonatal response to the extrauterine conditions. It should, however, be stressed that they may not only protect from infection but also participate in pathophysiological processes, including SIRS/sepsis (5).

Certain known, rare mutations of corresponding *COLEC10* and *COLEC11* genes were found to cause Malpuech facial clefting syndrome (3MC syndrome), with craniofacial, renal or genital abnormalities, growth and intellectual disability [reviewed in (11)]. In contrast, MBL deficiency is considered the most frequent human immunodeficiency. It is associated with several single nucleotide polymorphisms of the *MBL2* gene, localised to the promoter region and/or exon 1: -550 G>C (rs11003125, commonly termed H/L), -221 G>C (rs76206, Y/X), C4T (rs7095891, P/Q), C223T (R52C, rs5030737, A/D), G230A (G54D, rs1800450, A/B) and G239A (G57E, rs1800451, A/C). Variant alleles D, B, and C of exon 1 coding region are collectively called O. The MBL inborn deficiency is generally associated with O/O and LXPA/O (or LXA/O, XA/O) genotypes [reviewed in (5)].

The ficolin family is another group of PRMs and is structurally related to collectins by possessing a collagen-like region, but in this

case, with fibrinogen-like domain as recognition module. Recently, we reported associations of the *FCN2* gene polymorphisms regions and concentrations of its product, ficolin-2, in cord sera with clinical features of Polish preterm neonates (12–14). Here we extend those data with results concerning relationships between CL-10, CL-11, MBL, and selected clinical parameters as well as complications commonly associated with prematurity. Although numerous reports concerning the role of MBL in neonatal health and disease have been published, data are often inconsistent while the literature concerning CL-10 and CL-11 is still rather scarce and focused mainly on their crucial role in fetal development (5). Therefore, the aim of our study was to determine relationships between the mentioned complement-activating collectins and perinatal, prematurity-associated complications, including early-onset infections, respiratory distress, fetal growth restriction, preterm premature rupture of membranes and need for intensive care. To achieve those goals, concentrations of CL-10, CL-11 and MBL in cord sera as well as selected polymorphisms of corresponding genes were investigated in a relatively large cohort of preterm neonates. Additionally, as no data concerning associations of CL-10 or CL-11 with gestational age have been previously published, we tested a series of cord serum samples from term deliveries and determined expression of the *COLEC10*, *COLEC11* and *MBL2* in preterm and term placentas.

2 Materials and methods

2.1 Subjects

Cord blood samples from 535 preterm newborns (born between 24th and 37th weeks of pregnancy), including 112 born at gestational age (GA) <33 weeks, were obtained from the Department of Newborns' Infectious Diseases (Poznań University of Medical Sciences, Poland) (n=272), Department of Neonatology (Medical University of Gdańsk, Poland) (n=207) and Department of Perinatology (Medical University of Łódź, Poland) (n=56). This cohort included 504 babies previously investigated by us (12–14). Three hundred and forty two individuals came from singleton pregnancies, 187 from 106 twin pregnancies and 6 from 2 triple pregnancies. From 25 twin pairs, material from only one sibling was collected. Furthermore, cord blood samples from randomly selected 17 term neonates (GA 38–41 weeks, no symptoms of infection within 72 h after birth) and sections of placenta from randomly selected 35 live births (GA 30–41 weeks) were collected (Medical University of Łódź, Poland). The exclusion criteria included maternal COVID-19, HIV infection and viral hepatitis. The study was approved by the corresponding local ethics committees (Bioethics Committee of Poznań University of Medical Sciences, Independent Bioethics Committee for Scientific Research at Medical University of Gdańsk, Bioethics Committee of The Medical University of Łódź). Written informed parental consent was obtained. This work conforms to the provisions of the Declaration of Helsinki.

2.2 DNA, serum and mRNA samples

Cord blood samples for DNA extraction were taken into S-Monovette citrated tubes (Sarstedt, Germany) and stored at -20 °C. DNA was extracted with the use of GeneMATRIX Quick Blood DNA Purification Kit (EURx Ltd, Poland), according to the manufacturer's protocol. Total RNA was isolated from placenta sections, using the GeneMATRIX Universal RNA purification kit (EURx Ltd) according to the manufacturer's protocol. DNA and RNA concentrations were measured using a NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific, USA). The absorbance 260/280 nm and 260/230 nm ratios were evaluated to control residual contamination from the nucleic acids extraction procedure. Samples for serum isolation were taken into S-Monovette tubes with Z-Gel clot activator (Sarstedt). Sera were stored at -80°C until tested.

2.3 Genotyping

Single nucleotide polymorphisms (SNP) of the *MBL2* gene, localised to promoter (H/L, at position -550, rs11003125 and Y/X, at position -221, rs7096206) were analysed using allele-specific PCR, previously described (n=535) (14). Exon 1 (A/D, codon 52, rs5030737; A/B, codon 54, rs1800450 and A/C, codon 57, rs1800451) polymorphisms were investigated with the use of PCR-RFLP procedures, employing MluI, BshNI and MboII (all purchased from Thermo Fisher Scientific) enzymes, respectively (15). As mentioned above, those polymorphisms affect MBL expression and/or activity and LXA/O or O/O genotypes are associated with its primary deficiency.

COLEC10 and *COLEC11* polymorphisms were analyzed using TaqMan[®] SNP Genotyping Assays (Thermo Fisher Scientific). For *COLEC10*, rs149331285 (Assay ID: C_174416274_10) and rs148350292 (custom TaqMan probe), were genotyped. For *COLEC11*, rs3820897 (Assay ID: C_2040704_10) and rs7567833 (Assay ID: C:25989885_10) were investigated (n=420). The reactions were conducted in a final volume of 20 µl on QuantStudioTM 5 Real-Time PCR System (Applied Biosystems by Thermo Fisher Scientific), using a 2x TaqManTM Genotyping Master Mix. The reaction conditions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The results were analyzed using the QuantStudioTM Design & Analysis Software v1.5.1" (Thermo Fisher Scientific). *COLEC10* exon 5 rs149331285 (+36545 T>C, Arg125Trp) was previously found to affect concentration of CL-10 in serum whilst promoter five-nucleotide deletion rs148350292 (-161_-157 delAAAAAT) overlaps with transcription factors binding sites, affecting developmental processes (including liver differentiation) (16). *COLEC11* exon rs7567833 (+39618 A>G, His219Arg) was previously found not to influence CL-11 level but it was chosen as a non-synonymous polymorphism, affecting protein structure. Carrying of the promoter rs3820897 (-9570 C>T) variant allele was, in turn, demonstrated to be associated with a lower concentration of this lectin in serum (16).

2.4 Determination of concentrations of collectins in cord serum samples

CL-10 and -11 concentrations (in cord sera from 420 preterm and 17 term neonates) were determined in ELISA, as described by Axelgaard et al. (17) and Selman et al. (18), respectively. Briefly, MaxiSorp F96 immunoplates (NUNC, Denmark) were coated with murine anti-human CL-10 or CL-11 antibodies (clones 16-1 and 11-10, respectively, 5 µg/ml), in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). Plates were incubated overnight at 4°C. After blocking with TBS (10 mM Tris, 145 mM NaCl, pH 7.4) supplemented with 5 mM EDTA and 0.05% Tween (pH 7.4) and incubation with tested sera (prediluted 1:50 in sample buffer: TBS, supplemented with 5 mM EDTA, 0.05% Tween, 0.1% bovine serum and 50 µg/ml heat-aggregated human IgG), the bound proteins were detected with the help of biotinylated anti-CL-10 (clone 16-13) or anti-CL-11 (clone 16-25) (0.5 µg/ml) antibodies (diluted in TBS supplemented in 5 mM EDTA, 0.05% Tween and 1 mg/ml BSA) and HRP-labelled streptavidin (0.1 µg/ml in TBS supplemented with 5 mM EDTA, 0.05% Tween and 1 mg/ml BSA). Next, TMB One was added to each well, and the plate was incubated for 15 min in a dark chamber. A standard curve was constructed from serially diluted standard serum. For negative control, the addition of serum was omitted. CL-10 concentrations <462 ng/ml and CL-11 levels <223 ng (<10th percentile determined for preterms) were considered low.

The concentration of MBL in cord serum samples (n=448) was determined by ELISA based on binding to solid-phase yeast mannan (Sigma-Aldrich) and detection with anti-human MBL mAb (HYB131-1, Statens Serum Institut, Denmark) as described by Cedzynski et al. (19). Concentrations <150 ng/ml were considered low.

2.5 Expression of genes in placenta

The cDNA was obtained using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific). The expression of mRNA from *COLEC10*, *COLEC11*, as well as two housekeeping genes *YWHAZ* and *SDHA* (20), were analyzed using the primers listed in Table 1. Expression of mRNA from the *MBL2* gene was analysed with the use of commercial primers provided by Qiagen (PPH07136E).

The PCR mixture consisted of 10 µL of 2x miRCURY LNA SYBR Green PCR Kit (Qiagen), 1 µL of appropriate primers and 1 µL of cDNA template. The analyses were carried out using the QuantStudio™7 (Applied Biosystems, Thermo Fisher Scientific). All reactions were performed under the following conditions: an initial denaturation step at 95°C for 10 min, followed by 40 amplification cycles of denaturation (95°C, 15 s), a single annealing and extension step (60°C for 1 min), in duplicates.

2.6 Statistical analysis

The frequencies of genotypes were compared by Fischer's exact test (two-tailed) or χ^2 test when appropriate. The Shapiro-Wilk test

TABLE 1 Sequences of primers used for investigation of *COLEC10*, *COLEC11* and housekeeping *YWHAZ* and *SDHA* genes.

Gene	Primer sequence:	
<i>COLEC11</i>	Forward	5'-CTAGCGCGTGCTCAGGAGTT-3'
	Reverse	5'-CGGCGACAGCATTCTTGATG-3'
<i>COLEC10</i>	Forward	5'-AGCGCTCCTACCGCTGAAGT-3'
	Reverse	5'-GCGTCCCACTTGCCATGCT-3'
<i>YWHAZ</i>	Forward	5'-ACTTTTGGTACATTGTGGCTTCA-3'
	Reverse	5'-CCGCCAGGACAAACCAGTAT-3'
<i>SDHA</i>	Forward	5'-TGGAACAAGAGGGCATCTG-3'
	Reverse	5'-CCACCACTGCATCAAAATTCATG-3'

was used for the determination of normality of distributions of gestational age, birthweight as well as CL-10, CL-11 and MBL concentrations. The median levels of collectins were compared with Mann-Whitney *U* test with Bonferroni correction or Kruskal-Wallis test with *post-hoc* Dunn's test when appropriate. For comparisons between twins, Wilcoxon matched-pairs test was employed. For correlations, Spearman's coefficients were calculated. For multiple logistic regression analysis, each of the following parameters or clinical conditions: gestational age <33 weeks, birthweight <1500 g, 1' Apgar score <7, hospitalization >14 days, NICU stay >4 days, need for respiratory support, respiratory distress syndrome (RDS), fetal growth restriction (FGR), preterm premature rupture of membranes (pPROM), early-onset infection (EOI), pneumonia was analyzed as dependent variable with the use of the following set of independent variables: low (<462 ng/ml) CL-10, low (<223 ng/ml) CL-11, low (<150 ng/ml) MBL concentrations, presence of variant alleles for *COLEC11* rs3820897 and rs7567833, MBL deficiency-associated genotype. The Statistica (version 13.3, TIBCO Software) and SigmaPlot (version 12, Systat Software) software packages were used for data management and statistical calculations. Odds ratio was calculated using online MedCalc software (<https://www.medcalc.org>). P values <0.05 were considered statistically significant.

3 Results

3.1 Basic data

Both gestational age (GA) and birthweight (BW) were not normally distributed (medians: 35 weeks and 2320 g, respectively). As expected, the GA and BW were highly correlated ($r=0.687$, $p<0.000001$) (Supplementary Figure 1).

Polymorphisms of the *COLEC10* and *COLEC11* genes as well as serum concentrations of CL-10 and CL-11 were investigated in 420 preterm infants. As genotyping failed in 6 cases for *COLEC10* and in 5 instances for *COLEC11*, therefore data from 414 and 415 individuals were available for analyses of corresponding polymorphisms, respectively. For *COLEC10*, all babies were major

(C) allele homozygotes at rs149331285 and only 2 heterozygotes were found at rs148350292. These polymorphisms were, therefore, not submitted for further statistical analysis. For *COLEC11* rs3820897, 287 (69.2%) neonates had C/C, 107 (25.8%) had C/T and 21 (5.1%) had T/T genotypes, respectively. Analysis of rs7567833, revealed 382 (91.8%) A/A homozygotes and 34 (8.2%) A/G heterozygotes. No variant homozygote was found.

The median serum CL-10 level equalled 667 ng/ml, while that of CL-11 was 321 ng/ml. CL-10 concentrations in cord sera ranged from 172 ng/ml to 2273 ng/ml (IQR: 559–888 ng/ml). Corresponding values for CL-11 were found to be 78–1306 ng/ml and 270–402 ng/ml, respectively. Levels <10th percentile (462 ng/ml and 223 ng/ml for CL-10 and CL-11, respectively) were considered low.

No significant differences in CL-11 concentrations were found among carriers of different *COLEC11* genotypes at rs7567833 (+39618 A>G, His219Arg) or rs3820897 (-9570 C>T) (Supplementary Figures 2A, B, respectively). The concentrations of CL-10 and CL-11 were highly correlated ($r=0.912$, $p<0.000001$) (Supplementary Figure 3A). Furthermore, the levels of both collectins correlated weakly with ficolin-2 [data recently reported by Gajek et al. (14)]: $r=0.129$, $p=0.0098$ (Supplementary Figure 3D) and $r=0.123$, $p=0.014$ (Supplementary Figure 3E), respectively.

Among 535 premature newborns tested for *MBL2* gene polymorphisms, 352 (65.8%) had A/A genotypes, 162 (30.3%) were A/O heterozygotes, while 21 (3.9%) were O/O homo- or heterozygotes. In 40 A/O heterozygotes, the A allele was combined with the LX promoter haplotype; therefore, 61 (11.4%) altogether were considered MBL-deficient (LXA/O or O/O genotype).

Mannose-binding lectin concentrations were determined in cord serum samples from 448 babies. The median MBL concentration was 642 ng/ml (range: 0–3955 ng/ml, interquartile range (IQR): 183–1153 ng/ml). Ninety-four (21%) individuals had low (<150 ng/ml) concentrations. Typically, the highest median corresponded to A/A (903 ng/ml), while the lowest corresponded to XA/O and O/O genotypes (39 ng/ml), respectively ($p<0.000001$). No significant correlations with CL-10, (Supplementary Figure 3B), or CL-11 (Supplementary Figure 3C) or ficolin-2 (Supplementary Figure 3F) levels were observed ($r<0.1$).

As the concentrations of none of those serum collectins were normally distributed (not shown), non-parametric tests were used for statistical calculations.

3.2 Associations of collectins with gestational age and birthweight

The concentrations of CL-10, CL-11 and MBL correlated weakly but significantly with gestational age [$r=0.175$, $p=0.00035$ (Supplementary Figure 4A); $r=0.13$, $p=0.0081$ (Supplementary Figure 4B); $r=0.114$, $p=0.015$ (Supplementary Figure 4C), respectively]. Since, to our knowledge, no reports concerning associations of CL-10 or CL-11 with GA have been published, we measured their concentrations (as well as MBL levels) in cord serum samples from 17 term babies (GA 38–41 weeks). As expected, those

relationships were confirmed ($r=0.164$, $p=0.00064$; $r=0.145$, $p=0.0026$; $r=0.13$, $p=0.005$ for CL-10, CL-11 and MBL, respectively).

Babies born at gestational age (GA) ≤ 32 weeks had lower CL-10 concentrations compared with those born at GA 33–37 weeks but not from term newborns, probably due to the low number of subjects in the last mentioned group (Figure 1A). However, Kruskal-Wallis test demonstrated a significant association between gestational age and CL-10 concentration (Figure 1A). The frequency of low values differed significantly between babies born at GA ≤ 32 and 33–37 weeks: 17/88 (19.3%) vs. 25/325 (7.7%). The statistical analysis is summarised in Table 2.

In contrast to CL-10, the median CL-11 level determined for preterms with shorter GA was significantly lower compared with term babies but not with preterms born at GA 33–37 weeks (Figure 2A). When Kruskal-Wallis test was performed, the median CL-11 concentrations in cord sera were significantly different in regard to GA (Figure 2A). Low concentrations were found more frequent among neonates born at ≤ 32 GA compared with those born at GA 33–37 weeks: 16/88 (18.2%) vs. 24/326 (7.4%) (Table 2).

The difference between median serum MBL in babies born at GA 24–32 and those born at GA 33–37 did not reach statistical significance (Figure 3A), nor was there any significant difference in frequency of low (<150 ng/ml) concentrations between those groups: 22/94 (24.5%) vs. 72/354 (20.3%) (Table 2).

The frequency of MBL deficiency-associated genotypes (LXA/O plus O/O) was similar in both preterm groups [11/112 (9.8%) vs. 50/423 (11.8%), Table 2]. Similarly, the numbers of carriers of *COLEC11* C/T or C/C (rs3820897) and A/G (rs7567833) genotypes barely differed between babies born at GA ≤ 32 and ≥ 33 weeks [28/87 (32.2%) vs. 100/328 (30.5%), and 7/87 (8%) vs. 27/329 (8.2%), $p=1.0$, respectively (Table 2).

The concentrations of CL-10 in cord sera correlated significantly with birthweight in premature babies born at GA 24–37 weeks ($r=0.218$, $p=0.000008$) (Supplementary Figure 4D). Similar associations were found for CL-11 ($r=0.169$, $p=0.00058$) (Supplementary Figure 4E) but not MBL ($r=0.062$, $p=0.19$) (Supplementary Figure 4F). Corresponding differences in median levels of CL-10 and CL-11 were apparent when preterm neonates were classified into low (<1500 g), medium (1500 g – 2499 g) and high (≥ 2500 g) BW groups. For MBL, median values from babies born with the lowest BW differed significantly from those of the highest BW. Individual concentrations, of tested collectins, depending on birthweight ranges are presented in Figure 1B (CL-10), Figure 2B (CL-11) and Figure 3B (MBL).

The frequency of low levels of collectin-10 among newborns with body mass at birth <1500 g (8/42, 19%) was significantly higher compared with that among newborns with BW ≥ 2500 g (9/164, 5.5%) (Table 2). CL-10 concentrations <10th percentile were also found in 23/204 (11.3%) of babies born with BW 1500–2499 g (no significant difference in comparison with afore-mentioned groups).

The lowest CL-11 levels in cord sera were observed in 9/42 (21.4%), 19/204 (9.3%) and 10/165 (6.1%) neonates, depending on the BW groups as defined. The differences were statistically significant between the low and high BW groups (Table 2).

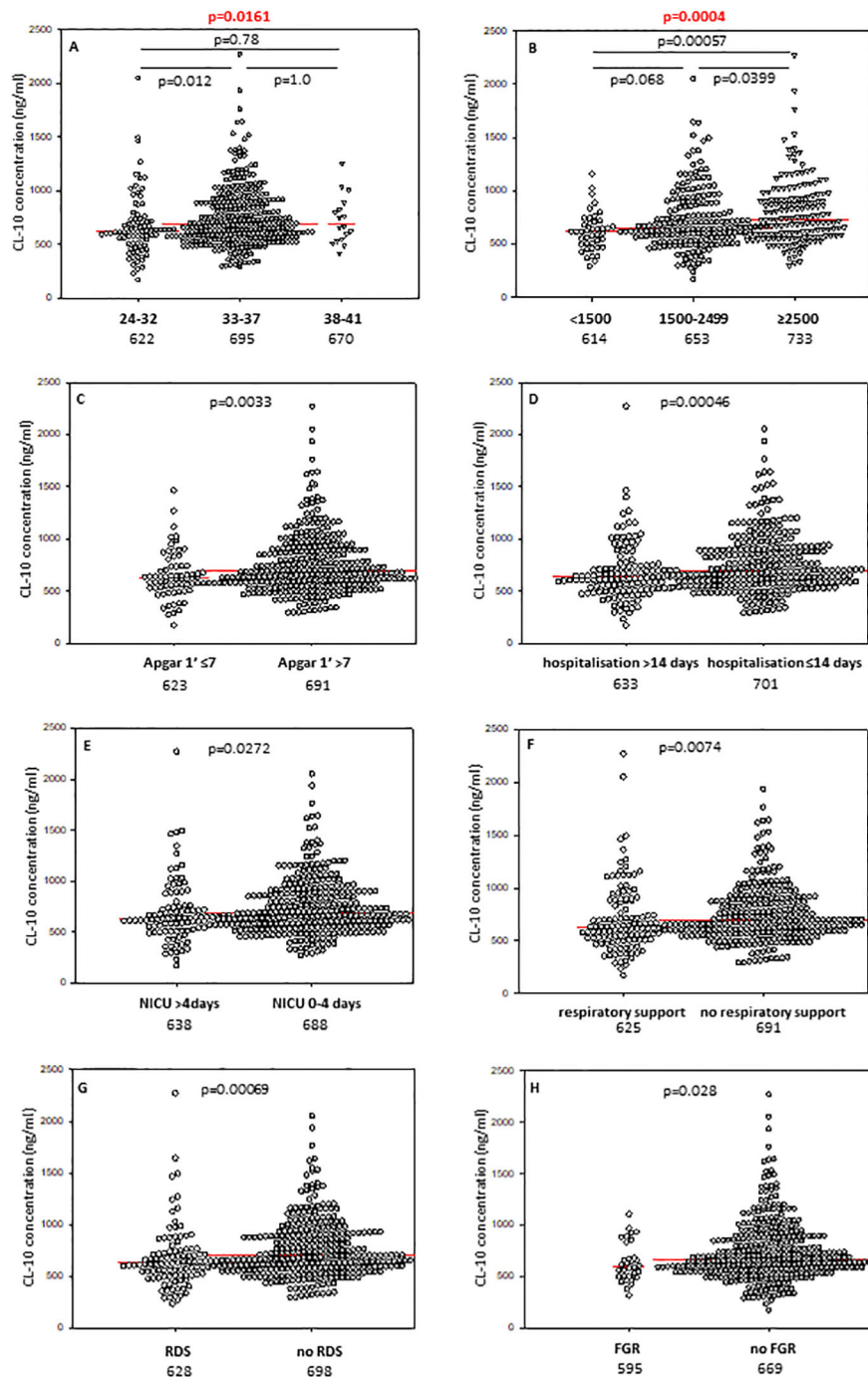


FIGURE 1

Individual concentrations of collectin-10, depending on gestational age (A), birthweight (B), 1' Apgar score (C), length of hospitalisation (D), length of intensive care (E), need for respiratory support (F), incidence of respiratory distress syndrome (G), and fetal growth restriction (H). Red bars represent median values (demonstrated as numbers below the graphs). Statistical significance values show comparisons with the use of Kruskal-Wallis test with *post hoc* Dunn test (A, B) or Mann-Whitney *U* test (C–H).

Moreover, low CL-11 was found more frequently among preterms with BW <1500 g than among those with BW 1500–2499 g [$p=0.033$, OR=2.66, 95% CI (1.11–6.37)]. No significant difference in the frequency of CL-11 concentrations <223 ng/ml was found between the medium and the high BW groups ($p=0.33$). However, multiple logistic regression analysis demonstrated an association of

low CL-11 in cord serum with low body mass at birth (OR>3.5) (Table 3).

No significant differences were observed for low MBL concentrations (<150 ng/ml) between low, medium and high BW groups: 27.7%, 21.1%, and 18.5% (statistical analysis concerning the first and the last mentioned groups is demonstrated in Table 2).

TABLE 2 Univariate analysis of associations of low concentrations of CL-10, CL-11 and MBL and selected polymorphisms of *COLEC11* and *MBL2* genes with clinical parameters and perinatal complications in preterm neonates (the significant associations are marked in red).

Clinical parameter/complication	Genotype/low collectin concentration in cord serum					
	Low CL-10 (<462 ng/ml vs. ≥462 ng/ml)	Low CL-11 (<223 ng/ml vs. ≥223 ng/ml)	<i>COLEC11</i> rs3820897 (C/T or T/T vs. C/C)	<i>COLEC11</i> rs7567833 A/G vs. A/A	Low MBL (<150 ng/ml vs. ≥150 ng/ml)	MBL primary deficiency (<i>MBL2</i> (XA/O or O/O vs. A/A or YA/O)
GA <33 weeks vs. ≥33 weeks	p=0.026 OR=2.87 (1.47-5.61)	p=0.0041 OR=2.8 (1.41-5.54)	p=0.79 OR=1.08 (0.65-1.8)	p=1.0 OR=0.98 (0.41-2.33)	p=0.57 OR=1.2 (0.7-2.06)	p=0.55 OR=0.81 (0.41-1.62)
BW <1500 g vs. ≥2500 g	p=0.0091 OR=4.05 (1.46-11.26)	p=0.0049 OR=4.23 (1.59-11.22)	p=0.26 OR=0.93 (0.45-1.93)	p=1.0 OR=1.11 (0.29-4.16)	p=0.22 OR=1.68 (0.8-3.55)	p=0.64 OR=0.73 (0.26-2.01)
1 st Apgar score <7 vs. ≥7	p=0.27 OR=1.56 (0.7-3.45)	p=0.25 OR=1.62 (0.73-3.6)	p=0.1 OR=1.52 (0.95-2.45)	p=0.68 OR=1.2 (0.55-2.61)	p=1.0 OR=1.0 (0.52-1.9)	p=1.0 OR=1.01 (0.45-2.24)
Hospitalisation >14 days vs. 0-14 days	p=0.045 OR=2.02 (1.05-3.9)	p=0.011 OR=2.4 (1.23-4.66)	p=0.25 OR=1.32 (0.85-2.06)	p=0.85 OR=1.1 (0.52-2.29)	p=1.0 OR=0.99 (0.61-1.61)	p=0.77 OR=1.09 (0.61-1.94)
NICU stay >4 days vs. 0-4 days	p=0.048 OR=2.09 (1.05-4.15)	p=0.16 OR=1.7 (0.84-3.46)	p=0.37 OR=1.28 (0.79-2.09)	p=0.67 OR=1.21 (0.55-2.69)	p=0.5 OR=1.2 (0.71-2.02)	p=0.23 OR=1.44 (0.8-2.62)
Respiratory support vs. no support	p=0.0019 OR=2.85 (1.48-5.48)	p=0.01 OR=2.41 (1.24-4.66)	p=0.046 OR=1.59 (1.01-2.49)	p=0.84 OR=1.04 (0.48-2.25)	p=0.44 OR=1.21 (0.74-1.99)	p=0.79 OR=0.92 (0.51-1.61)
RDS vs. no RDS	p=0.01 OR=2.46 (1.26-4.82)	p=0.051 OR=2.04 (1.02-4.05)	p=0.019 OR=1.76 (1.1-2.81)	p=0.68 OR=0.78 (0.33-1.86)	p=0.077 OR=1.59 (0.96-2.64)	p=0.059 OR=1.72 (0.97-3.05)
FGR vs. no FGR	p=0.35 OR=1.71 (0.62-4.72)	p=0.54 OR=1.37 (0.45-4.12)	p=0.24 OR=1.56 (0.74-3.27)	p=0.73 OR=1.25 (0.36-4.34)	p=1.0 OR=1.0 (0.48-2.09)	p=0.39 OR=1.4 (0.65-3.0)
pPROM vs. no pPROM	p=0.67 OR=1.23 (0.56-2.7)	p=0.83 OR=1.1 (0.49-2.49)	p=0.59 OR=1.67 (0.99-2.81)	p=0.039 OR=2.28 (1.06-4.89)	p=0.89 OR=1.04 (0.59-1.83)	p=0.88 OR=1.05 (0.54-2.06)
EOI vs. no EOI	p=0.17 OR=1.84 (0.86-3.97)	p=1.0 OR=0.94 (0.18-2.33)	p=0.77 OR=1.09 (0.62-1.91)	p=0.46 OR=1.43 (0.6-3.45)	p=0.29 OR=1.25 (0.67-2.32)	p=0.43 OR=0.67 (0.27-1.62)
Pneumonia vs. no pneumonia	p=0.43 OR=1.45 (0.53-3.94)	p=0.77 OR=1.13 (0.38-3.36)	p=0.58 OR=1.7 (0.84-3.45)	p=0.76 OR=0.62 (0.14-2.68)	p=0.41 OR=1.38 (0.64-2.95)	p=0.097 OR=0.32 (0.07-1.33)

It should be mentioned, that when associations of BW with levels of tested collectins in cord sera were analyzed separately in subgroups including neonates born at GA 24-32 weeks and 33-37 weeks, median CL-10 and CL-11 differed significantly in the second mentioned, between babies with GA <1500 g and ≥1500 g [507 ng/ml vs. 699 ng/ml, p=0.023 (Supplementary Figure 5D) and 253 ng/ml vs. 359 ng/ml, p=0.03 (Supplementary Figure 5E)]. However, no significant differences were found within shorter GA group (Supplementary Figures 5A, B). Furthermore, median MBL concentrations did not differ between babies born with body mass <1500 g and ≥1500 g either at GA 24-32 (Supplementary Figure 5C) or 33-37 weeks (Supplementary Figure 5F).

No appreciable differences were found for *COLEC11* polymorphisms. C/T or T/T genotypes (rs3820897) had 13 of 41

(31.7%) babies with BW <1500 g, 57/204 (27.9%) of those with BW 1500-2499 g and 55/165 (33.9%) newborns with body mass ≥2500 g. Correspondingly, frequencies of A/G heterozygosity at rs7567833 equalled 3/41 (7.3%), 19/205 (9.3%) and 11/165 (6.7%) (Table 2).

The distributions of LXA/O plus O/O *MBL2* genotypes were also similar in the BW groups [BW <1500 g: 5/55 (9.1%); BW 1500-2499 g: 32/278 (11.5%) and BW ≥2500: 24/199 (12.1%) (Table 2).

3.3 Concentrations of collectins in cord sera from twins

The concentrations of tested collectins were compared between twins. Despite no differences in birthweight (p>0.05), median levels

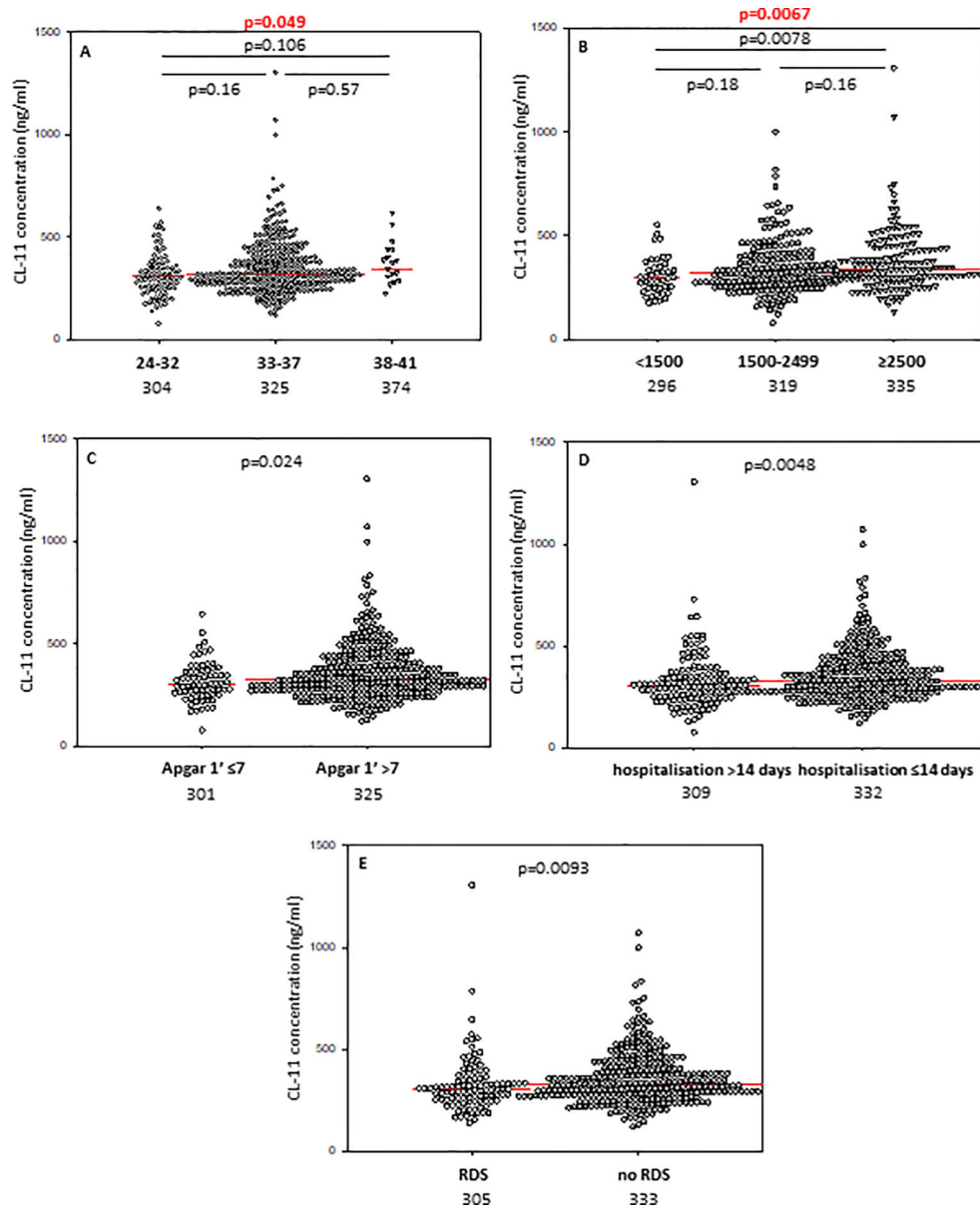


FIGURE 2

Individual concentrations of collectin-11, depending on gestational age (A), birthweight (B), 1' Apgar score (C), length of hospitalisation (D), and incidence of respiratory distress syndrome (E). Red bars represent median values (demonstrated as numbers below the graphs). Statistical significance values show comparisons with the use of Kruskal-Wallis test with *post hoc* Dunn test (A, B) or Mann-Whitney *U* test (C–E).

of CL-10, CL-11 and MBL differed significantly between twins of different sex (data from 12, 12 and 10 pairs, respectively) (Figure 4A) and of the same sex (37, 37, 27 pairs, respectively) (Figure 4B). The differences were still significant between monozygotic-diamniotic (MCDA) twins (9, 9, and 6 pairs, respectively) (Figure 4C).

3.4 Associations of collectins with 1' Apgar score

Median concentrations of both collectin-10 and collectin-11 in cord sera were found to be lower in preterms with 1' Apgar scores

<7 compared with babies scored ≥7 ($p<0.05$) (Figures 1C, 2C, respectively). However, when Bonferroni correction was applied, the difference for CL-11 occurred insignificant ($p>0.0167$) (Figure 2C). Despite a similar trend, MBL levels did not differ significantly between those groups (Supplementary Figure 6A).

When proportions with low concentrations were compared, there were no significant differences: 9/69 (13%) vs. 29/329 (8.8%) (CL-10); 9/69 (13%) vs. 28/330 (8.5%) (CL-11); 14/68 (20.6%) vs. 69/335 (20.6%) (MBL) (more details are presented in Table 2).

Thirty-eight/102 (37.3%) and 10/103 (9.7%) babies scored <7 carried at least 1 variant (T) allele at rs3820897 and/or were A/G heterozygotes (rs7567833), respectively, while corresponding frequencies in preterms with higher scores equalled 82/292

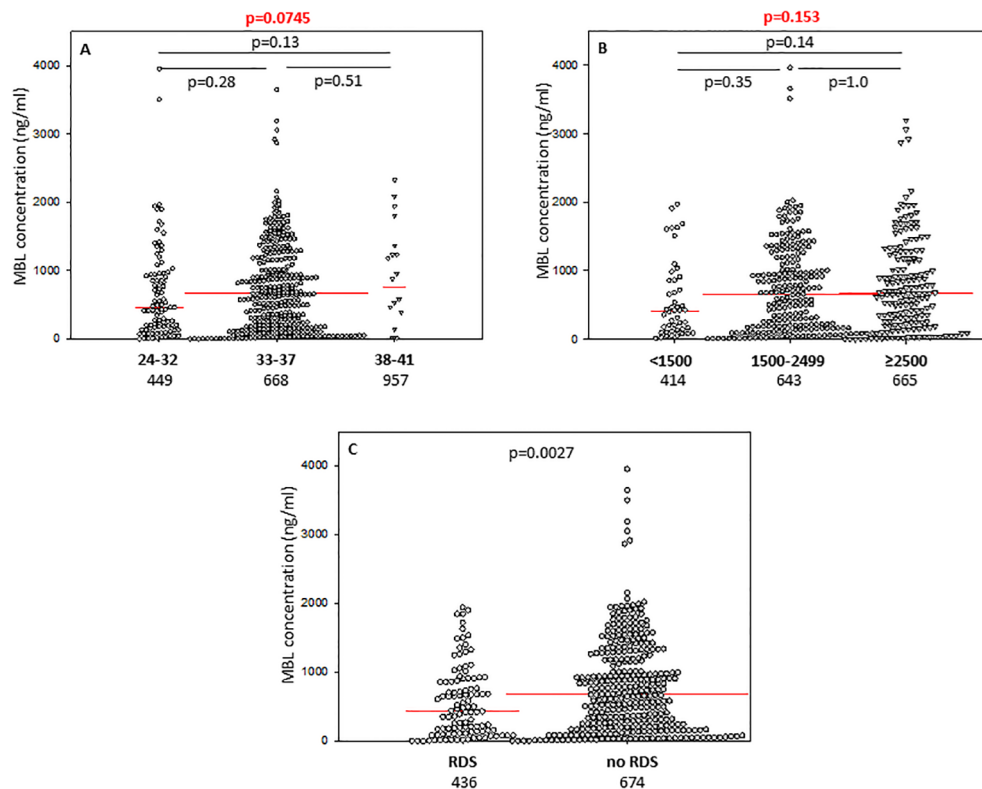


FIGURE 3

Individual concentrations of mannose-binding lectin, depending on gestational age (A), birthweight (B), and incidence of respiratory distress syndrome (C). Red bars represent median values (demonstrated as numbers below the graphs). Statistical significance values show comparisons with the use of Kruskal-Wallis test with *post hoc* Dunn test (A, B) or Mann-Whitney U test (C).

(28.7%) and 24/292 (8.7%) (no significant differences, Table 2). It should be stressed, however, that multiple logistic regression analysis revealed a significant relationship of rs3820897 with low 1' Apgar score (OR>1.8) (Table 3). No impact of genetically-determined MBL deficiency was found: XA/O or O/O *MBL2* genotypes were carried in 8/78 (10.3%) subjects who scored 1-6 and 41/402 (10.2%) of those who scored 7-10 (Table 2).

3.5 Associations of collectins with length of hospitalisation and need for intensive care

The median concentrations of both CL-10 and CL-11 in preterm neonates who stayed in hospital for more than two weeks were significantly lower in comparison with those who left the neonatal ward earlier (Figures 1D, 2D, respectively). Furthermore, low (<462 ng/ml) collectin-10 levels were observed more frequently among babies hospitalised for >14 days [19/135 (14.1%) vs. 21/280 (7.5%), Table 2]. Similarly, a significant difference was observed for collectin-11 [20/135 (14.5%) vs. 19/281 (6.8%), Table 2].

In contrast, MBL concentrations seemed not to be associated with a need for prolonged hospitalisation (Supplementary Figure 6B). Frequency of low MBL (<150 ng/ml) levels did not

differ markedly between those groups [31/145 (21.4%) vs. 63/293 (21.5%), Table 2].

Interestingly, CL-10 (but not CL-11 or MBL) concentrations in cord sera were associated with need-for-stay in the NICU. The median CL-10 level in babies who required intensive care was markedly lower compared with newborns who did not. The difference, however, lost significance when Bonferroni correction was applied (Supplementary Figure 7A). No similar relationship was noted in the case of CL-11 (Supplementary Figure 7B) or MBL (Supplementary Figure 7C). The differences in frequency of low concentrations for CL-10, CL-11, and MBL were not statistically significant: 20/146 (13.7%) vs. 20/262 (7.6%), 18/146 (12.3%) vs. 21/263 (8%), 35/160 (21.9%) vs. 60/284 (21.1%), respectively (Table 2).

When data from preterms who required prolonged (>4 days) intensive care were compared with results from newborns who spent 0-4 days in NICU (all survivors), a similar association of CL-10 was found (Figure 1E). Furthermore, low CL-10 concentrations were more common among patients requiring prolonged NICU stay when compared with the reference group [15/97 (15.5%) vs. 25/311 (8%), Table 2]. That association has moreover been confirmed by multiple logistic regression analysis (Table 3). However, no similar relationships were found with CL-11 (Supplementary Figure 8A) or MBL (Supplementary Figure 6C). None of the genetic polymorphisms investigated were associated with length of hospitalisation or with stay in NICU. The frequencies of

TABLE 3 Multiple logistic regression analysis of associations of low concentrations of CL-10, CL-11 and MBL and selected polymorphisms of *COLEC11* and *MBL2* genes with clinical parameters and perinatal complications in preterm neonates (the significant associations are marked in red).

Dependent variables	Independent variables					
	Low CL-10 (<462 ng/ml vs. ≥462 ng/ml)	Low CL-11 (<223 ng/ml vs. ≥223 ng/ml)	<i>COLEC11</i> rs3820897 (C/T or T/T vs. C/C)	<i>COLEC11</i> rs7567833 A/G vs. A/A	Low MBL (<150 ng/ml vs. ≥150 ng/ml)	MBL primary deficiency (<i>MBL2</i> (XA/O or O/O vs. A/A or YA/O)
GA <33 weeks	p=0.37 OR=1.55 (0.6-3.99)	p=0.11 OR=2.2 (0.84-5.78)	p=0.75 OR=1.09 (0.63-1.88)	p=0.88 OR=1.07 (0.44-2.62)	p=0.28 OR=1.45 (0.74-2.82)	p=0.51 OR=0.75 (0.32-1.77)
BW <1500 g	p=0.91 OR=0.93 (0.26-3.33)	p=0.04 OR=3.55 (1.06-11.92)	p=0.91 OR=0.96 (0.45-2.03)	p=0.93 OR=0.95 (0.27-3.34)	p=0.29 OR=1.6 (0.67-3.84)	p=0.52 OR=0.68 (0.21-2.22)
1 st Apgar score <7	p=0.95 OR=1.04 (0.33-3.25)	p=0.3 OR=1.82 (0.59-5.63)	p=0.043 OR=1.81 (1.02-3.2)	p=0.42 OR=1.45 (0.59-3.54)	p=0.54 OR=1.27 (0.59-2.7)	p=0.8 OR=0.88 (0.34-2.31)
Hospitalisation >14 days	p=0.67 OR=1.21 (0.5-2.97)	p=0.057 OR=2.43 (0.97-6.06)	p=0.65 OR=1.12 (0.7-1.78)	p=0.88 OR=0.94 (0.43-2.07)	p=0.97 OR=0.99 (0.54-1.82)	p=0.89 OR=1.05 (0.5-2.21)
NICU stay >4 days	p=0.028 OR=2.86 (1.12-7.34)	p=0.68 OR=0.8 (0.29-2.25)	p=0.39 OR=1.26 (0.75-2.12)	p=0.48 OR=1.35 (0.59-3.06)	p=0.44 OR=1.29 (0.68-2.48)	p=0.81 OR=1.1 (0.5-2.43)
Respiratory support	p=0.005 OR=3.72 (1.5-9.27)	p=0.89 OR=0.93 (0.35-2.49)	p=0.035 OR=1.68 (1.04-2.72)	p=0.73 OR=1.15 (0.52-2.57)	p=0.64 OR=1.16 (0.62-2.17)	p=0.91 OR=1.04 (0.49-2.22)
RDS	p=0.003 OR=4.24 (1.63-11.05)	p=0.57 OR=0.74 (0.26-2.11)	p=0.01 OR=1.95 (1.18-3.24)	p=0.8 OR=0.89 (0.36-2.18)	p=0.93 OR=1.03 (0.53-1.99)	p=0.026 OR=2.33 (1.11-4.89)
FGR	p=0.3 OR=2.2 (0.5-9.73)	p=0.99 OR=0.988 (0.2-4.95)	p=0.3 OR=1.53 (0.68-3.43)	p=0.86 OR=0.87 (0.2-3.91)	p=0.13 OR=0.39 (0.11-1.34)	p=0.11 OR=2.59 (0.81-8.23)
pPROM	p=0.48 OR=1.47 (0.51-4.24)	p=0.87 OR=0.91 (0.29-2.83)	p=0.35 OR=1.3 (0.76-2.24)	p=0.027 OR=2.41 (1.11-5.28)	p=0.83 OR=0.92 (0.45-1.9)	p=0.63 OR=1.23 (0.53-2.86)
EOI	p=0.034 OR=3.07 (1.09-8.67)	p=0.15 OR=0.41 (0.12-1.39)	p=0.69 OR=1.13 (0.63-2.04)	p=0.68 OR=1.22 (0.48-3.11)	p=0.41 OR=1.37 (0.66-2.84)	p=0.35 OR=0.62 (0.23-1.68)
Pneumonia	p=0.84 OR=1.15 (0.29-4.61)	p=0.99 OR=0.993 (0.23-4.33)	p=0.63 OR=1.2 (0.57-2.51)	p=0.56 OR=0.65 (0.15-2.84)	p=0.15 OR=1.94 (0.8-4.71)	p=0.11 OR=0.28 (0.06-1.32)

COLEC11 C/T or T/T genotypes, A/G heterozygosity and MBL deficiency-associated genotypes were 47/136 (34.6%), 12/136 (8.8%) and 20/171 (11.7%) when preterms were hospitalised for more than 2 weeks and 77/270 (28.5%, $p=0.25$), 22/271 (8.1%, $p=0.85$) and 38/351 (10.8%, $p=0.77$), when their stay in hospital was shorter (Table 2).

Among babies who underwent intensive care, 52/143 (36.4%) carried C/T or T/T genotypes (*COLEC11* rs3820897), 15/143 (10.5%) – A/G genotype (rs7567833) and 22/190 (11.6%) had MBL-deficient genotypes. Corresponding frequencies in newborns who did not stay in NICU equalled 73/261 (28%, $p=0.092$), 19/262 (7.3%, $p=0.266$) and 36/341 (10.6%, $p=0.72$). No significant

differences in distribution of studied genotypes were observed in regard to prolonged intensive therapy: 33/94 (35.1%) vs. 92/310 (39.7%), $p=0.37$ (*COLEC11* C/T or T/T); 9/94 (9.6%) vs. 25/311 (8%), $p=0.67$ (*COLEC11* A/G); 18/130 (13.8%) vs. 40/399 (10%), $p=0.23$ (*MBL2* XA/O or O/O) (Table 2).

3.6 Associations of collectins with need for respiratory support

Cord serum levels of CL-10 were significantly lower in preterm newborns requiring respiratory support compared with

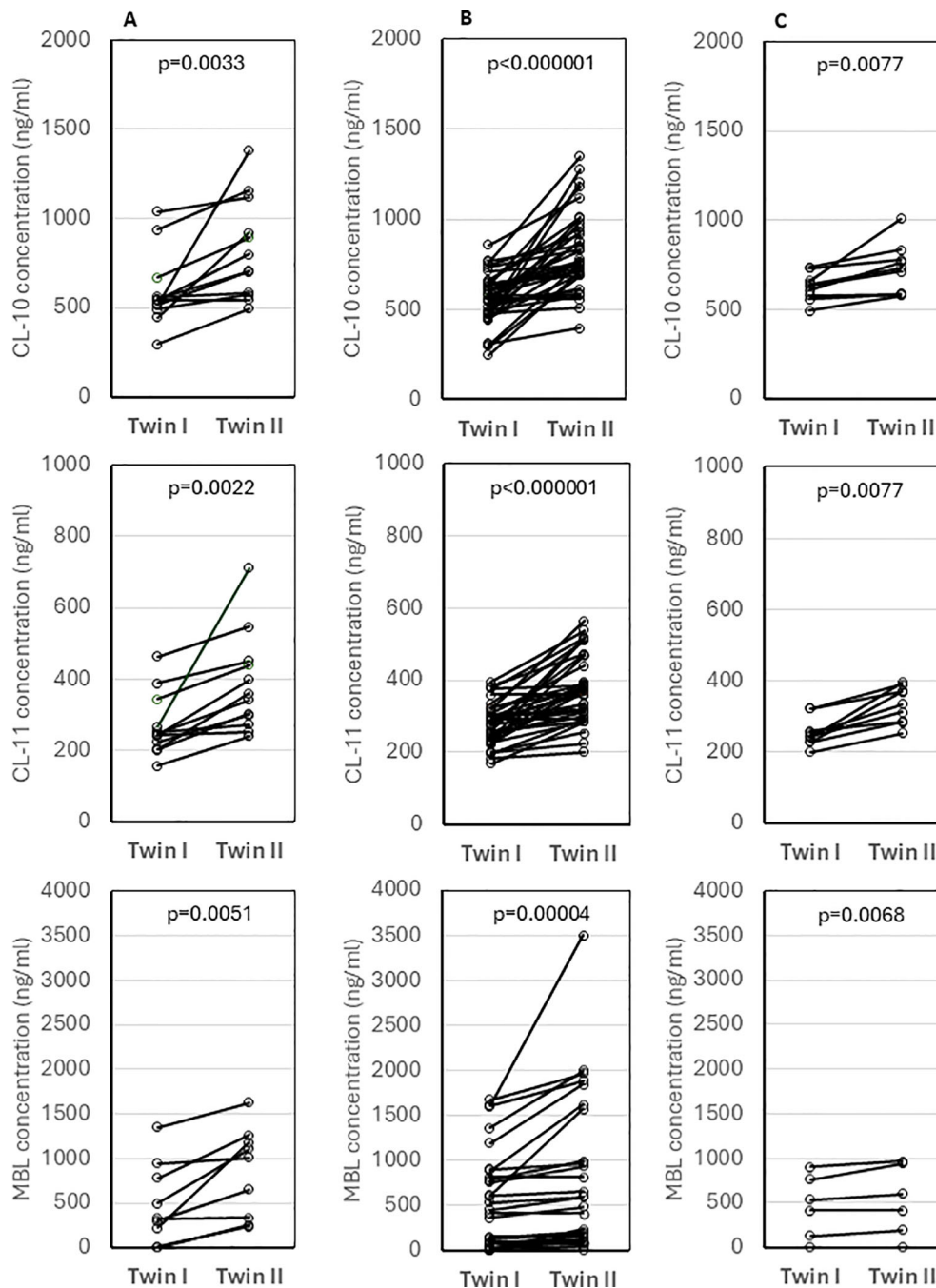


FIGURE 4

Comparison of concentrations of collectin-10, collectin-11 and mannose-binding lectin between twins of different sex (A), twins of the same sex (B) and monozygotic-dizygotic twins (C). Statistical significance values show comparisons with the use of Wilcoxon matched-pairs test.

spontaneously breathing neonates (Figure 1F). A similar, but statistically non-significant, trend was observed for CL-11 (Supplementary Figure 8B) and MBL (Supplementary Figure 6D).

Low concentrations of CL-10 were more common among babies who needed respiratory support compared with those who did not [21/122 (17.2%) vs. 20/294 (6.8%), Table 2]. This relationship was confirmed by multiple logistic regression analysis ($OR>3.7$) (Table 3). A similar association was found for CL-11 [19/122 (15.6%) vs. 21/295 (7.1%)] but not MBL [30/128 (23.4%) vs. 64/318 (20.1%)] (Table 2). Consequently, neonates with low CL-10 or

CL-11 needed longer respiratory support [mean 4.7 days vs. 2.2 days for low CL-10 ($p=0.0059$) and 4.3 days vs. 2.3 days for low CL-11 ($p=0.026$)]. Low MBL concentration in cord serum had no impact on the length of respiratory support (2.8 days vs. 2.3 days, $p=0.22$).

The *COLEC11* rs3820897 SNP could be weakly associated with the need for respiratory support: the C/T or T/T genotypes were found more frequently in the affected, compared with the reference group [45/117 (38.5%) vs. 83/294 (28.2%), Table 2]. Again, this finding was confirmed in multiple logistic regression ($OR=1.69$, Table 3). No such relationship was found in the case of *COLEC11*

rs7567833 heterozygosity [10/118 (8.5%) vs. 24/294 (8.2%)] or MBL primary deficiency [17/156 (10.9%) vs. 44/376 (11.7%)] (Table 2).

3.7 Associations of collectins with respiratory distress syndrome

Preterm newborns who suffered from RDS had significantly lower median concentrations of CL-10, CL-11 and MBL in cord sera than babies without that complication. Those associations remained significant after correction for multiple comparisons. The individual values, depending on RDS incidence are presented in Figure 1G (CL10), Figure 2E (CL-11) and Figure 3C (MBL). Intriguingly, median CL-10 and CL-11, differed significantly in babies born at GA ≥ 33 weeks but not in those born at GA < 33 weeks (Supplementary Figures 7D, E). An opposite relationship was found for MBL (Supplementary Figure 6F). Corresponding associations were observed depending on birthweight: RDS patients with BW ≥ 1500 g had lower levels of both CL-10 and CL-11 compared with unaffected babies (Supplementary Figures 7G, H) while MBL concentration differed in the other (BW < 1500 g) group (Supplementary Figure 7I).

Low CL-10 concentrations were observed more frequently among neonates with than without RDS [17/104 (16.3%) vs. 23/313 (7.3%), Table 2] and multiple regression analysis confirmed low CL-10 to be a risk factor for disease development (OR > 4.2) (Table 3). Similar, but statistically non-significant, trends were found for CL-11 [15/104 (14.4%) vs. 24/314 (7.6%)] and MBL [29/107 (27.1%) vs. 64/338 (18.9%)] (Table 2).

Interestingly, as many as 41/101 (40.6%) preterm newborns who developed RDS were carriers of the variant T allele at *COLEC11* rs3820897 polymorphism, compared with 87/311 (29%) of those who did not (significant difference, see Table 2). That association was confirmed in multiple logistic regression analysis (OR = 1.69, Table 3), but G allele carriers at rs7567833 did not differ significantly in that regard [7/101 (6.9%) vs. 27/311 (8.7%), Table 2]. MBL deficiency-associated genotypes were commoner among babies who developed RDS [21/131 (16%) vs. 40/401 (10%), Table 2]. That relationship reached statistical significance after multiple analysis (OR = 2.3, Table 3). Conversely, A/A genotypes were significantly less common in RDS compared with the reference group [57.3% vs. 68.3%, OR = 0.62, 95 CI (0.41–0.93), $p = 0.021$].

3.8 Associations of collectins with fetal growth restriction

The median concentration of collectin-10 in cord sera from preterm neonates diagnosed with fetal growth restriction was significantly lower than in eutrophic newborns (Figure 1H). However, the difference lost statistical significance when correction for multiple comparisons was applied ($p > 0.0167$). Although a similar trend was observed in the case of CL-11, the difference was not statistically significant (Supplementary Figure 8C). Median MBL was slightly higher in the grow

restriction group, in comparison with the reference group (Supplementary Figure 6E).

No significant differences were found when the distribution of low serum levels was compared with regard to FGR: 5/32 (15.6%) vs. 36/369 (9.8%) (CL-10); 4/32 (12.5%) vs. 35/370 (9.5%) (CL-11); 10/47 (21.3%) vs. 85/399 (21.3%) (MBL) (Table 2).

Similarly, genotype frequencies of both *COLEC11* or *MBL2* did not differ significantly between preterms diagnosed with FGR and those with no FGR: 13/32 (40.6%) vs. 111/364 (30.5%), (C/T or T/T, *COLEC11* rs3820897); 3/32 (9.4%) vs. 28/365 (7.7%) (A/G, *COLEC11* rs7567833); 9/62 (14.5%) vs. 51/470 (10.9%) (XA/O or O/O, *MBL2*) (Table 2).

3.9 Associations of collectins with preterm premature rupture of membranes

The median concentrations of CL-10, CL-11 and MBL were similar in babies from pregnancies with and without premature rupture of membranes (Supplementary Figures 7J–L, respectively). Also, the proportions of low serum values for all three collectins did not differ appreciably between the groups: 9/79 (11.4%) vs. 32/339 (9.4%) (CL-10); 8/79 (10.1%) vs. 32/340 (9.4%) (CL-11); 19/88 (21.6%) vs. 75/358 (20.9%) (MBL) (Table 2).

However, heterozygosity for *COLEC11* rs7567833 was carried more frequently by babies born with pPROM compared with newborns without [11/77 (14.3%) vs. 23/337 (6.8%), Table 2]. That association was confirmed in multiple logistic regression analysis (OR > 2.4 , Table 3). No such relationships were found for the other collectin genotypes investigated [26/77 (33.8%) vs. 102/336 (30.4%), (*COLEC11* rs3820897); 12/101 (11.9%) vs. 49/432 (11.3%) (*MBL2* XA/O or O/O)] (Table 2).

3.10 Associations of collectins with early-onset infections

The concentrations of CL-10, CL-11 and MBL seemed not to be associated with susceptibility to early-onset perinatal infections (Supplementary Figures 7M–O, respectively). No appreciable differences were noted in frequencies of low concentrations of tested proteins, although a trend was apparent for CL-10 [10/66 (15.2%) vs. 31/351 (8.8%), Table 2]. Moreover multiple regression analysis confirmed that association (OR > 3) (Table 3). Corresponding frequencies for CL-11 [6/66 (9.1%) vs. 34/352 (9.7%)] or MBL 16/67 (23.9%) vs. 70/348 (20.1%)] occurred virtually the same (Table 2).

No significant differences were observed in the distribution of genotypes investigated [21/65 (32.3%) vs. 106/347 (30.5%) (C/T or T/T, *COLEC11* rs3820897); 7/65 (10.8%) vs. 27/348 (7.8%) (A/G, *COLEC11* rs7567833); 6/76 (7.9%) vs. 48/421 (11.4%) (XA/O or O/O, *MBL2*)] (Table 2).

Additionally, neither concentrations of collectins nor frequencies of variant alleles of corresponding genes differed significantly between preterms with pneumonia and those without (Table 2).

3 11 Expression of collectins in placenta

The expression of *COLEC10*, *COLEC11* and *MBL2* genes was analysed in 35 (GA 30–41, mean 36.5) placenta samples from live births. *MBL2* mRNA was found in 27, while the others tested genes were expressed at detectable levels in all placentas. The expression of *COLEC10* and *COLEC11* (but not *MBL2*) was significantly higher in the maternal, in comparison with the fetal part of the placenta. Detailed data are presented in Table 4. Significant correlations were observed between mRNA levels in maternal and fetal parts [$r=0.44$, $p=0.0091$ (Supplementary Figure 9A), $r=0.483$, $p=0.0038$ (Supplementary Figure 9B) and $r=0.676$, $p=0.00008$ (Supplementary Figure 9C), respectively].

4 Discussion

Both CL-10 and CL-11 are thought to be important for fetal development as mutations of *COLEC10* and *COLEC11* genes have been reported in patients diagnosed with 3MC syndrome [reviewed in (10)]. However, no data have been published previously concerning possible relationships of those collectins with spontaneous preterm births and their associated complications. Much more data are available about MBL in neonatal health and disease [reviewed in (5, 6)].

The well-established relationship between *MBL2* genotype and MBL concentration (21) was confirmed. We found no such relationship concerning the *COLEC11* rs3820897 (promoter, -9570 C>T) SNP, previously suggested to influence the serum level of CL-11 (16). The difference might reflect age-dependent mechanisms of regulation of gene expression, including the effect of sex hormones. We also found no influence on the serum concentration of another polymorphism (rs7567833, +39618 A>G, His219Arg), and in that regard, our two studies are in agreement (16). Although PolyPhen-2 tool (<http://genetics.bwh.harvard.edu/pph2/>) classified that SNP as “benign”, it cannot be excluded that amino acid exchange affects CL-11 binding affinity (16). Despite no impact of tested polymorphisms on collectin-11 levels, their clinical significance cannot be excluded: the minor alleles at rs7567833 and rs3820897 were more frequent among pPROM cases and among preterms requiring respiratory support, respectively.

Intriguingly, the median concentration of CL-11 in cord sera from preterm neonates was exactly the same as in sera from healthy

adults (0.32 µg/ml) while CL-10 levels differed markedly: 0.63 µg/ml reported here for babies and 1.87 µg/ml for blood donors (16), despite highly significant CL-10/CL-11 correlation in both studies ($r=0.91$ and $r=0.741$, respectively). That may suggest age-dependent differences in *COLEC10* gene expression. Such differences have previously been reported for other complement-activating lectins as MBL, ficolins as well as for associated serine protease MASP-2 (23, 28). Vinuela et al. identified as many as 137 genes expressed differentially with age (independently of environmental factors) what, among others, may reflect different genetic regulation or mRNA splicing (24). As mentioned, *COLEC10* rs149331285 and rs148350292 were not submitted for statistical analysis due to the lack or low frequency of variant alleles, respectively. Based on data from 296 Danish blood donors, their corresponding MAF were previously reported as 0.007 (rs149331285) and 0.005 (rs148350292) (16). Here, we analyzed a larger, specific group (preterm neonates) from another (Polish) population which might be considered a reason for the differences noted (MAF<0.001 and MAF=0.002, respectively).

It has long been known that twins may have dramatically different cord MBL concentrations (25), as illustrated in Figure 4. In dizygotic siblings, this is obviously due to the inheritance of different *MBL2* genotypes. This could be considered evidence that cord MBL is derived exclusively from the fetus and not from the maternal circulation. However, marked differences in MBL, CL-10 and CL-11 concentrations in monozygotic-diamniotic twin pairs were also noted, therefore, epigenetic mechanisms of regulation of their expression cannot be excluded. Even in monozygotic pregnancies, the umbilical cords may, for example, differ, to some extent, in maternal blood flow rate what may in turn affect transportation of nutrients, hormones, etc. It should be however stressed that Husby et al. (26) reported highly significant correlation between MBL levels in both monozygotic and dizygotic twins of the same sex, aged 6–9 years ($r=0.97$, $p<0.01$ and $r=0.22$, $p<0.01$, respectively). They suggested the importance of non-additive genetic and non-shared environmental factors, and estimated heritability to equal 0.96 [95% CI (0.92–0.97)] (26).

Despite significant correlations of CL-10, CL-11 and MBL concentrations with gestational age, only CL-10 differed significantly between preterms with shorter (<33 weeks) and longer GA (33–37 weeks). On the other hand, low CL-11 concentrations were almost as common as low CL-10 amongst babies born at GA ≤32. The majority of, but not all, earlier studies concerning MBL demonstrated a significant correlation between MBL levels and gestational age and/or lower average concentrations in preterm compared with term neonates (22, 27–31). In contrast, in our previous investigation, neither significant correlation of MBL with GA nor a greater difference in median MBL concentration in cord sera between term and preterm newborns was noted (21). Probably, reported here and elsewhere, the correlations reflect rather differences in ability of fetal liver to produce collectins and release them into the blood, depending on gestational age than direct cause-and-effect relationship between low protein concentration and shortened GA. However, the median CL-10 concentration in cord sera was found to be relatively low within the group of preterms affected by fetal growth restriction.

Here, we found no impact of *COLEC10*, *COLEC11* or *MBL2* polymorphisms on gestational age. Only *MBL2* has been previously

TABLE 4 The comparison of expression of mRNA from the *COLEC10*, *COLEC11* and *MBL2* genes in maternal and fetal parts of placentas from live births.

Gene	mRNA expression level (median $2^{-\Delta C_t}$)		Statistical significance
	maternal part	fetal part	
<i>COLEC10</i>	1.232×10^{-2}	6.39×10^{-3}	$p=0.005$
<i>COLEC11</i>	1.253×10^{-3}	5.265×10^{-4}	$p<0.0001$
<i>MBL2</i>	7.533×10^{-3}	1.318×10^{-3}	$p>0.05$

studied in that respect. Some papers reported differences in allele distribution between preterm and term newborns (21, 32, 33), while others indicated no such relationship (30, 34). Our data presented here, concerning preterms only, seem to confirm those published by Frakking et al. (30) and Grumach et al. (34).

For the first time, we found significant relationships between concentrations of CL-10 and CL-11 in cord sera and birthweight (Figures 1B, 2B), but not with *COLEC10* or *COLEC11* polymorphisms. That, at least to some extent, may reflect differences in size of liver, being the main site of synthesis of investigated collectins. However, MBL concentration did not correlate with birthweight and this adds to an inconsistent literature. For example, Hartz et al. (35) found no association of MBL with very low birthweight (VLBW), while Cakmak et al. (36) found the opposite.

We report here associations of CL-10 and, to a lesser extent, CL-11 with a poor Apgar score and an extended hospital stay. There is no proof that those relationships are causal, but they could be an indication of an important role in embryogenesis and/or early ontogenesis.

A major finding of this study has been the clear association of CL-10, CL-11 and MBL with respiratory distress syndrome (RDS). RDS is considered to be a leading aetiology of neonatal respiratory failure (NRF) and the main cause of NRF-related deaths (37). It is a multifaceted and intricate disease that typically manifests in newborns with lung underdevelopment and deficiency of lung surfactant. It is closely associated with the onset of bronchopulmonary dysplasia (BPD) and numerous other adverse events (38), and may even lead to respiratory problems in adulthood. Ours is the first study to relate CL-10 and CL-11 to RDS, but MBL has previously been investigated by others (39, 40). Since we previously also found low concentrations of ficolin-2 in RDS patients (from the same cohort), it may be supposed that impaired activation of complement *via* the lectin pathway contributes to the development of this complication. On the other hand, we cannot exclude the consumption of collectins/ficolins due to the involvement of complement in RDS pathogenesis or (considering the relative high frequency of primary MBL deficiency in patients), both mentioned mechanisms. The different role of MBL compared with CL-10 or CL-11 might be assumed based on different GA and BW ranges in which their significance is observed. Lower concentrations of intact C4 and higher - of complement activation products in neonatal RDS were reported and proposed to associated with poor response to surfactant treatment (41–43).

Numerous studies have addressed *MBL2* genotypes and/or serum MBL concentrations in relation to perinatal infections, including sepsis. Diversely designed studies of various cohorts/populations often led to contradictory conclusions: some suggested MBL deficiency was a risk factor, while others found no role for that collectin [reviewed in (5, 6)]. We found no association between *MBL2* polymorphisms or MBL in cord sera and early onset infections (including several cases of sepsis) in preterms. The multicentre studies, with the use of an unified method for measurement of MBL concentration and determination of the same set of polymorphisms might address the mentioned inconsistencies and lead to *consensus*. However, differing frequencies of variant alleles for commonly tested *MBL2*

polymorphisms in various ethnic groups may complicate it. Although a possible association with low CL-10 concentration was noted, we found little evidence for an anti-microbial role for those collectins before birth. On the other hand, low level of CL-10 seemed to predict the need for prolonged hospitalisation (including NICU), not necessarily associated with infection, and the need for respiratory support. That may suggest its particularly important role in keeping neonatal homeostasis. Although, in general, complement-activating collectins studied here are closely related both structurally and functionally, they may play distinct roles (and therefore different clinical associations) due to their different affinity, hepatic and extra-hepatic expression, ability (or its lack) to form heterocomplexes, *etc.*

The complement system is thought to be important for placental and fetal development from implantation (44). Here, we have reported expression of *COLEC10*, *COLEC11* and *MBL2* genes at the mRNA level in both preterm and term placentas. This confirms the work of others (45–47) who described the expression of *COLEC10* and *COLEC11* genes as well as corresponding proteins in full-term placentas. However, for the first time, we reported significant differences between expression levels of the mentioned genes in maternal and fetal parts. At least in the case of *COLEC10* gene that might be a reflection of generally higher expression in adults compared with neonates (*vide* difference in median concentration of CL-10 in the sera, not noted for CL-11). According to data from Genotype-Tissue Expression (GTEx) Portal (www.gtexportal.org), *COLEC11* expression (in contrast to *COLEC10*) is relatively high in the ovary (but, on the other hand, low in the uterus in both cases). Verification of the hypothesis whether the mentioned phenomena explain our findings needs however further investigation. Our data are moreover consistent with the observations of Kilpatrick et al. (48) that MBL protein can be detected in first-trimester placenta and Yadav et al. (49), who found *MBL2* transcript in term placentas. The presence of *COLEC10* and *COLEC11* (in contrast to *MBL2*) transcripts in all tested samples seems to confirm particular importance of collectin-10 and collectin-11 during embryogenesis.

5 Conclusions

Both CL-10 and CL-11 may be important for human embryo-/ontogenesis, based on associations of their gene mutations with 3MC syndrome. We have found for the first time a relationship between low concentrations of CL-10 in cord sera and very premature births at GA ≤ 32 weeks. Both CL-10 and CL-11 were found to be associated with very low birthweight, low Apgar 1' score, and prolonged hospitalisation. Lower median CL-10 was associated with fetal growth restriction, while *COLEC11* heterozygosity for a polymorphism affecting CL-11 activity was more common in pPROM, compared with the corresponding reference groups. Both CL-10 and CL-11 were found to be associated with RDS, and CL-10 may influence susceptibility to early-onset infections. MBL was also associated with RDS, but did not share the other clinical correlates of CL-10/CL-11. These complement-activating collectins [as well as ficolin-2 (12–14)]

may be important for keeping homeostasis in preterms. The mentioned numerous associations have, however, to be confirmed in an independent study to verify the usefulness of those innate immunity factors as prognostic/diagnostic markers, possibly in a common panel.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Bioethics Committee of Poznań University of Medical Sciences, Independent Bioethics Committee for Scientific Research at Medical University of Gdańsk, Bioethics Committee of The Medical University of Łódź. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

GG: Conceptualization, Funding acquisition, Investigation, Project administration, Writing – review & editing. SH: Investigation, Methodology, Validation, Writing – review & editing. DJ: Investigation, Methodology, Writing – review & editing. MK-B: Investigation, Writing – review & editing, Data curation. AS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. PK: Data curation, Investigation, Writing – review & editing. AS-P: Investigation, Writing – review & editing. KC: Data curation, Investigation, Writing – review & editing. KS: Data curation, Investigation, Writing – review & editing. ID-P: Data curation, Investigation, Writing – review & editing. JM: Data curation, Investigation, Writing – review & editing. JK: Data

curation, Investigation, Writing – review & editing. ST: Investigation, Methodology, Writing – review & editing. MC: Conceptualization, Data curation, Formal analysis, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1463651/full#supplementary-material>

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Africanized honeybee venom (*Apis mellifera*) promotes human complement activation split products storm

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Introduction: Complement activation split products are signatures of many immunopathological disorders. Among the laboratory findings observed in these diseases, a reduction in the level of circulating intact complement components can be mentioned, and this change has also been detected in envenomation by multiple Africanized honeybee (*Apis mellifera*) stings. Although envenomation by these animals elicits diverse life-threatening reactions, the capacity of bee venom (AmV) to activate the human complement system remains elusive.

Methods and findings: By coupling immunochemical and functional approaches, it was observed that AmV strongly consumes components of the alternative pathway (AP) of the complement system in normal human serum (NHS). Additionally, AmV interfered with classical (CP) and lectin pathways (LP) activities. In parallel, a high increase in Ba fragment levels was detected, suggesting that the changes in AP activity were due to its activation. Furthermore, an increase in the level of the C1s-C1INH complex and a decrease in the physiological level of MASP1-C1INH suggested that CP and LP were also activated in the presence of AmV. Strikingly, NHS exposed to increasing AmV concentrations varying from 5 to 1000 µg/mL presented a high generation of C3a, C4a and C5a anaphylatoxins, and sC5b-9 complexes assembly, thus reinforcing that AmV triggers complement activation.

Conclusion: These results show that AmV is a strong complement activator. This activation presents a mixed profile, with a predominance of AP activation. This suggests that complement split products can play important roles in the envenomation by Africanized honeybee, as they could induce diverse immunopathological events observed in patients and may also dictate patient clinical prognosis.

KEYWORDS

complement pathways activation, anaphylatoxins, Africanized honeybee, *Apis mellifera* venom, immunopathology

1 Introduction

The complement system (C) is a crucial component of the innate immune response, playing a vital role in the organism's immunosurveillance against infections, tissue injury, and malignancies (1–3).

C is composed of over 50 components, including proenzymes, proteases, regulators, and receptors, which are distributed across various body compartments (such as plasma, lymph, cell membranes, mitochondrial membranes, and lysosomes). These components primarily function to eliminate microorganisms and dead cells, clear immune complexes, and induce a wide range of inflammatory responses. Generally, complement components exist in a non-activated state; however, in the presence of Microorganism-Associated Molecular Patterns (MAMPs) and/or Damage-Associated Molecular Patterns (DAMPs), these components become activated through proteolytic cascades (1–3).

Complement activation can be initiated through three pathways: the alternative pathway (AP), the lectin pathway (LP), and the classical pathway (CP), each triggered by different stimuli. The CP is activated via the C1 complex in the presence of acute phase reactants (APRs) such as C-reactive protein, pentraxin 3 (PTX3), serum amyloid protein (SAP), and IgG and IgM antibodies bound to antigens. It can also be triggered by DAMPs like phosphatidylserine on apoptotic cells and HMGB-1, as well as various MAMPs, including different types of lipopolysaccharides (LPS) and lipoteichoic acid. The LP is initiated when pattern recognition molecules (PRMs), such as mannose-binding lectin (MBL) and ficolins, bind to high-density mannose, N-acetylglucosamine, N-acetylmannosamine, and fucose carbohydrate residues found on the surface of microorganisms or host-altered cells. Additionally, APRs and several DAMPs can act as triggers for the LP. The AP is continuously activated through tick-over mechanisms and on unprotected surfaces exposed to microorganisms and DAMPs, such as free heme. Furthermore, the AP can be initiated through C3 component activation via the LP and CP, or through its own activity in a feedback loop (1–3). Interestingly, several authors have described additional processes of complement activation mediated by various molecular mechanisms (e.g., lysosomal cathepsins, animal venoms, leukocytes, coagulation proteases, and free radicals), which are highlighted as significant contributors to various biological and pathological processes (4–13).

Regardless of the pathway or trigger, complement activation converges to assemble multimolecular enzymatic complexes known as C3 and C5 convertases. These convertases activate the core complement proteins C3 and C5, respectively, resulting in the generation of opsonins and anaphylatoxins. Additionally, the cleavage of C5 initiates the terminal pathway of complement activation, culminating in the formation of the membrane attack complex (MAC) or soluble terminal complement complexes (sTCC).

If such activation occurs in a fine-tuned way (14, 15), the products generated, e.g., anaphylatoxins (C3a, C4a, C5a), opsonins (C3b, iC3b, C3d, C3dg, C4b), sTCC and MAC (16–21), will be crucial for inducing, maintaining, and regulating immune reactions as well as to promote tissue healing.

Interestingly, complement system activation can induce the generation of a large group of inflammatory mediators (13, 16,

22–24); cell degranulation (25); diapedesis (9, 13); cell debris and immunocomplex clearance (26–28); potentiation of antibody production (29); and T-cell polarization (10, 22, 23). Due to its capacity to orchestrate a multitude of cellular and molecular immunological events, the actions of the complement system must be minutely controlled since exacerbated complement activation or deficiencies of its components can be life-threatening (14, 15, 24).

Complement-mediated diseases involve a broad range of physiopathological events, which can include cell function impairment, tissue injury, organ dysfunction, multiorgan failure, and, in some cases, death (13, 24, 30–32). Interestingly, in blood samples from complementopathy carriers, several clinical laboratory findings have been reported, including increased levels of complement activation split products (i.e., anaphylatoxins, opsonins, inactivation fragments) and a reduction in C-pathways activity, as detected in systemic erythematosus lupus, polytrauma and sepsis (9, 24, 33–38).

Among the C-components associated with a significant reduction in these complementopathies, the C3 protein, a central complement component, can be highlighted. This reduction has also been observed in envenomations by Africanized honeybee (*Apis mellifera*) (AHB) (39). Accidents involving AHB (Order Hymenoptera) stings have been reported in Brazil since the late 1950s in parallel with the development of national beekeeping activities. Due to their intense swarming capacity, these bees spread across Brazil, reaching other countries in South and Central America as well as the United States. Thus, large number of human, and domestic and wild animal attacks cases have been reported by health authorities (40–45). During the last two decades, the numbers of such attacks have increased significantly in Brazil injuring more than 24,350 people in 2022 (46).

The clinical manifestations after stings depend on several factors in bees and patients, including sting numbers, venom amounts inoculated, and allergic sensitization state (47–49). Patients sensitized to venom toxins can present with severe local oedema, which affects a large body area and lasts more than 24 h. Systemic mast cell degranulation, bronchospasms, and cardiovascular impairment characterize an anaphylactic reaction that can lead to death; Hymenoptera allergens are the main cause of anaphylaxis worldwide (50–54).

Patients who are nonsensitized to venom and are attacked by one or a few bees generally experience local inflammatory reactions characterized by swelling, redness, itching, papules, pain and sometimes epithelial and subcutaneous necrosis. Conversely, individuals who suffer a swarm attack and thus receive multiple stings (>200) can experience systemic toxic reactions. These patients can develop systemic rhabdomyolysis, disseminated intravascular hemolysis, acute lung injury and direct liver impairment. Additionally, these patients can develop hyper/hypotension, myocardial infarction, stroke, acute renal failure, acute respiratory distress syndrome, multiple organ failure and death (39, 46, 47, 55–58).

Among the laboratory findings in patients who suffer multiple stings, an increase in the levels of C-reactive protein (CRP) and intense neutrophilia, which persists for several days after a

swarming attack, suggest that the inflammatory reaction is a signature of Africanized bee envenomation (39, 58). Interestingly, several authors have reported that patients affected by multiple stings present high *A. mellifera* venom (AmV) levels in the bloodstream, urine and peritoneal fluids (39, 58). Notably, venom toxins were detected in patient blood samples more than ten days after a swarming attack (58). Nonetheless, the effects of AmV on blood components, in addition to erythrocytes, have been underexplored. França and colleagues (39) reported that a patient attacked by > 800 bees presented a decrease in complement C3 component levels days after the accident. However, the consequences of such complement alterations (i.e., activation or inhibition) were not evaluated.

Thus, considering that (i) a reduction in complement component levels is an immunopathological signature in several clinical conditions, (ii) envenomation by AHB results in hallmark inflammatory events, and (iii) the complement system could impact the clinical manifestations of Africanized bee envenomation, here, by coupling several immunochemical and functional strategies, the ability of AmV to trigger human complement system activation and the profile of such activation were investigated.

2 Material and methods

2.1 Venom collection

AmV was obtained from the experimental apiary of the Núcleo de Ensino, Ciência e Tecnologia em Apicultura Racional (Center of Education, Science and Technology in Rational Beekeeping, NECTAR, Botucatu, São Paulo, Brazil) according to standard procedures (40). Briefly, colonies of AHB were maintained in Langstroth hives, and venom collection was performed with an electronic microprocessor-controlled device. For this purpose, the device was positioned on a sterile glass sheet at the hive entrance and turned on to produce electrical stimulation. The bees who touched the device received an electrical shock, and the venom was released onto the glass sheets. Once the AmV was composed of >80% water (47), the glass sheets containing the venom were dried in an air-drying oven at 34°C for 24 h. Finally, the venom was scraped, and the powder was stored at -80°C until solubilization.

2.2 AmV reconstitution and toxins analysis

AmV powder was solubilized in cold sterile saline at 3 mg/mL, and the protein concentration was evaluated by a Pierce BCA Protein Assay Kit (Thermo Scientific, Wisconsin, USA) following the manufacturer's recommendations. The venom stock solution was aliquoted and maintained at -80°C until further use. To assess venom integrity, several assays were performed. The presence of endotoxins in the AmV samples was evaluated by the Microbiological Quality Control Sector of the Butantan Institute, São Paulo, Brazil, using Limulus Amoebocyte Lysate (LAL) test (Pyrogen™-5000 Kinetic Turbidimetric LAL Assay Test Kit, Basel, Switzerland). The results were expressed as Endotoxin

Units (EU)/mL and values obtained was < 1.0 EU/mL, which were considered negative, and they did not cause any interference in biological/immunological assays (59).

The electrophoretic profile of the venom proteins was evaluated by 15% SDS-PAGE (60) under nonreducing conditions, and the protein bands were revealed by silver staining (61). Additionally, the enzymatic activity of hyaluronidase and phospholipase A2 (PLA₂) was determined by turbidimetric (62) and fluorescence resonance energy transfer (FRET) assays, respectively. The FRET assay for detecting PLA₂ activity was performed by using an EnzChek™ Phospholipase A2 Kit (Thermo Scientific™, Massachusetts, USA) in accordance with the manufacturer's guidelines. For both enzymatic activities, the results are expressed as the specific activity obtained by standard calculations, as presented by Silva de França et al. (62).

2.3 Ethics statement

Experimental approaches performed with human serum samples were evaluated and approved (n. 4.214.120; CAEE 35572620.0.0000.5464) by the Human Research Ethics Committee of the Municipal Health Secretary of São Paulo (CEP-CONEP system, Plataforma Brasil). Blood samples were obtained from healthy donors after they agreed to participate in the study and signed an informed consent document.

2.4 Human blood drawing and serum collection

Human blood samples (50 mL) were collected from individuals (n= 4) by venipuncture into glass tubes in the absence of anticoagulant. Initially, these tubes were maintained at room temperature for 20 min to start clot formation. Then, the samples were incubated at 4°C for 2 h to allow total blood coagulation and to minimize the basal index of complement activation. In sequence, the tubes were centrifuged at 400 × g for 10 min at 4°C, after which the normal human serum (NHS) was collected. Serum samples were centrifuged again, and the supernatants were obtained, aliquoted and stored at -80°C.

2.4.1 Determination of anti-AmV IgG presence in NHS samples

To evaluate the level of circulating IgG antibody against AmV in donor sera, NHS samples were subjected to ELISA. ELISA plates (Corning® - New York, USA) were sensitized overnight with 1 µg/well AmV diluted in sterile saline (100 µL). Then, the plates were washed once with PBS-Tween 20 0.05% (PT) (300 µL) and blocked with 5% PBS-BSA for 2 h at 37°C. Afterwards, the plates were washed three times with PT and incubated with NHS samples diluted in 0.01% PBS-BSA (1:100–1:1,600) for 1 h at room temperature. In sequence, the plates were washed again, and goat anti-human IgG-HRP antibodies (Sigma/Merck, Taufkirchen, Germany) diluted 1:10,000 in 100 µL 0.01% PBS-BSA were added to the plates for 1 h. Finally, the plates were washed with PT, and the

reactions were incubated with 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide (H_2O_2) substrate (TMB) (BD Biosciences, New Jersey, USA) (50 μ L) for 30 min. The reactions were subsequently disrupted with H_2SO_4 4 N solution (50 μ L). The plates were subjected to spectrophotometric reading on a Cytation 3 Cell Imaging Multi-Mode reader (BioTek Santa Clara- CA, USA) at 492 nm. The ELISA controls included wells containing TMB + stop solution (blank) and AmV + Block + detection antibodies. The absorbance values of the experimental samples were subtracted from those of the controls. The samples were considered positive/reactive agents if the absorbance values were three times greater than those of the blank wells.

2.4.2 Serum exposure to AmV

NHS samples were exposed to vehicle (sterile saline) or to increasing concentrations of AmV (5 to 1000 μ g/mL) for 30, 60 or 120 min under constant shaking at 37°C. After incubation, these mixtures were aliquoted and stored at -80°C for further analyses of complement activation-related molecular markers and complement pathway functionality. Before freezing, the samples used to determine complement activation products were subjected to treatment with 20 mM ethylenediaminetetraacetic acid (EDTA) to stop the reactions and prevent further generation of such molecular markers.

2.4.2.1 AP, CP, and LP functional analysis

The effects of AmV on complement system pathway activity in NHS were investigated by functional ELISA according to the methodologies established by Seelen et al., 2005 (63) and Kotimaa et al., 2016 (64), with some modifications. ELISA plates (Corning®- New York, USA) were coated overnight at 4°C with 3 μ g of LPS from *Salmonella enteritidis* (Sigma/Merck- Taufkirchen, Germany) diluted in PBS/10 mM $MgCl_2$, 1 μ g of purified human IgM (Sigma/Merck- Taufkirchen, Germany) or 10 μ g of Mannan (Sigma/Merck- Taufkirchen, Germany) from *Saccharomyces cerevisiae* diluted in carbonate buffer (100 mM Na_2CO_3 , 100 mM $NaHCO_3$, pH 9.6). Then, the plates were washed three times with PT (300 μ L) and blocked with 1% PBS-BSA for 1 h at 37°C. Afterwards, the plates were again washed with PT, diluted serum samples (100 μ L) were added, and the plates were incubated at 37°C for 2 h to allow complement components binding to their targets.

For AP evaluation, serum samples were diluted 1:10–1:80 in APB (5 mM barbital sodium, 7 mM $MgCl_2$, 150 mM NaCl, and 10 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), pH 7.2) and exposed to the wells coated with LPS. For CP analysis, sera were diluted 1:100–1:800 in VBS²⁺ (2.8 mM barbituric acid, 0.9 mM sodium barbital, 0.3 mM $CaCl_2$, 145 mM NaCl, 0.8 $MgCl_2$, pH 7.2) and incubated in plates containing human IgM. The changes in LP were characterized in plates sensitized with mannan and experimental serum samples diluted 1:100–1:800 in BVB²⁺ (VBS²⁺, 0.1% BSA, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$). In sequence, the plates were washed five times with APB-Tween 0.05% (APBT), VBS²⁺ (VBST) or BVB²⁺ (BVBVT) (300 μ L) and incubated for 1 h at 37°C with capture goat anti-human C3 (COMPTECH - Texas, USA) (AP) or goat anti-human C4

polyclonal antibodies (COMPTECH - Texas, USA) (CP and LP), both of which were diluted 1:128,000 in the specific pathway buffer mentioned above. Subsequently, the plates were washed and incubated with rabbit anti-goat IgG-HRP conjugate detection antibodies (Sigma/Merck-Taufkirchen, Germany) diluted 1:40,000 in APBT, VBST or BVBVT for 1 h at 37°C. Finally, the plates were washed, and the reactions were developed with TMB solution (50 μ L) (BD Biosciences-New Jersey, USA). These reactions were allowed to develop for 15 min and stopped by the addition of H_2SO_4 4 N (50 μ L). The absorbance (ab) values were determined using a Cytation 3 Cell Imaging Multi-Mode reader (BioTek- Santa Clara- CA, USA) at 450 nm. As plate controls, specific wells were incubated with TMB and Stop solution (blank) or complement target + blockage solution + conjugate, and the absorbance values were subtracted for all the samples. The specific complement reaction controls included NHS samples (serum exposed to sterile saline) (positive control) and IHS samples (human serum inactivated at 60°C for 45 min) (negative control). The results generated were expressed as AP activity C3 [%] or CP/LP activity C4 [%] and were calculated using the following formula: $(abAmV - abIHS)/(abNHS - abIHS) \times 100$. All the experimental samples and complement controls were tested in duplicate for all the dilutions.

2.4.2.2 Complement activation split products measurement

To confirm the activation of each complement pathway, several specific molecular markers were quantified by immunoassays. Ba fragment (AP), C1s-C1INH (CP), and MASPI-C1INH (LP) complexes were measured using the MicroVue Ba EIA kit from QUIDEL (San Diego, USA), C1s/C1-INH and MASP-1/C1-INH ELISA kits from HycultBiotech (Uden, The Netherlands), respectively. Anaphylatoxins C3a, C4a, and C5a, as well as sC5b-9 complexes, were evaluated using the BD Cytometric Bead Array (CBA) Human Anaphylatoxin Kit (BD Biosciences, New Jersey, USA) and MicroVue SC5b-9 ELISA. All assays were performed according to the manufacturers' recommendations. The obtained results were expressed as μ g/mL for C3a and ng/mL for all other split products.

2.5 Statistical analyses

All assays were performed in triplicate and repeated four times. The generated results were expressed as mean and standard deviation ($SD \pm$). Statistical analyses were performed using a *t* test or two-way ANOVA followed by Sidak's multiple comparison test. Spearman's rank correlation coefficients (*rs*) were determined to estimate the relationship between complement pathways functions and changes in its specific molecular markers. The correlation strength between variables was determined in accordance with Cohen's cutoff points (65), on which *rs* values ± 0.10 indicate a weak correlation; ± 0.30 a moderate correlation; and ± 0.50 a strong correlation. All the analyses were performed using GraphPad Prism 8 software, and differences were considered significant when $p \leq 0.05$.

3 Results

3.1 AmV toxins analysis

By using a colorimetric assay, it was determined that the protein concentration in AmV was 2.79 mg/mL. SDS-PAGE electrophoretic analysis revealed that AmV samples presented several protein bands, with molecular weights varying from < 10 to 100 kDa (Supplementary Figure S1A). Moreover, the AmV samples used in our experiments were enzymatically active. Incubation of AmV with hyaluronan, an important extracellular matrix and serum component that drives a plethora of biological and pathological processes (66), promoted its depolymerization, indicating that venom hyaluronidases were preserved in our samples (Supplementary Figure S1B). In addition, strong PLA₂ activity was also detected in the AmV samples, as demonstrated by its capacity to degrade phosphatidylcholine and phosphatidylglycerol membrane phospholipids (Supplementary Figure S1C). Moreover, diverse concentrations of AmV and its dilution vehicle were analyzed at the Microbiological Quality Control Sector from Instituto Butantan, and these samples did not show the presence of LPS/endotoxins (Supplementary Figure S2). Thus, given that our AmV samples were highly active and free of endotoxins, which are important features for investigating the inflammatory effects of AmV in human serum, subsequent experiments were performed to determine its effects on the human complement system.

3.2 AmV interferes with human complement pathways activity

To analyze the effects of AmV on human complement system, we tested increased venom doses considering the quantity of venom toxins injected by an AHB, which corresponds to 100 µg released per sting (54, 67). Thus, we defined the following doses for the experiments, i.e., 100 µg: corresponding to one sting, 500 µg: five stings, and 1000 µg: 10 stings. Additionally, experiments were also conducted by stimulating NHS with 10 µg of AmV, which is a dose 10 times less than the possible amount of AmV injected by one bee, to determine whether low venom doses could also cause C-activation.

Exposure of NHS to 10 µg of AmV significantly reduced the functional binding of the C3 complement component to the plates sensitized with LPS (Figure 1A). This reduction occurred over time in all donor samples. After incubating the serum samples with 100 µg of venom toxins (Figure 1C), which is the dose that just one bee can inject, C3 binding on the plates was completely abrogated, and this event was also detected in the serum samples stimulated with 500 and 1000 µg of AmV/mL (Figures 1E, G) during all the periods evaluated. Thus, AHB venom is capable of strongly interfering with complement AP activity. In a dose- and time-dependent fashion, these changes in AP function were accompanied by increased levels of Ba fragments (Figures 1B, D, F, H), showing that such impairment of complement activity was due to its activation. Such results were reinforced by Spearman's rank correlation analysis. Through this evaluation, a strong correlation was

detected between impairment in C3 binding to the plates and FBa generation (Table 1), independently of AmV amounts.

In addition to the modifications of AP activity, we also investigated the effects of AmV on CP and LP. Initially, given that CP is initiated by immunocomplexes (2) and that the presence of antibodies against bee venom could result in C4 component consumption, we performed an analysis to identify the presence of specific IgG antibodies against the AmV toxins in donors' serum samples. No donor presented antivenom antibodies (Supplementary Figure S3); thus, experiments to evaluate the effects of AmV on CP and LP were conducted. Figures 2, 3 show that increased venom concentrations interfered with human serum CP and LP activities, as determined by the reduced binding of C4 on plates coated with human IgM or mannan, for the experiments. Nonetheless, over time, we detected an increase in the abundance of C1s-C1INH complexes, thus confirming that CP (Figures 2B, D, E, G) was activated in the serum samples. In contrast, we observed a decrease in the physiological levels of MASP1-C1INH complexes, which may be related to the activity of LP on NHS exposed to venom (Figures 3B, D, E, G).

Interestingly, the interference with CP functions demonstrated a strong correlation with elevated levels of C1s-C1INH complexes, particularly at high venom doses (Table 2). While exposure of the normal human serum (NHS) to 10 µg of venom showed a moderate correlation with the assembly of CP molecular markers, it still yielded significant results. Additionally, the reduced levels of C4 protein binding to mannose-coated plates, along with the decrease in MASP1-C1INH complexes, also exhibited a correlation that appears to depend on the venom dose used in the assays. The strength of this correlation varied from weak (AmV 10 µg/mL) to strong (AmV 1000 µg/mL); however, all venom doses elicited a significant correlation between the reduction in LP activity C4 [%] and its associated molecular markers (Table 3).

3.3 AmV is a potent anaphylatoxin inducer

The main consequence of complement activation, whether through intrinsic or extrinsic routes, is the generation of split products, such as the anaphylatoxins C3a, C4a and C5a, which are important immunological drivers of a plethora of physiological and pathological conditions (3, 13). Several authors have identified in clinical reports that patients who suffer swarming attacks have a broad range of venom amounts, both acutely and for more than ten days, that can be detected in diverse biological fluids, including serum (39, 58). Thus, we investigated whether AmV doses ranging from 5 µg to 1000 µg/mL could elicit the generation of anaphylatoxins. NHS contact with increasing AmV promoted robust generation of C3a (Figure 4). Venom toxins triggered a peak in C3a anaphylatoxin release at 30 min, with 100 and 1000 µg/mL being the most potent venom doses that caused C3a generation (Figures 4E, I). In addition to C3a, all AmV doses were capable of stimulating C4a anaphylatoxin generation. The peak in the C4a generation occurred 30 min after AmV (5, 10, 25, or 100 µg/mL) incubation with serum (Figures 5A–C, E), while at other venom concentrations, the peak C4a release

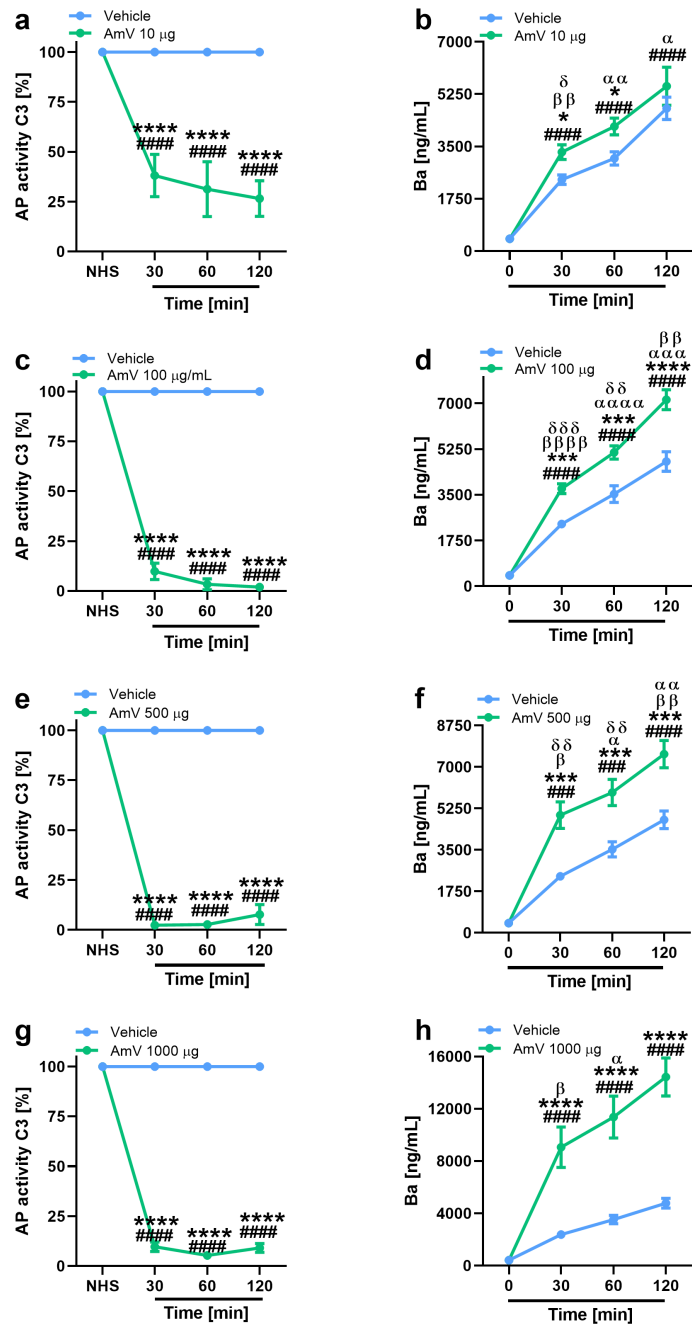


FIGURE 1
AHB venom triggers complement AP activation. Impairment in AP activity was determined by functional assays with experimental envenomed serum samples diluted 1/10 (A, C, E, G). AP activation was confirmed by MicroVue Ba EIA kit, provided by QUIDEL Corporation, on which experimental samples were diluted 1/6,000 (B, D, F, H). The results are expressed as the means $\pm$ SDs and were statistically analyzed with GraphPad PRISM 8 by two-way ANOVA followed by Sidak's multiple comparison test ($n = 4$). Differences were considered significant at $p \leq 0.05$. # T0/NHS, x all times; * AmV x Vehicle; α 30 min x 60 or 120 min; β 60 min x 30 or 120 min; δ 120 min x 30 or 60 min.

TABLE 1 AP activity C3 [%] versus FBa levels.

AmV [$\mu\text{g/mL}$]	r_s	p value
10	+ 0,5428	<0.0001
100	+ 0,5831	<0.0001
500	+ 0,6147	<0.0001
1000	+ 0,6039	<0.0001

Spearman's correlation coefficients (r_s).
 ± 0.10 = weak correlation, ± 0.30 moderate correlation.
 ± 0.50 strong correlation.

occurred at 60 min (Figures 5D, F–I). In contrast to what was observed for C3a and C4a generation, all the venom doses stimulated C5a anaphylatoxin production without a subsequent decrease (Figure 6). Strikingly, during the different incubation times, independent of the AmV dose, there was a constant increase in the generation of this peptide. High amounts of C5a were detected in NHS samples treated with venom, with 100 and 1000 $\mu\text{g/mL}$ being the concentrations that induced more than 300 and 900 ng/mL of C5a generation, respectively (Figures 6E, I).

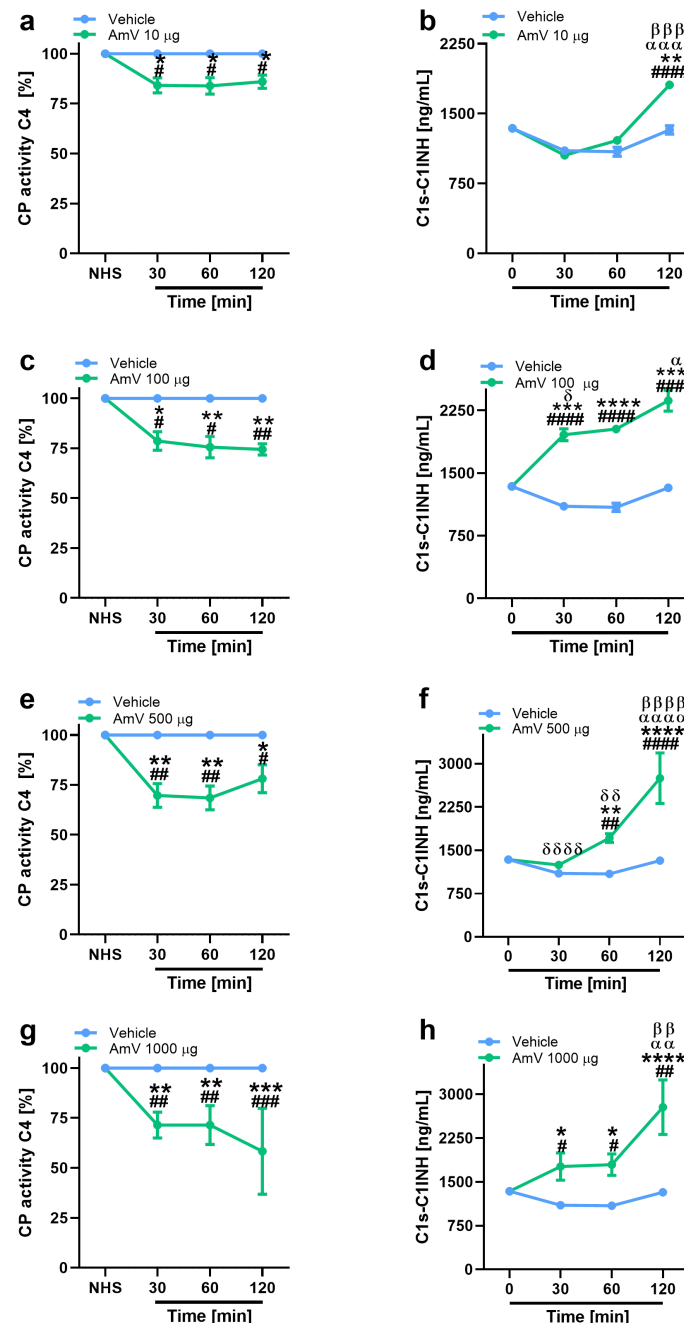


FIGURE 2

AHB toxins induce complement CP activation. To access AmV effects upon CP, experimentally envenomed serum samples, diluted 1:100-800 in VBS²⁺, were evaluated to C4 binding in plates coated with human IgM (A, C, E, G). CP activation was confirmed by analysis of levels of C1s-C1INH complexes (B, D, F, H) by immunoassays (serum samples diluted 1/800) by using the C1s/C1INH, human, ELISA kit purchased from HycultBiotech. The results are presented as the means $\pm$ SDs, and statistical analysis was performed with GraphPad PRISM 8 by two-way ANOVA followed by Sidak's multiple comparison test ($n = 4$). Differences were considered significant at $p \leq 0.05$. # T0/NHS, all times; * AmV x Vehicle; α 30 min x 60 or 120 min; β 60 min x 30 or 120 min; δ 120 min x 30 or 60 min.

3.4 Honeybee venom stimulates sC5b-9 complex assembly

In line with the purpose of anaphylatoxin evaluation, the same rationale was used to determine the assembly of the sC5b-9 complexes. The formation of the sC5b-9 complex starts with the

cleavage of the C5 complement component (68, 69). The sC5b-9 complex was induced by all the venom doses tested, and the amount of the complex increased significantly over time (Figure 7). All AmV concentrations induced the formation of sC5b-9 complexes, however, 100 and 1000 µg/mL AmV (Figures 7E, I) were the strongest inducers of these high-molecular-weight complexes.

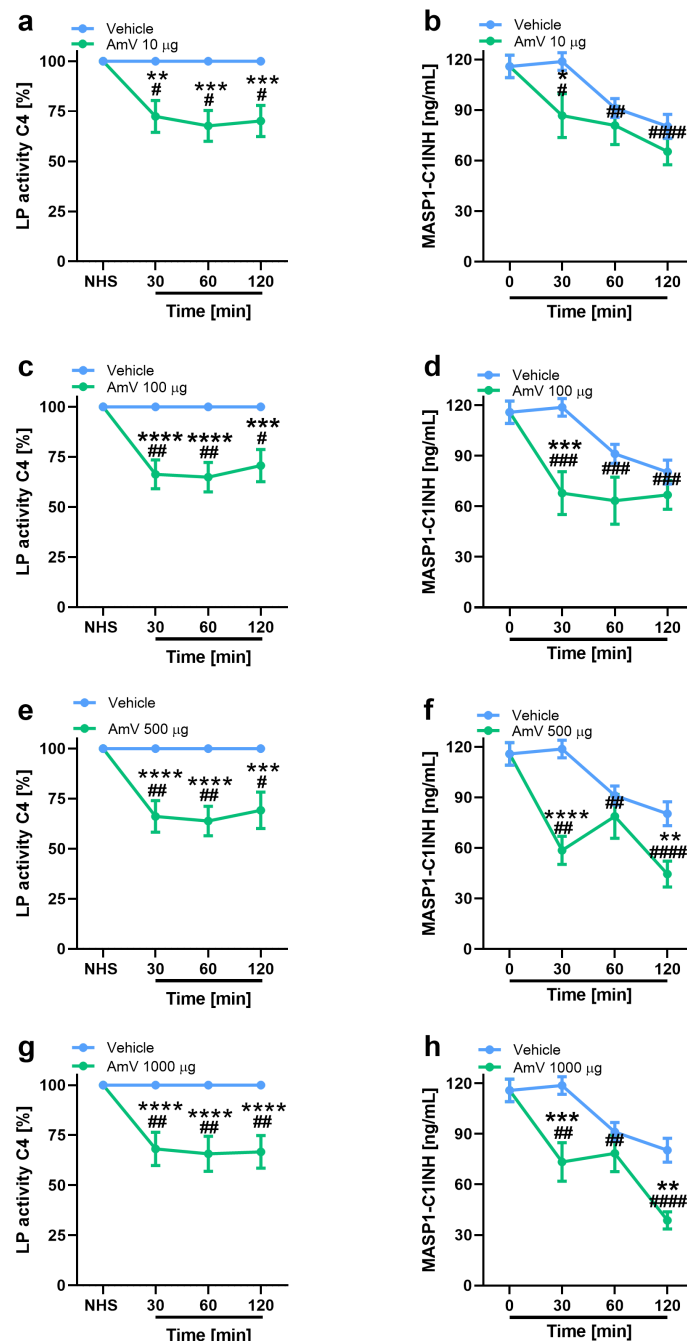


FIGURE 3

Bee venom toxins modify complement LP functionality. Using a functional ELISA, the effects of AmV toxins upon LP functions were analyzed (A, C, E, G). The changes in LP were confirmed through the measurement MASP1-C1INH complexes in serum samples diluted 1/100 (B, D, F, H). These assays were performed by MASP-1/C1INH ELISA kit manufactured by HycultBiotech. The results are shown as the means $\pm$ SDs. The statistical analysis was performed by GraphPad PRISM 8 through two-way ANOVA followed by Sidak's multiple comparison test ($n=4$). Differences were considered significant at $p \leq 0.05$. # T0/NHS x all times; * AmV x Vehicle; α 30 min x 60 or 120 min; β 60 min x 30 or 120 min; δ 120 min x 30 or 60 min.

4 Discussion

Here, we analyzed the effects of AHB venom on the human complement system. Strikingly, we demonstrated that a broad range of venom doses (5-1000 $\mu\text{g/mL}$) could cause the generation of biologically active complement split products, i.e., anaphylatoxins and sTCC. In 1982, De Carolis and colleagues (70) showed that

European honeybees (*A. mellifera*) venom elicits complement activation only at high doses, i.e., 1000 $\mu\text{g/mL}$. In addition, these authors found only AP activation and C3a anaphylatoxin release since no CP or LP split products or impaired functions were identified. Although the Africanization process of *A. mellifera* on the American continent did not cause the emergence of a new species, differences in the abundance of some venom toxin classes

TABLE 2 CP activity C4 [%] versus C1s/C1INH complex levels.

AmV [µg/mL]	rs	p value
10	+ 0,340	0.0159
100	+ 0,6142	<0.0001
500	+ 0,6034	<0.0001
1000	+ 0,6064	<0.0001

Spearman's correlation coefficients (r_s).
± 0.10= weak correlation.
± 0.30 moderate correlation.
± 0.50 strong correlation.

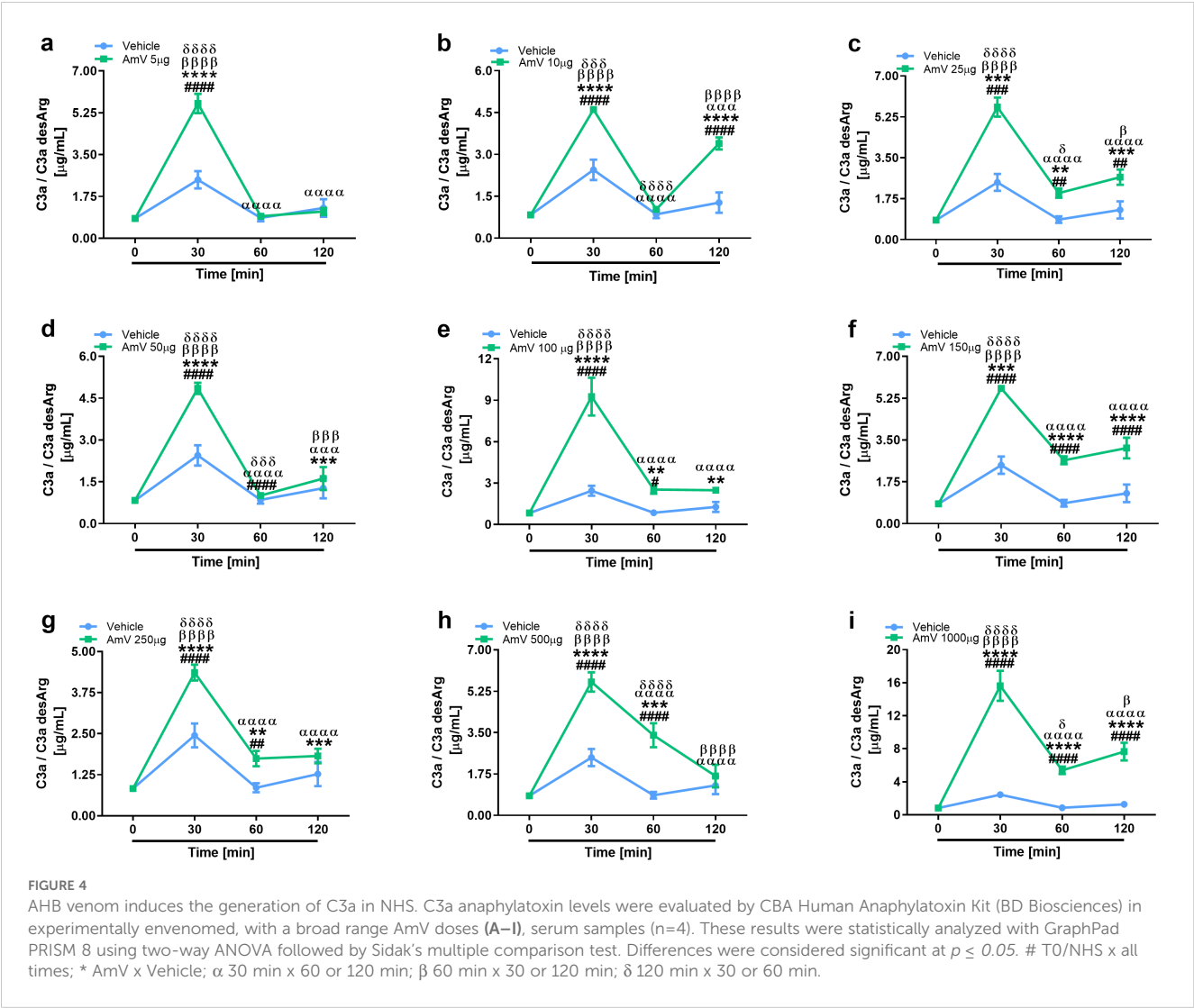
TABLE 3 LP activity C4 [%] versus C1s/C1INH complex levels.

AmV [µg/mL]	rs	p value
10	- 0,2361	0.0302
100	- 0,354	<0.0021
500	- 0,2915	<0.0433
1000	- 0,6487	<0.0001

Spearman's correlation coefficients (r_s).
± 0.10= weak correlation.
± 0.30 moderate correlation.
± 0.50 strong correlation.

(71) may be responsible for the capacity of these species to elicit complement activation independent of the dose. In line with these ideas, we determined that incubating AmV with NHS impaired C4 binding to plates sensitized with mannose and increased C4a generation. These findings suggest that the sugar groups containing Mannose and N-acetylglucosamine residues present in AmV (72), as well as the DAMPs released by venom

toxin actions, could trigger complement activation by LP, since such carbohydrates are stronger activators of this pathway. Independent of the venom concentration, an important decrease in the physiological levels of the MASP1-C1INH complexes was detected. C1INH is able to control MBL-associated serine protease 1 (MASP1) activities, which is responsible for the initial enzymatic events of complement activation by LP (73, 74). Based on



our findings, it is possible that, during the envenomation process by Africanized bees, an imbalance in the physiological maintenance of the MASP1-C1INH complex occurs, which could lead to accidental LP activation, resulting in the consumption of its components, such as C4, leading to C4a anaphylatoxin generation, as detected here. Interestingly, previous studies identified that in a cohort of patient's carriers of Hereditary Angioedema (HAE), presenting both type I or II phenotypes of the disease, a fall in the levels of MASP1-C1INH complexes, which was correlated with several aspects of the disease, including, C4 consumption and the number of attacks through the year of blood samples drawing (75). Strikingly, in our evaluations, the impairment in LP functions, along with the decrease in MASP1-C1INH complex levels, also presented a significant correlation. Thus, it's important to highlight that in both clinical illness (HAE and Bee envenoming) there are important vascular dysfunctions which culminates in multiple edema sites, which suggest that such molecular alterations together with C4a peptide release could be clinically important to envenomed patients.

Interestingly, a reduction in CP activity was induced by AmV, and considering that this complement pathway shares several components with LP it is possible that molecular events in the Africanized bee envenomation process, which interfere with one of these pathways, impact the other. We observed a dose- and time-dependent increase in C1s-C1INH complex levels, which showed significant and strong correlations with the reduction in classical pathway (CP) activity, as indicated by C4 [%]. These findings confirm the activation of the CP during the envenomation process.

In our experiments, we showed that NHS donors were negative for the IgG antibodies against AmV. Thus, taking these findings, it is possible to consider that following envenomation by Africanized bees, CP activation is triggered in the absence of immunocomplexes. In accidents involving Africanized bees, strong inflammation and cell damage are observed since AmV causes rhabdomyolysis, hemolysis, and renal and peripheral blood mononuclear cell necrosis and elevates CRP levels (39, 47, 49, 55–58, 76, 77). Necrotic cells and CRP are notorious CP starters (26, 27), which suggests that in

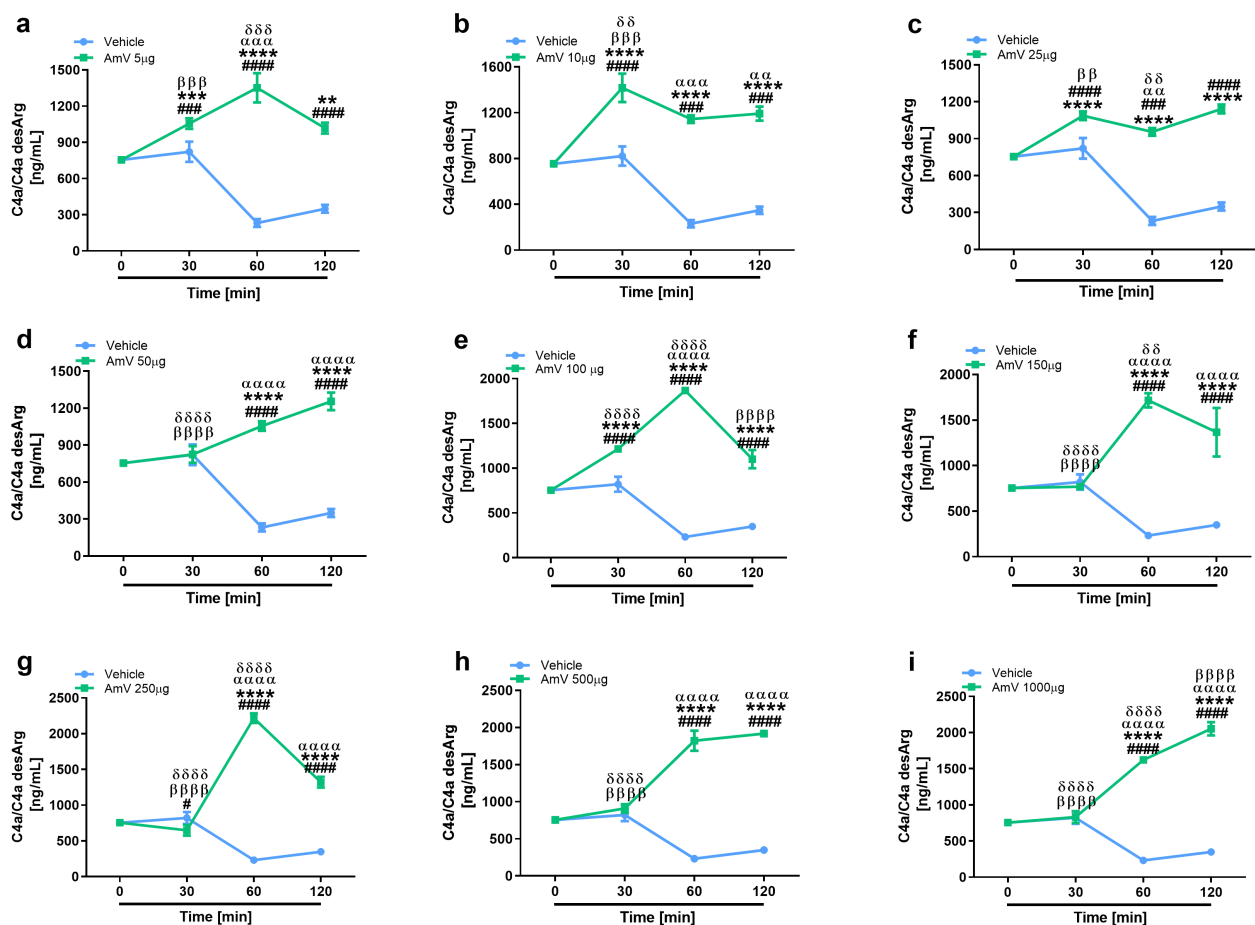


FIGURE 5

AHB venom stimulates C4a peptide release. C4a anaphylatoxin dosage was made by CBA Human Anaphylatoxin Kit (BD Biosciences) in NHS exposed to increasing AHB venom doses (A–I). Data obtained were analyzed with GraphPad PRISM 8 by two-way ANOVA followed by Sidak's multiple comparison test. Differences were considered significant at $p \leq 0.05$. # T0/NHS x all times; * AmV x Vehicle; α 30 min x 60 or 120 min; β 60 min x 30 or 120 min; δ 120 min x 30 or 60 min.

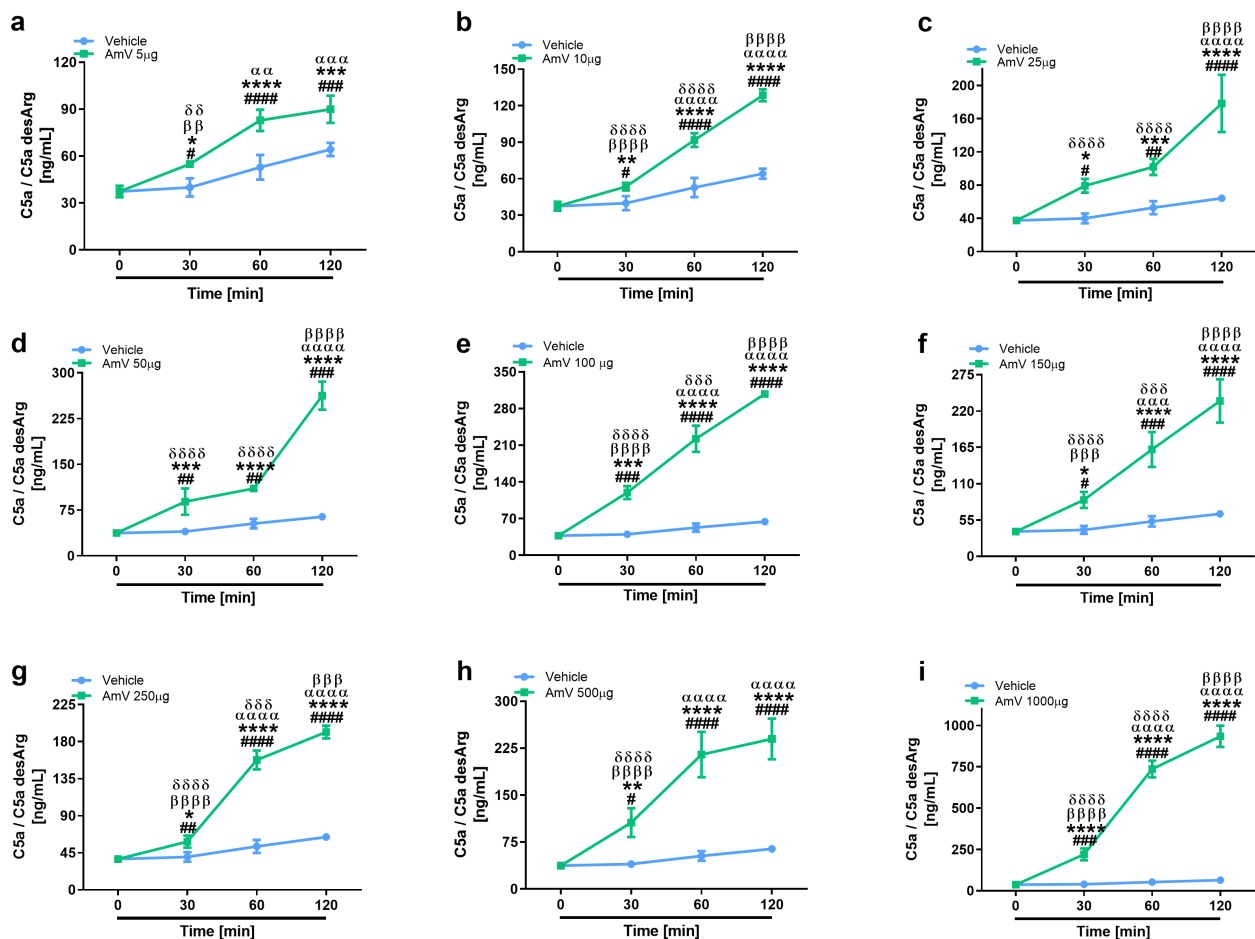


FIGURE 6

AHB venom toxins are strong C5a anaphylatoxin inducers. C5a anaphylatoxin was detected in NHS samples incubated with AmV amounts varying of 5–1000 µg/mL (A–I) by using CBA and evaluated statistically by two-way ANOVA followed by Sidak's multiple comparison test in GraphPad PRISM 8. $p \leq 0.05$ was considered to indicate statistical significance. # T0/NHS x all times; * AmV x Vehicle; α 30 min x 60 or 120 min; β 60 min x 30 or 120 min; δ 120 min x 30 or 60 min.

addition to direct CP activation by AmV, the consequences of its toxins effects in the body can potentiate the activity of such complement pathway.

The contact between the different AmV concentrations and NHS caused the strong consumption of AP complement factors, as indicated by an abrupt reduction in C3 protein binding to the LPS-coated plates. Interestingly, the reduction was venom dose dependent since the low dose used here reduced AP activity but maintained ~25% of the pathway functionality. However, the other doses completely abolished the AP reactions.

Although the 100, 500 and 1000 µg/mL venom doses were able to abolish AP activity, the intensity of AP activation elicited by each of these doses could only be determined by Ba fragment generation assessment via ELISA. Strikingly, depending on the AmV concentration and incubation time, different amounts of Ba were released. This finding suggested that AP activation by Africanized bee envenomation is dependent on how much venom/how many stings the individuals received.

In line with the clinical illness developed by patients envenomated by AHB, AP activity as well as the split products generated by its action have been presented in recent decades as crucial to the clinical outcomes of several pathological conditions in which inflammatory reactions are uncontrolled, including sepsis and polytrauma. These investigations revealed that strong C3 component consumption and concomitant increases in C3a anaphylatoxin levels were correlated with poor patient prognosis and death. Additionally, a decrease in intact C3 levels was also correlated with uncontrolled systemic and pulmonary inflammation and hemostatic disorders, thus indicating that complement can act mutually with other systems in diverse diseases (31, 35–37, 78), including in bee envenomation (39). Thus, considering that AmV is an AP activator, as demonstrated here and that patients exhibiting severe envenomation by Africanized bees are predisposed to several organ dysfunctions we can hypothesize that (1) AP activation is an important immunopathological event that exacerbates *A. mellifera* envenomation; (2) AP activation could lead to physiological dysfunctions, as detected in patients; and (3) molecules released

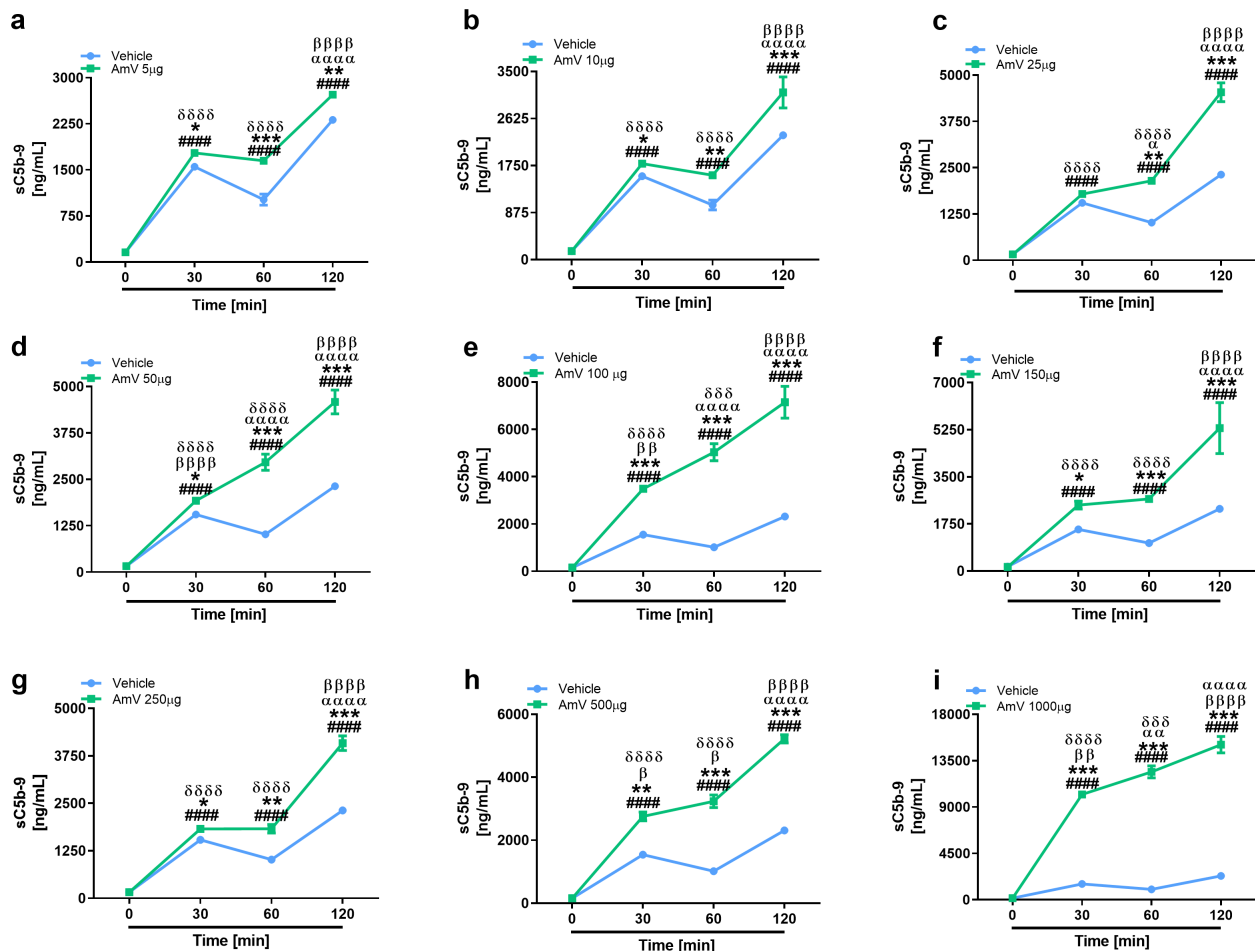


FIGURE 7

Assembly of the sC5b-9 complex is stimulated by bee venom components. Soluble Terminal Complement Complexes were detected in all serum samples exposed to increased venom amounts (A–I) by ELISA (MicroVue sC5b-9 kit – QUIDEL), and the results were statistically analyzed with GraphPad PRISM 8 by two-way ANOVA followed by Sidak's multiple comparison test. Differences were considered significant at $p \leq 0.05$. # T0/NHS x all times; * AmV x Vehicle; α 30 min x 60 or 120 min; β 60 min x 30 or 120 min; δ 120 min x 30 or 60 min.

during AP activation, such as the Ba fragment, could be used in clinical settings to monitor patient health conditions and for prognosis and decisions regarding therapeutic interventions. Notably, in our correlation analysis, the alternative pathway (AP) activity C3 [%] showed a strong correlation with FBa fragment release, regardless of the experimental envenomation degree (AmV doses). This finding reinforces the questions raised earlier regarding the activation of the AP during the envenomation process.

We showed that all AmV concentrations used in the experiments were able to cause C3a generation, which can be correlated with the strong impairment of AP activity due to consumption and conversion of the C3 component. Interestingly, some venom doses, such as 100 and 1000 $\mu\text{g/mL}$, were able to induce excessive amounts of these peptides, which induced increases of 9 and 16 $\mu\text{g/mL}$, respectively. Notably, C3 consumption and consequent C3a formation can represent harmful characteristics of AHB envenomation since both molecular events have been correlated with poor prognosis in some

inflammatory diseases (31, 35–37, 78). Interestingly, among the physiological dysfunctions presented by the envenomated Africanized bee patients, hypertension was detected (39). The C3a-C3aR axis has been described in the literature as a strong hypertensive agent because of its capacity to induce intensive TXA_2 generation and systemic vasoconstriction (79). In addition, several reports have shown that such signaling is an important driver of thromboinflammatory events. Both hypertensive events and thromboinflammatory reactions are risk factors that predispose individuals to stroke and myocardial infarction (80), which was detected as pathological consequence of bee envenoming (81).

An interesting molecular event detected here was C5a anaphylatoxin generation at all venom doses and at all times analyzed. C5a anaphylatoxin generation is an important molecular event in complement activation due to its strong proinflammatory properties (13, 24). Notably, this is the first study in which C5a anaphylatoxin generation was reported because of bee venom action,

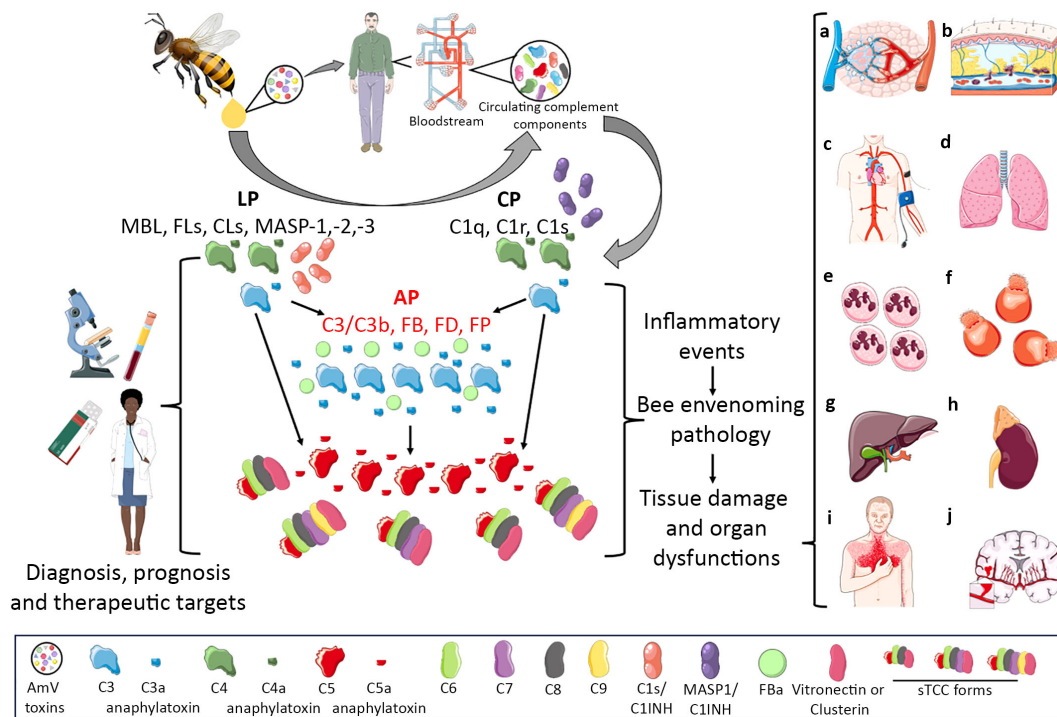


FIGURE 8

Complement Activation in Africanized *A. mellifera* Honeybee Attacks. The venom of Africanized *A. mellifera* honeybees (AHB) contains several molecules with toxic and allergenic properties. When exposed to normal human serum, these venom toxins can directly or indirectly trigger the activation of the complement system. This activation occurs predominantly through the alternative pathway (highlighted in scientific drawing by red letters) (AP), although the classical (CP) and lectin (LP) pathways are also activated. Through this mixed activation profile, large amounts of the C3a, C4a, C5a anaphylatoxins and sC5b-9 (sTCC) complexes are generated. These biologically active complement split products are known to promote a variety of biological and pathological reactions, including vascular dysfunctions (a,b,c) which can evolve to swelling (A, B) or hypo/hypertension (C); acute lung injury/respiratory distress syndrome (ALI/ARDS) (D), leukocytosis/neutrophilia (E), disseminated intravascular hemolysis (F), liver injury (G), acute kidney failure (H), heart attack (I), and stroke (J). Notably, all the immunopathological conditions mentioned are identified in patients who have suffered multiple or even few Africanized bee stings. Therefore, it is possible that the complement system is an important inflammatory driver of these pathological settings identified in patients attacked by AHB swarms become such a potential therapeutic target as well diagnostic/prognostic molecular markers which could help physicians in the management of envenomed patients. This Figure was partly produced using Servier Medical Art (SMART), licensed under CC BY 4.0. Of note, some icons in this figure were adapted for this article purposes.

and this molecular envenomation feature deserves attention since this peptide is linked to several immunopathological disorders and fatal consequences of such as identified in diverse experimental models of pathological conditions (13, 24, 82–84).

In the NHS samples exposed to AmV, soluble C5b-9 complexes were also observed to form in a time- and dose-dependent manner. This complex, unlike the MAC, which is assembled and inserted into the cell membrane, is present in the fluid phase, can interact with diverse cell and molecular systems and can cause several inflammatory events (11). The sC5b-9 and C5a generation were detected in all time of NHS exposure to AmV, suggesting that the late phases of complement activation are a constant and possibly dangerous inflammatory event in bee envenomation. The sC5b-9 complex, like C5a, is a potent endothelial disrupting agent through its capacity to induce NLRP3 inflammasome activation, IL-1 β release, eicosanoids release, platelet activating factor (PAF) and bradykinin generation *in*

vivo. Additionally, sC5b-9 complexes are linked to thrombotic events in various pathologies (13, 68, 85, 86). In conclusion, the formation of sC5b-9 together with other complement activation split products could account for the vascular impairment and other pathological reactions observed in stung patients.

In this article, we showed that complement activation may constitute an important molecular immunopathological signature of envenomation by AHB. Therefore, considering that complement activation split products are the main drivers of various inflammatory disorders, the role of this system in the imbalance induced by bee venom toxins should be further analyzed in detail. Perhaps drugs already in use in the clinic for complement-mediated diseases could be applied for the treatment of individuals who suffer few or multiple stings, as well as for anaphylaxis triggered by venom allergens. Additionally, complement activation products could be used in clinical settings as biomarkers of envenomation severity

since some complement activation products are strongly dependent on the AmV concentration, which could reflect the number of stings as well as the amount of venom injected (Figure 8).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Human Research Ethics Committee of the Municipal Health Secretary of São Paulo (CEP-CONEP system, Plataforma Brasil). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

FS: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing, Investigation. Rd: Formal analysis, Methodology, Writing – review & editing, Resources. DF: Formal analysis, Writing – review & editing, Data curation, Investigation. TL: Data curation, Formal analysis, Investigation, Writing – review & editing. DT: Data curation, Formal analysis, Writing – review & editing, Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft.

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Supplementary material

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The complement system in lipid-mediated pathologies

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The complement system, a coordinator and facilitator of the innate immune response, plays an essential role in maintaining host homeostasis. It promotes clearance of pathogen- and danger-associated molecular patterns, regulates adaptive immunity, and can modify various metabolic processes such as energy expenditure, lipid metabolism, and glucose homeostasis. In this review, we will focus on the intricate interplay between complement components and lipid metabolism. More precisely, we will display how alterations in the activation and regulation of the complement system affect pathological outcome in lipid-associated diseases, such as atherosclerosis, obesity, metabolic syndrome, age-related macular degeneration, and metabolic dysfunction-associated steatotic liver disease. In addition to that, we will present and evaluate underlying complement-mediated physiological mechanisms, observed both *in vitro* and *in vivo*. Our manuscript will demonstrate the clinical significance of the complement system as a bridging figure between innate immunity and lipid homeostasis.

KEYWORDS

innate immunity, complement, lipid metabolism, lipid-mediated pathologies, complement system

Introduction

Living organisms require immune homeostasis - a balance between the immune tolerance to self and immunogenicity to exogenous challenges deleterious to the host. This equilibrium is achieved through coordinated interplay between tissues with proteins and cells of the immune system. In contrast to pathogen-induced inflammation, metabolic changes caused by abnormal amounts of nutrients lead to sterile low-grade inflammation (1). This kind of inflammation can be initiated within various organs, and if not resolved by immune system action, it can drive disease development.

One of the essential energy sources, lipids, are fundamental for building cell structures, cellular signaling, and the generation of physiologically active compounds. They affect immunity in a bidirectional manner, e.g., through the balance between pro- and anti-inflammatory lipid-derived mediators modulating cellular and humoral immune response by shaping repertoires of immune cells, circulating antibodies and complement system

components. At the same time, the immune system can steer lipid metabolism and determine the fate of lipid derivatives affecting the general metabolic homeostasis. Next to native lipids, modified lipids and lipoproteins are increasingly recognized as drivers in many cellular and immune processes and disease pathogenesis such as atherosclerosis, metabolic dysfunction-associated steatotic liver disease (MASLD), etc. (1–3).

Lipid-mediated pathologies comprise a broad spectrum of diseases in which abnormal lipid metabolism, signaling and storage affect various organs and systems. The most prevalent are obesity, atherosclerosis, type 2 diabetes mellitus (T2DM), age-related macular degeneration (AMD), and MASLD (4). This group of non-communicable diseases reduces the quality of life and is responsible for 20 million deaths annually (<https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases>). Several factors, including genetics, diet, and lifestyle contribute to these complex conditions. The diagnosis is usually established by anthropometric measurements, biochemical, genetic, liver function tests, inflammatory markers, and imaging techniques.

In this mini-review, we will report recent knowledge on the interaction between lipids, lipid metabolism within tissues and the complement system.

Complement system – an overview

The complement system, a humoral part of innate immunity, is a network of proteolytic cascades exerting its function in extracellular and intracellular fluids and on cellular surfaces. Most of its constituents are synthesized in the liver, whereas some are produced in immune or non-immune cells. It is an ancient defense system consisting of pattern recognition receptors (PRRs) and regulatory proteins, organized into three different pathways: classical (CP), lectin (LP) and alternative (AP). The CP is initiated by binding of its PRR - C1q, to antigen/antibody complexes, some pentraxins, apoptotic bodies and amyloid fibrils. Furthermore, repeating carbohydrates or acetylated residues, as well as aberrant glycocalyx patterns, engage recognition by PRRs of the LP (5–7). In contrast to that, the AP has a continuous low level of activation on self and non-self surfaces by the tick-over mechanism (8). Recently it was shown that complement can be activated in a non-canonical manner, by certain proteases from coagulation and fibrinolysis pathways (9–12). Cascades of all complement pathways conduct and control deposition of the central effector molecule C3 (13). Once deposited, and if not inactivated, C3 can guide the recruitment and formation of C5-convertase and terminal complement complex (C5b-C9) with a lytic function (8). Moreover, C3 and C5 cleavage generates anaphylatoxins that can drive chemotaxis and activation of immune cells, further propagating damage and inflammation (14, 15). Due to their strong auto-damaging potential, complement cascades are controlled by multiple regulators and inhibitors present in the fluid phase and on host cells (7, 16).

Novel evidence demonstrated that complement acts intracellularly. The intracellular complement, complosome, plays a role in cellular responses to the environment or the homeostatic

balance maintenance, by regulating many cellular functions, e.g., cell metabolism, autophagy, survival, signaling, response to infections, and efferocytosis capacity (17–21).

Finally, the complement system functions are versatile: it eliminates pathogens and altered self-structures, coordinates innate and adaptive immune responses, controls tissue reorganization, instructs clearance of metabolic waste, and responds to metabolic alternations on intra- and extracellular levels (7, 17, 22).

The complement system in atherosclerosis

Atherosclerosis is a chronic inflammatory disease characterized by the deposition and oxidation of low-density lipoprotein (LDL) particles in the vessel wall, followed by immune cell infiltration, leading to the formation of fatty streaks that can progress to plaques. If untreated, plaques become larger, more fibrous, calcified and prone to rupture. According to WHO, atherosclerosis is a major cause of mortality worldwide and is responsible for most myocardial infarctions (MI), strokes, and peripheral artery disease (PAD) (23).

The relevance of the complement system in atherosclerosis was demonstrated in the 1970s (24). This was followed by detection of complement proteins C3, C1q, C4, C9, C-reactive protein (CRP), C5b-C9, CD55, CD35, C3aR1, C5aR1, factor B (FB), factor H (FH), C1-inhibitor (C1-INH), C4-binding protein (C4BP), as well as active degradation products of some, within atherosclerotic plaques (25–32). Various clinical or genome-wide association studies (GWAS), demonstrated that components of the LP and CP have both proatherogenic and atheroprotective effects. More precisely, ficolin-1 and -2, pentraxin 3 (PTX3), mannan-binding lectin serine protease 2 (MASP2) and MASP3 have predictive value towards adverse cardiovascular events (33–35). The absence of mannan-binding lectin (MBL) predisposes to atherogenesis, but there are some controversies over its serum level effect on atherosclerotic cardiovascular disease (ACVD) (36–41). *C1Q* has been identified as a risk gene; in advanced lesions its local production is higher, but clear mechanisms of how its serum levels affect disease outcome are unknown (42–49). Predisposition for MI or cerebrovascular episodes was seen in hereditary C2 deficiency (50). Also, elevated levels of C4 have been associated with cardiovascular disease (CVD) or diabetic stroke, independently of traditional risk factors (51–55). In contrast to CP or LP components, elevated C3 levels correlated with classical risk factors and worsened CVD outcomes (56–63). Similarly, components of the C5b-C9 complex have a strong association with pathogenicity (60, 64–69). Serum levels of C4BP can be predictive of the severity of the PAD (70, 71). Although AP is proatherogenic, there is no conclusion about the effect of *CFH* polymorphisms on coronary heart disease (CHD) (72–76). On the contrary, plasma concentrations of factor H-related protein 1 (FHR1) were elevated in patients with ACVD and correlated with the expression of the inflammation markers (77).

The mechanistic role of complement in atherosclerosis was explored *in vitro* and *in vivo*. High-fat diet (HFD) feeding led to elevated amounts of circulating lipids and increased levels of circulating C3, C4 and C1q (78, 79). Oxidatively modified LDL (OxLDL), located within the vessel, presents various damage-associated molecular patterns (DAMPs) on its surface, which are recognized by natural IgM antibodies, CRP, C1q, MBL, C3a, FH, FHR1, FHR3, FHR5 or scavenger receptors on macrophages (1, 2, 80–86). Due to its damage potential, if not neutralized by the immune system, OxLDL can activate endothelial cells. It alters their phenotype to procoagulatory and induces their secretion of C3a, C5a and other chemokines. This further propagates endothelial distress and activates immune response, partially through C3a/C3aR, C5a/C5aR axis, or by deposition of sublytic C5b-C9 (87–92). Consequently, monocytes are recruited to the intima, where they become foam cells (23). Macrophage uptake of OxLDL, cholesterol efflux and foam cell transformation is affected by PTX3, C3a, C1q, factor D (FD), FH and MBL (81, 84, 93–97). If the amount of engulfed lipids is too excessive, cholesterol crystals (CCs) build up and trigger NLR family pyrin domain containing 3 protein (NLRP3) activation, resulting in macrophage death. At this stage, IgM, C1q, MBL-A, MBL-C, and C3b play a protective role – guiding their removal of dying cells by macrophages, employing complement receptor 3 (CR3) and V-set and immunoglobulin domain containing 4 protein (VSIG4) receptors (98–102). Next to it, a balance between intracellular C3 activation and repression by FH controls the efferocytosis rate of lesional macrophages and affects necrotic core formation (19). The impaired clearance rate of lipid-overloaded macrophages leads to the release of DAMPs resulting in the recruitment of additional immune cells and generation of C3a and C5a, propagating inflammation, and smooth muscle cell expansion (103). This leads to the necrotic core formation filled with CCs, cellular debris, monocytes/macrophages rich in tissue factor and erythrocytes. Cholesterol crystals are recognized by C1q, C3c, ficolin-2, MBL, PTX3, and CRP. When cleared by CR3-rich monocytes, opsonized CCs activate the inflammasome in a C5a-dependent manner and enhance IL-1 β secretion (104–108). Later, it was clarified that the metabolic switch required for IL-1 β production by macrophages requires mitochondrial C5aR1 ligation generated by cell-intrinsic C5a (109). Furthermore, C5a makes plaques unstable and prone to rupture by affecting the senescence and death rate of smooth muscle cells (110, 111). Upon plaque rupture, released CCs activate complement and drive thrombus formation (112).

The presence of components of the C5b-C9 complex within lesions is shown to be proatherogenic *in vivo*. This observation was further supported by the finding that the absence of CD59, a C5b-C9 inhibitor, accelerated advanced atherosclerosis (113, 114). The previously mentioned proatherogenic effect of C5a was further confirmed by findings that inhibition of surface-expressed C5aR1 and deficiency of C5aR2 resulted in smaller lesions using animal models (111, 115–117).

The discrepancy in complement inhibition effectiveness between human trials and animal studies in atherosclerosis highlights the need for further research on the complement system function to develop novel therapeutic strategies (118–120).

The complement system in metabolic syndrome

Metabolic syndrome (MetS) represents a cluster of several disorders, including insulin resistance (IR), obesity, dyslipidemia, hypertension and hyperglycemia. It contributes to the development of ACVD and T2DM (121–124). Even though increased C3 and C4 levels have been associated with the risk of developing MetS, metabolic alterations such as IR, obesity, inflammation, and neurohormonal dysfunction are pivotal initiators of this pathogenic cascade (123, 125–130).

Insulin resistance is characterized by the loss of sensitivity to insulin within insulin-dependent tissues such as adipocytes, muscles and liver. Observational studies have shown that C4 levels are associated with the homeostasis model assessment (HOMA) index, the parameter for IR (131). Although C1qA deficiency protects from HFD-induced IR, conflicting findings for the involvement of CP have been reported (132–135). In contrast to CP, LP is predominantly protective. Independently of multiple metabolic features, MBL correlated with insulin sensitivity and its levels were low among obese individuals (136–139). Additionally, low ficolin-3 was independently associated with IR and predicted type 2 diabetes mellitus (T2DM) (140). Baseline C3 levels and level changes correlated with HOMA, multiple organ IR and T2DM independently of obesity, metabolism- and inflammation-related risk factors (131, 141–149). Mechanistically, C3 influence on IR may be linked to the activity of C3a and C5a and their receptors. Studies in mice show that lack of C3aR or C3 can increase insulin sensitivity, although human studies did not find a connection between C3a and IR or T2DM (147, 150, 151). Furthermore, *in vivo* data on the role of C3adesArg – acylation stimulating protein (ASP) in IR are inconsistent (152, 153). In humans, higher ASP levels correlate with increased IR through altered lipid and glucose metabolism (154). Downstream proteins of the AP have been negatively correlated with insulin sensitivity. Weight loss and treatment of IR with rosiglitazone decreased FH concentrations in plasma, although conflicting results were found in SLE patients (126, 155). Additionally, the association between properdin, FH and Bb with HOMA was observed (156). However, data on the role of properdin obtained *in vivo* differ from human ones, as properdin deficiency did not affect insulin-mediated glucose uptake (157). Mechanistic data confirm the relevance of FB in MetS, as *Cfb*^{-/-} mice exhibit increased insulin sensitivity and decreased inflammation (158). For more downstream components, *in vitro* and *in vivo* data have shown C5aR1 to contribute to IR development (159, 160). Furthermore, the role of C5b-C9 complex in IR is still unclear. For instance, it does not correlate with IR nor influence the incidence of T2DM (147). However, in chronic heart failure, a positive correlation with HOMA-IR, fasting glucose and insulin level was seen (161).

Obesity is a low-grade inflammatory disease defined by excessive fat accumulation in visceral and subcutaneous fat depots (BMI ≥ 30 kg/m²) (162, 163). It is driven by lifestyle, genetic, environmental and cultural factors and is considered endocrine and metabolic disease (162, 164–166). Excessive nutrient intake or

low energy expenditure causes lipid accumulation, leading to adipocyte hypertrophy or hyperplasia, consequently driving inflammation that further exacerbates obesity and associated health issues (166–168). Adipose tissue produces many complement proteins including C3, FB, FH, CR1, C1q, C1r, C1s and properdin, and is the predominant source of key players in adipose tissue biology such as ASP, FD, and adiponectin (155, 169–173). In obesity, serum levels of C3, FB, FH and factor I (FI), but not FD, were elevated when compared to normal weight controls (174, 175). Similarly, analysis of BMI-discordant monozygotic obese twin pairs demonstrated that levels of FHR5, C4, C1qA, C1-INH, MASP1, FH, FI, C3 and C8 were elevated in a twin with higher BMI (176). Moreover, visceral and subcutaneous adipose tissue of a heavier twin had increased expressions of C1, C2, C3, FB, FI, properdin, FH, FHR2, C3aR, C5aR1, VISIG4, CD59, in contrast to FD and components of the C5b-C9 (177). In most rodent models of obesity, decreases in FD levels, induction of C1q, and inconsistency in overproduction and secretion of C3 or FB by adipose tissue were seen (170, 173, 178–180). Different production rate of certain complement proteins between subcutaneous and visceral fat of obese subjects, or between dissimilar stages of adipocyte maturation was reported in mice and men (172, 181–185). Within adipose tissue, by binding to the C5L2 receptor, ASP stimulates TG synthesis, increases glucose transport through GLUT1 and GLUT4, fractional free fatty acids re-esterification and inhibits lipolysis (186–194). In line with this, C3-deficient mice have a reduction in fat mass and are resistant to diet-induced obesity. Exogenous ASP administration to C3-/- animals on a standard diet led to a weight increase of a fat pad (193, 195–199). Overexpression of FB in preadipocytes boosts their lipid accumulation and maturation (185, 200). Additionally, through C3a/C3aR axis, FD regulates glucose uptake, increases TG synthesis and inhibits lipolysis (169, 172, 201–203). However, animals deficient in FD had no abnormality in development or body weight (204). Another adipokine similar to C1q is adiponectin, with an anti-inflammatory and anti-fibrotic function. It enhances insulin sensitivity and is downregulated in obesity (205–208).

Diabetes mellitus is the dysregulation of blood glucose levels due to insufficient insulin secretion by pancreatic beta cells, insensitivity of peripheral tissues to insulin, or a combination of both. Type 2 DM, an inflammatory disease, represents 90% of newly diagnosed cases. It is related to obesity and multiple metabolic disturbances, e.g., IR leading to hyperinsulinemia, beta cell exhaustion and finally insulin insufficiency (209). The complement system was shown to have critical metabolic functions within the beta cells. It affects insulin secretion, substrate and metabolite processing and regulates inflammatory processes within islets. Elevated C3 is associated with an increased risk of developing diabetes, independently of demographic, hereditary, metabolism- and inflammation-related factors (141, 147, 210). Interestingly, C3 was associated with insulin secretion, even after adjustment for insulin sensitivity index (211). Moreover, C3a, C3c and C3d correlated with T2DM, although for C3a these associations were attenuated after adjustment for confounding factors (147, 212, 213). Decreased levels of FD were observed in T2DM patients (174, 203). In one study, levels of properdin and soluble C5b-C9 were associated with

a family history of T2DM, although the effect of C5b-C9 niveau was not confirmed in others (147, 156, 214, 215).

Since insulin infusion did not affect C3 expression within adipocytes, this implicated that C3 levels might affect insulin secretion *in vivo* (216). In line with this, increased C3 expression in T2DM pancreatic cells demonstrated that intracellular C3 has protective effects on islet beta cells in stress conditions, through interaction with ATG16L1 (18, 20). The highest expressed complement gene in human beta cells, *CD59*, was shown to control glucose-mediated insulin secretion (217–219). This effect is further promoted by ASP (220). On the contrary, beta cell FH suppresses insulin secretion via adrenomedullin (221). Additionally, C3a and C5b-C9 have been identified as potent inflammasome activators, suggesting their role in insulinitis (222, 223). Interestingly, C3a and C5a generated by FD activity, and through the activity of their receptors, play a key role in adipose tissue-pancreas axis in murine models, by inducing insulin production and dampening beta cell death and dedifferentiation (201, 203, 224). Accordingly, C3aR and C5aR1 agonists improved glucose-dependent insulin production (225).

Dyslipidemia represents abnormal levels of lipids and lipoprotein particles in circulation and is a crucial risk factor for ACVD. It accounts for nearly 50% of deaths due to ischemic heart disease (226). Dyslipidemia is characterized by abnormal lipid levels, e.g., increased TGs, and decreased high-density lipoprotein (HDL). Many complement proteins have been shown to bind lipoprotein particles in plasma and are essential for their metabolic turnover. For instance, C3, acidic form of C4 (C4-A), basic form of C4 (C4-B), and C9 were detected on very low-density lipoprotein (VLDL) and LDL particles, while FHR3 appeared only on LDL (227, 228). Moreover, HDL was associated with C3, C4-A, C4-B, C9, vitronectin and clusterin in coronary artery disease and cholesterol ester protein transfer deficiency (229, 230). Additionally, FH, FD, properdin and MASP3 showed associations with lipoprotein particle concentration and size (228). Apolipoprotein E, a major protein component of lipoproteins, binds to FH, and C1q (97, 231, 232). In observational studies, levels of TGs, adverse lipoprotein subclass profile and enrichment in TGs in all lipoprotein subclasses are associated with higher levels of circulating C3; however, contradictory opinions were reported (129, 144, 216, 228, 233–238). Accordingly, ASP and TG levels correlate, although significance is lost when adjusted for waist/hip ratio and LDL size (195, 239). Elevated expression of C1S, C5aR1, CD59 and CD55 in subcutaneous adipose tissue was seen in patients with familial combined hyperlipidemia (240). Experimental data further underscores the significance of complement-lipid interaction. For instance, postprandial C3 and ASP secretion by adipocytes is shown to be stimulated by chylomicrons (190, 241). Additionally, male C3-/- mice (ASP deficient) display delayed postprandial clearance of TGs and increased fasting and postprandial free fatty acids levels (198). Properdin-deficient animals have increased fat accumulation on HFD, impaired postprandial TG clearance and decreased energy expenditure (157, 242).

As demonstrated above, the complement system controls insulin production and resistance, adipose tissue remodeling, and

lipoprotein metabolism, consequently affecting chronic low-grade inflammation.

The complement system in metabolic dysfunction-associated steatotic liver disease

The liver, a primary site for complement protein synthesis, is particularly susceptible to complement-mediated damage (243, 244). Dysregulation of the complement system can exacerbate liver inflammation and fibrosis (245).

Metabolic dysfunction-associated steatotic liver disease (MASLD) is characterized by excess fat accumulation in hepatocytes, without significant alcohol intake and is among the most commonly diagnosed liver disorders (246, 247). A progressive and inflammatory form of MASLD, known as metabolic dysfunction-associated steatohepatitis (MASH), features hepatic steatosis, inflammation, and fibrosis (248, 249). Predisposing factors for MASLD and MASH are components of MetS, oxidative stress, and lipid peroxidation. This condition can further advance to cirrhosis and hepatocellular carcinoma (250, 251).

A positive correlation between high C3 in serum and the prevalence and severity of MASLD was demonstrated in clinical studies (252–255). In addition to this, ASP, involved in adipocyte lipid metabolism, was increased in MASLD patients (255). Histological analyses demonstrated that in 74% of patients, cleaved C3b and C4d were deposited in liver tissue, with more

than 50% of C3-positive livers also showing C1q and MBL deposits, and exhibiting C5b–C9 formation. Additionally, these components were more frequently detected in MASH, demonstrating the involvement of both CP and LP in liver inflammation (256). However, in some patients, C3 activation was not associated with C1q, MBL, or C4d deposition, suggesting that the AP may also play a role in complement activation in the MASLD. This was confirmed by the positive correlation of properdin and the C3c deposits with liver inflammation. Additionally, levels of FH were downregulated in MASH subjects (257). C3a and C5a were identified to promote hepatic inflammation by attracting immune cells (258). Additionally, blocking or removing C3aR and C5aR1 might offer protection against steatosis, fibrosing MASH, inflammation, and metabolic dysfunction (17, 259–261). However, while some evidence points to C3a/C3aR and C5a/C5aR1 as a potential therapeutic approach, clinical trials targeting the complement system are still lacking, making its effectiveness uncertain.

The complement system and age-related macular degeneration

Age-related macular degeneration (AMD) is a chronic, inflammatory disease of the retina, and it is the most common cause of blindness in the elderly in developed countries (262). It is characterized by the accumulation of lipid-rich drusen between the Bruch membrane (BrM) and retinal pigment epithelium (RPE), resulting in degeneration of RPE, photoreceptors and consequently loss of vision. Joint action of aging, genetic and environmental

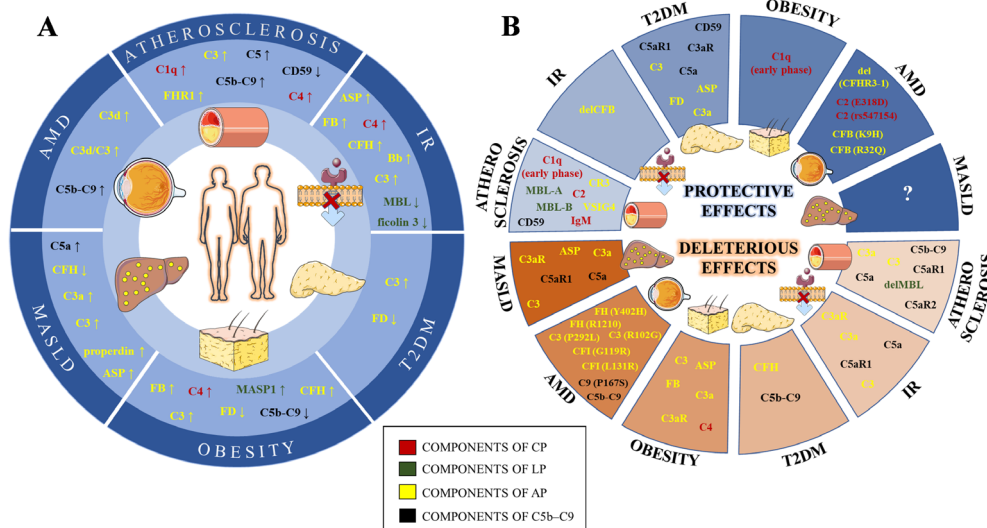


FIGURE 1

Schematic illustration of complement levels alterations (A) and their effects (B) in lipid-mediated pathologies. The components of the classical (CP), lectin (LP), alternative (AP) and terminal (C5b–C9) pathways are labeled in red, green, yellow and black, respectively. IR, Insulin resistance; T2DM, Type 2 diabetes mellitus; MASLD, Metabolic dysfunction-associated steatotic liver disease; AMD, Age-related macular degeneration; IgM, Immunoglobulin M; C1q, Complement Component 1q; C2, Complement Component 2; C4, Complement Component 4; MBL, Mannan binding lectin; MASP1, MBL Associated Serine Protease 1; C3, Complement Component 3; Asp, C3adesArg – acylation stimulating protein; FB, Factor B; FD, Factor D; FH, Factor H; FHR1, Factor H related protein 1; FI, Factor I; C3aR, Complement C3a Receptor; VSIG4, V-Set And Immunoglobulin Domain Containing 4; CR3, Complement Component Receptor 3; C5, Complement Component 5; C5aR1, Complement C5a Receptor 1; C5aR2, Complement C5a Receptor 2; C9, Complement Component 9; del, gene deletion; gene variants are given in the bracket after affected gene.

factors play a significant role in the disease onset and progression (263).

Lipids within the retina are crucial players in the pathogenesis of AMD. Due to photo-oxidative stress, they are highly susceptible to lipid peroxidation and the generation of oxidized and reactive breakdown products (1, 264–267). These degradation products can initiate sterile inflammation and stimulate immune responses (1, 2, 266, 268–270).

Seminal studies discovered 52 variants with genome-wide association (GWA) significance, among which *CFH* variant rs1061170 (Y402H), and variants within *ARMS2-HTR1*, *C2-CFB-SKVI2L* and *C3* had the strongest associations, confirming the key role of complement in the AMD pathogenesis (271–273). Today, Y402H is recognized as the major susceptibility variant. Heterozygous carriers of the minor C allele have a 2–4-fold, and homozygous carriers have a 3–7-fold increased risk for developing AMD (274). Moreover, it is important to note that this variant also affects the splice variant of FH, FHL-1, a main regulator of AP within BrM and RPE (275–277). Y402H decreases the binding ability of FH to heparin sulfate, CRP and malondialdehyde (MDA), thereby affecting its availability to act and regulate AP on surfaces decorated with these ligands (85, 276, 278–281). A similar aftermath was observed for the rare variant R1210C located within the C-terminal part of FH (273, 282). Deletion of *CFHR3-1* genes decreases the risk of AMD development (283–285). Moreover, deleterious effects were reported for some variants within *CFI*, *C3* and *C9* (274, 286–296). On the other hand, loss-of-function variants within complement activators *C2* and *CFB* are mostly protective (293, 297, 298).

Accumulation of oxidative damage shown by intensive MDA staining in BrM and choroid which co-stained with FH, implicated the binding of FH to MDA-adducts. Additionally, FH promoted the generation of iC3b on MDA surfaces and suppressed the proinflammatory effects of MDA *in vitro*. Due to the weaker binding to MDA, Y402H carriers have diminished regulation of AP and attenuated anti-MDA inflammatory properties (85). This was supported in a murine model, expressing chimeric FH, containing a human Y402H variant (299). Loss of endogenous FH in RPE cells renders them more susceptible to oxidative stress and reduces their viability (300, 301). Furthermore, we have demonstrated that FHR1 and FHR3 bind to MDA and thereby compete with FH for it, affecting FH AP regulatory function. These findings mechanistically explain the protective properties of *CFHR3-1* deletion (80).

Next to AP, the relevance of locally produced C5b-C9, deposited in choriocapillaris during the photoreceptor outer segment recycling by RPE was reported (302–304). In the aging retina, recycling capabilities decrease, resulting in enhanced C5b-C9 formation, pronounced in the presence of AMD-predisposing *CFH* variants (303, 305). Apart from locally produced FHL-1 and C5b-C9, increased systemic C3d/C3 ratio and enhanced local C3d deposition are implicated in AMD (302, 306, 307).

Activation of the AP, dysfunctional FH, together with aging, oxidative damage and disturbed lipid metabolism, are identified as critical steps in the development and progression of AMD (7).

Concluding remarks

Experimental evidence presented in this review confirms that the functions of the complement system in lipid homeostasis are versatile (Figure 1). Certain complement proteins sense and control lipid homeostasis locally or systemically. However, in case of excessive lipid accumulation or oxidation, complement proteins, if unable to neutralize it, can also act as initiators or propagators of lipid-driven inflammation. Therefore, understanding all mechanisms involved in cross-talk between lipid metabolism and complement system should be of importance for developing better diagnostic or therapeutic approaches.

Author contributions

LA: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. KD: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. NP-M: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

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C3 deficiency promotes pulmonary inflammation in AT1R-induced mouse model for systemic sclerosis

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Introduction: Autoantibody-mediated complement activation plays an essential role in a variety of autoimmune disorders. However, the role of complement in systemic sclerosis (SSc) remains largely unknown. In this study, we aimed to determine the role of complement C3 in the development of a recently described SSc mouse model based on autoimmunity to angiotensin II receptor type 1 (AT1R).

Methods: Mice were immunized with cell membrane extract isolated from Chinese hamster ovary (CHO) cells overexpressing AT1R or non-transfected CHO cells as a control. Peripheral blood, dorsal skin and the lung were then collected to evaluate disease characteristics. Apoptotic cells in the lung of mice were detected using the DeadEnd™ Fluorometric TUNEL System.

Results: Our results showed that experimental SSc in this model was featured by the deposition of IgG, but not of complement C3, in the lung. After immunization with AT1R, C3-deficient mice developed more severe pulmonary inflammations than wild type controls, whereas skin inflammation and fibrosis were not different as well as the anti-AT1R ab levels. Further, C3-deficient mice showed an increased rate of pulmonary cell apoptosis as compared to controls. The apoptosis rate correlated with the corresponding degree of lung inflammation.

Discussion: Taken together, our findings suggest an anti-apoptotic and anti-inflammatory role of complement C3 in pulmonary autoimmune inflammation.

KEYWORDS

systemic sclerosis, inflammation, mouse model, complement, pulmonary inflammation

Introduction

Systemic sclerosis (SSc) is an autoimmune connective tissue disease with multiple organ involvements and heterogeneous clinical manifestations (1). It is characterized by autoimmunity, inflammation, vasculopathy and fibrosis in skin and visceral organs (2). Although the disease prevalence is relatively low compared to other autoimmune disorders, SSc is featured by a high mortality rate mainly due to disease manifestations in the lung (3). Multiple autoantibodies (abs) are present in sera of patients with SSc. Some of them have been suggested to play a role in the development of SSc (4, 5). For example, high levels of abs against angiotensin II receptor type 1 (AT1R) are present in the serum of SSc patient. They are associated with fibrotic and vascular SSc manifestations and predict disease-related mortality (6). *In vitro*, anti-AT1R abs are able to induce the expression of chemokines and adhesion molecules in endothelial cells and collagen type-1 in fibroblasts (6–8), suggesting a potentially pathogenic role of these abs. Recently, we have verified this notion by immunizing mice with membrane extract from Chinese Hamster Ovary (CHO) cells overexpressing AT1R. After the immunization, C57BL/6J mice generated receptor-activating functional abs against AT1R as well as fibrosis and inflammation in the skin and lung, resembling multiple SSc features (9).

Autoantibodies are capable of inducing pathological alteration via multiple pathways, which generally can be categorized into 2 groups: Fc-dependent and Fc-independent (10). The Fc-dependent pathways stimulate immune cells by their Fc receptors and activate the complement system via immune complexes. Here, complement activation by autoantibody-antigen complexes lead to the deposition of a sublytic membrane attack complex and release of chemotactic and inflammatory factor such as C5a. This essential mechanism of autoantibody-mediated pathology was shown in various autoimmune diseases including myasthenia gravis (11), pemphigoid diseases (12) and rheumatic disorders (13). In contrast, the Fc-independent pathways are mediated solely by antigen binding domain of abs (10). It has been reported that SSc is featured by decreased circulating levels of C3 and C4 (14, 15). Moreover, biomarkers of complement system activation including C3d and C4d are increased in SSc patients as compared to healthy controls. The levels of those biomarkers are associated with disease manifestations such as scleroderma renal crisis (16, 17). Despite these clinical findings suggest that complement activation is associated with the development of SSc or at least of some SSc manifestations, the contribution of complement to the pathogenesis of SSc has not been investigated (18). Animal models are excellent research tool for investigating the pathogenesis of human disorders (19). They have been extensively applied to study the role of complement in experimental models for various autoimmune diseases, but not SSc (18, 20). Thus, although the complement system is thought to contribute to the pathogenesis of various autoimmune rheumatic diseases, its role in the development of SSc remains largely unknown (21).

In this study, we aimed to determine the role of complement system in SSc using the AT1R-induced mouse model for SSc. The complement system, a central component of innate immunity, is

essential for defense against pathogens and maintaining host homeostasis (22). It can be activated through three distinct pathways—classical, alternative, and lectin—all of which require C3 for proper function (22). Therefore, we determined the role of C3 in the mouse model for SSc using C3-deficient mice. Our results revealed an unexpected anti-inflammatory role of C3 in pulmonary inflammation.

Methods

Mice

Seven-week old female wild type C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, US). Complement C3-deficient mice (B6.129S4-C3^{tm1Crr}/J, C3 KO) were obtained from University of Lübeck, Lübeck, Germany. All mice were housed under specific pathogen free conditions with 12-hour light/darkness cycles at the animal facility of Research Center Borstel, Germany. All animal experiments were reviewed and approved by the Animal Research Ethics Board of the Ministry of Energy Change, Agriculture, Environment, Nature, and Digitalization, Kiel, Germany (Number 104-8/17).

Immunization with membrane extract

Cell membrane extract (ME) isolated from Chinese Hamster Ovary (CHO) cells overexpressing AT1R or non-transfected CHO cells were kindly provided by CellTrend (Luckenwalde, Berlin, Germany). Eight to ten weeks old female mice were immunized with 0.2 mg of control or AT1R ME in 50 µl of PBS emulsified with complete Freund adjuvant (CFA, Sigma-Aldrich, USA) by subcutaneous injection into the footpad as described previously (9). Three weeks after the immunization, the mice were boosted with the same amount of antigen emulsified with incomplete Freund adjuvant (IFA, Sigma-Aldrich, USA). Nine weeks after the first immunization, peripheral blood, dorsal skin and the lung were harvested for the evaluation of disease characteristics.

Detection of deposition of IgG and C3

The deposition of IgG and C3 in the murine lung were determined by immunofluorescence staining. Briefly, the lung samples were collected from mice and cryosection was performed at thickness of 3 µm, after fixation in pre-cold acetone and blocking in blocking buffer (Carl Roth, Germany), IgG binding was detected using DyLight 649-labeled goat anti-mouse IgG antibody (polyclonal, BioLegend, USA), complement 3 was detected with rat anti-mouse C3 antibody (RMC11H9, Cedarlane, Canada) and Alexa488 labeled-goat anti-rat IgG antibody (polyclonal, Invitrogen, USA). Coverslips were mounted with Gold mounting agent (Thermo Fisher, USA) that contains 4', 6-diamidino-2-phenylindole (DAPI) for nuclear staining, the fluorescence was evaluated by confocal microscopy at a magnification indicated in

the legends to figures (Leica SP5, Germany). The intensity of the fluorescent signals was analyzed and quantified using Image J software (version 1.53t, USA).

Quantification of anti-AT1R antibodies

The level of anti-AT1R autoantibodies in the mouse serum samples were detected by using enzyme-linked immunosorbent assay (ELISA) as previously described (9). Levels of anti-AT1R antibodies were defined as the dilution at which the OD value reached the half of maximal OD values of the curve.

HE staining

H&E staining was performed to determine the inflammation in tissues. Paraffin-embedded sections from mouse skin and lung were deparaffinized in xylene and rehydrated in ethanol series with decreasing concentrations, then stained with hematoxylin and eosin solutions. Thereafter, the stained sections were dehydrated and then mounted with mount medium. Severity of pulmonary and skin inflammation was quantified by the size and number of infiltrates in the lung and skin, and scored in a double-blinded fashion as described previously (9).

Masson's Trichrome staining

To evaluate tissue fibrosis, paraffin-embedded tissue sections of mouse skin and lung were stained with Masson's Trichrome staining kit (Sigma-Aldrich, USA). Tissue sections were deparaffinized in xylene and rehydrated in ethanol series with decreasing concentrations. Rehydrated sections were fixed in Bouin's solution at RT overnight, counterstained with Hematoxylin Gill II solution, washed and incubated with Biebrich scarlet-Acid Fuchsin, and subjected to phosphotungstic/phosphomolybdic acid solution. Phosphoric acid treated sections were then incubated with aniline blue solution, differentiated in 1% acetic acid, rinsed with deionized water, dehydrated in ethanol, and finally mounted with mounting medium. Skin thickness was measured as the thickness of the blue collagen layer.

Measurement of skin collagen content

The collagen content of the skin was determined by using the Sircol collagen detection kit (Biocolor, UK) according to the manufacture's instruction. Briefly, punches of skin tissues (9 mm²) were cut into pieces and digested in 0.5 M acetic acid solution containing porcine pepsin (Sigma, USA) at 4°C overnight on the shaker. In the next day, supernatant was collected and was mixed with Sircol dye solution and incubated on shaker. Afterwards, samples were centrifuged and pellets were washed with ice cold acid salt wash reagent to remove unbound dyes. Dye bound collagen pellets were dissolved in Alkali buffer was

added to each sample to release collagen. To record the OD values, samples were transferred into a plate and measured on TECAN microplate reader at a wavelength of 550 nm. Amount of collagen in samples were calculated according to the standard curve generated with gradient diluted collagen.

Immunohistochemistry

Immunohistochemistry staining was conducted on murine tissue sections to assess the cellularity of infiltrated cells. Lung paraffin-embedded sections underwent deparaffinization in xylene followed by rehydration in a gradient of ethanol. Subsequently, antigen retrieval was achieved by microwave heating with 1× citrate buffer (pH=6) (ZytoMed, BE, Germany, Cat#ZUC028-500), and sections were blocked using 3% H₂O₂. They were then incubated with specific primary antibodies for 1 hour at room temperature, including rabbit anti-CD20 (Invitrogen, MA, USA, Cat# PA5-16701, 1:500), rabbit anti-CD3 (ZytoMed, Cat# RBK024-05, 1:100), or rat anti-mouse neutrophil antibodies (Cedarlane, ON, Canada, Cat# CL8993AP, 1:800). Following washing steps, the sections were treated with ImmPRESS-HRP goat anti-rat IgG polymer kit (VECTOR, Cat# MP-7404-50) or ZytoChem Plus-HRP polymer kit (ZytoMed, Cat#POLHRP-100), followed by incubation with 3-Amino-9-ethylcarbazole (AEC) (ZytoMed, Cat#ZUC054-200) to visualize immunoreactivity. Finally, counterstaining with hematoxylin was performed, and bright-field microscopy was used to capture images.

Assessment of cell apoptosis in the lung tissue

The apoptotic cells in the lung of mice were detected with DeadEndTM Fluorometric TUNEL System (Promega, USA) according to the manufacturer recommended procedures. Briefly, the deparaffinized and rehydrated lung sections were permeabilized with 0.2% Triton X-100 and equilibrated in equilibration buffer. Immediately before the reaction, the TUNEL mixture was freshly prepared and added to the sections. After incubation with rTdT incubation buffer, the reactions were stopped by SSC buffer. Sections were finally mounted with mounting medium containing DAPI for counterstaining. To quantify the level of apoptotic cells, six to ten random confocal images were recorded and the number of apoptotic cells and total cells were measured, and the rate of apoptotic cells were calculated as number of apoptotic cells divided by total number of cells.

Statistics

All data was analyzed by using Graphpad Prism software (version: prism 10.2). Statistical differences of quantitative data between two groups were determined by two-tail unpaired Student's t test. Two-way ANOVA was used to determine the

statistical significance among multiple groups with two category variables. Significant differences between qualitative datasets were determined by Fisher-exact test. A p value below 0.05 was considered as statistically significant.

Results

AT1R immunization induces IgG deposition but not C3 deposition in murine lung

To investigate the role of complement activation in the AT1R-induced mouse model for SSc, we first determined the deposition of IgG and complement C3 in the lung. Direct Immunofluorescence staining revealed that mice immunized with AT1R showed significantly higher levels of IgG deposition in the lung than mice immunized with control ME (Figures 1A, B). By contrast, no significant difference in C3 deposition was observed between

AT1R-immunized mice and controls suggesting the lack of IgG-mediated complement activation (Figures 1C). In contrast, C3 deposition was effectively detected in a mouse model of epidermolysis bullosa acquisita (Supplementary Figure 1), where complement activation is a crucial step in disease progression (23).

C3 deficiency promotes AT1R-induced pulmonary inflammation

To better examine the role of complement C3 in this mouse model, C3 deficient mice and wild type (WT) control mice were immunized with AT1R ME or control ME. Nine weeks after the first immunization, blood, lung and skin were collected for further evaluation (Figure 2A). As shown in Figure 2B, immunizations with AT1R induced comparable levels of anti-AT1R IgG in C3 deficient and WT mice, suggesting that C3 deficiency does not affect the production of abs against AT1R.

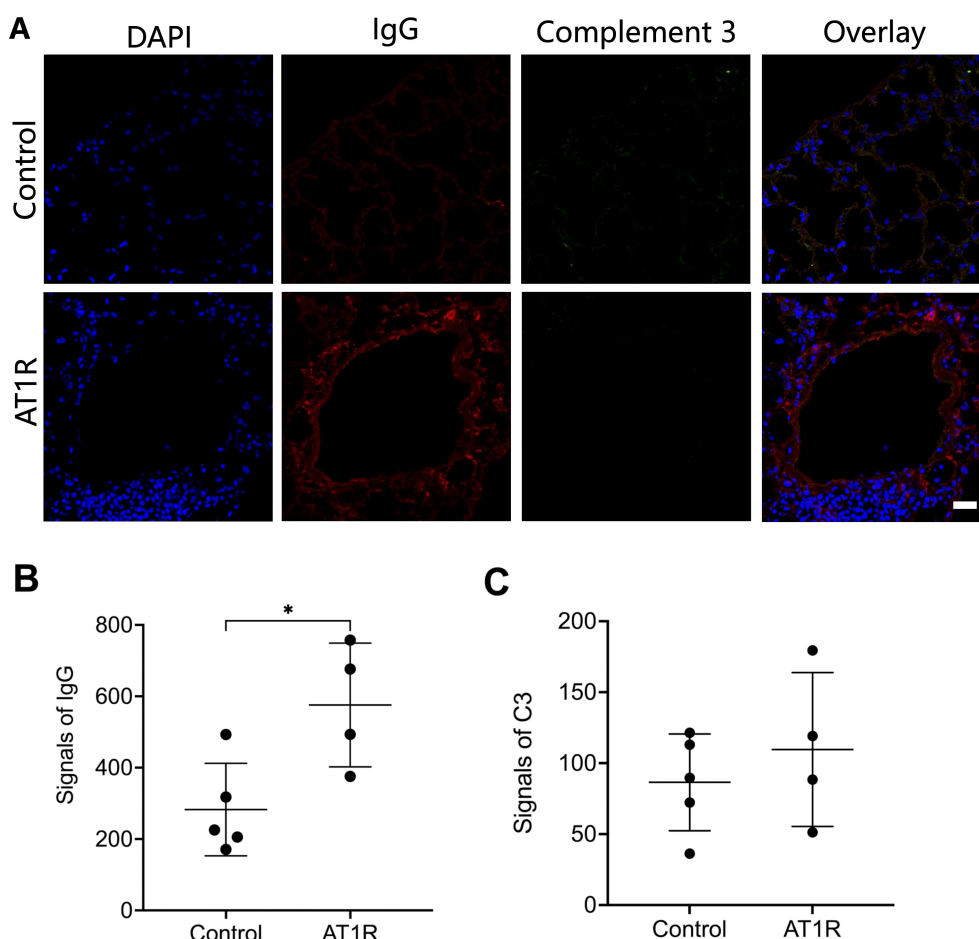


FIGURE 1

Deposition of IgG but not complement 3 in lung tissue of hAT1R-immunized mice. (A). Cryosections of lung tissue from hAT1R-immunized mice or control mice were incubated with DyLight 649-labeled goat anti-mouse IgG antibody to detect IgG deposition (red), complement 3 was stained with rat anti-mouse C3 antibody and Alexa488 labeled-goat anti-rat IgG antibody (green), nucleus was stained with DAPI (blue). The representative micrographs were shown (630x, scale bar= 25mm). (B, C). The quantitative analysis of the fluorescent signals of IgG and complement 3 was performed with pictures of representative fields of each group (n=4~5) using ImageJ software. The results are presented as the mean $\pm$ SD, and the statistical analysis was performed by using Student's t-test (*: $p < 0.05$).

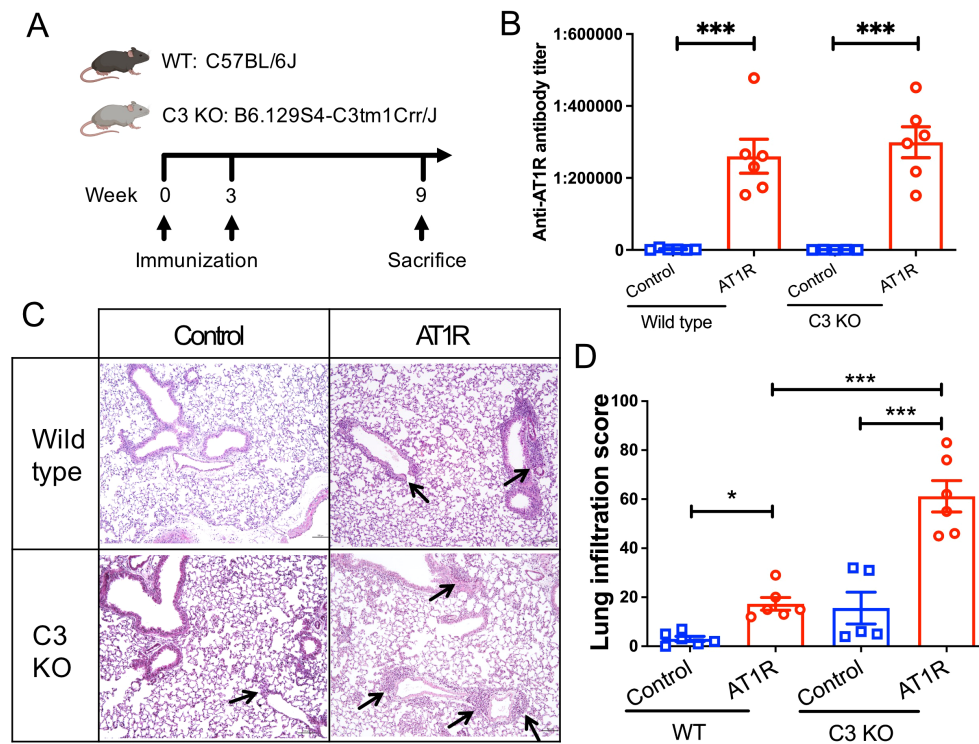


FIGURE 2

C3 deficiency promotes the AT1R immunization-induced lung inflammation. (A). Schematic diagram of immunization protocol and groups. Mice were immunized at week 0 with membrane extract (AT1R ME or control ME) and boosted 3 weeks later. Nine weeks after the first immunization, mice were sacrificed, and blood and tissue samples were collected for evaluation. Five to six mice were analysed in each group. (B). Levels of anti-AT1R IgG were determined in sera by ELISA. (C). Representative micrographs of H&E-stained lung sections of control ME- or AT1R ME-immunized mice. Black arrows indicate inflammatory cell infiltration in the lung. Scale bar = 100 μ m. (D). Quantitative analysis of pulmonary inflammation in mice. Statistical analysis was performed by using two-way ANOVA test. * $p < 0.05$, ***, $p < 0.001$.

Histological examination of lung tissue showed that AT1R immunization induced pulmonary inflammation in both C3 deficient mice and WT controls. Notably, C3-deficient mice developed a more severe AT1R-induced inflammation in the lung than WT mice (Figures 2C, D, $p < 0.001$). Immunohistochemistry analysis revealed that lung inflammation primarily consisted of T cells, with neutrophils and B cells also present, and the composition of lung infiltrates was comparable between AT1R-immunized C3 deficient and WT mice (Supplementary Figure 2). In addition, a trend of increased pulmonary inflammation, although not significant, in C3-deficient mice as compared to WT mice was also observed after immunization with control ME.

C3 deficiency is dispensable for AT1R-induced disease manifestation in the skin

We next examined the histopathological changes in the skin, another tissue involved in this experimental model (9). AT1R immunization induced comparable incidence of skin inflammation between WT controls and C3 deficient mice (Figures 3A, B). Furthermore, both skin thickness and collagen

content were not significantly different between C3-deficient mice and WT controls immunized with AT1R (Figures 3C–E), indicating that C3 is dispensable for disease manifestations in the skin and indicate organ-specificity of the protective C3 effects for the lung.

C3-deficiency promotes pulmonary cell apoptosis

In the next step we aimed to explore the mechanism underlying the effect C3 on pulmonary inflammation. Since it has been reported that C3 plays a protective role in apoptosis human airway epithelial cells and β -cells (24, 25), a common trigger of inflammation, we determined apoptosis of pulmonary cells in immunized mice. Compared to WT controls, C3-deficient mice showed a significantly increased pulmonary cell apoptosis rate after AT1R immunization ($p < 0.01$) (Figures 4A, B). However, increased pulmonary cell apoptosis rate in C3-deficient mice as compared to corresponding WT controls was also observed in animals which received control ME ($p < 0.01$). Finally, we evaluated the relationship between cell apoptosis and pulmonary inflammation. As shown in

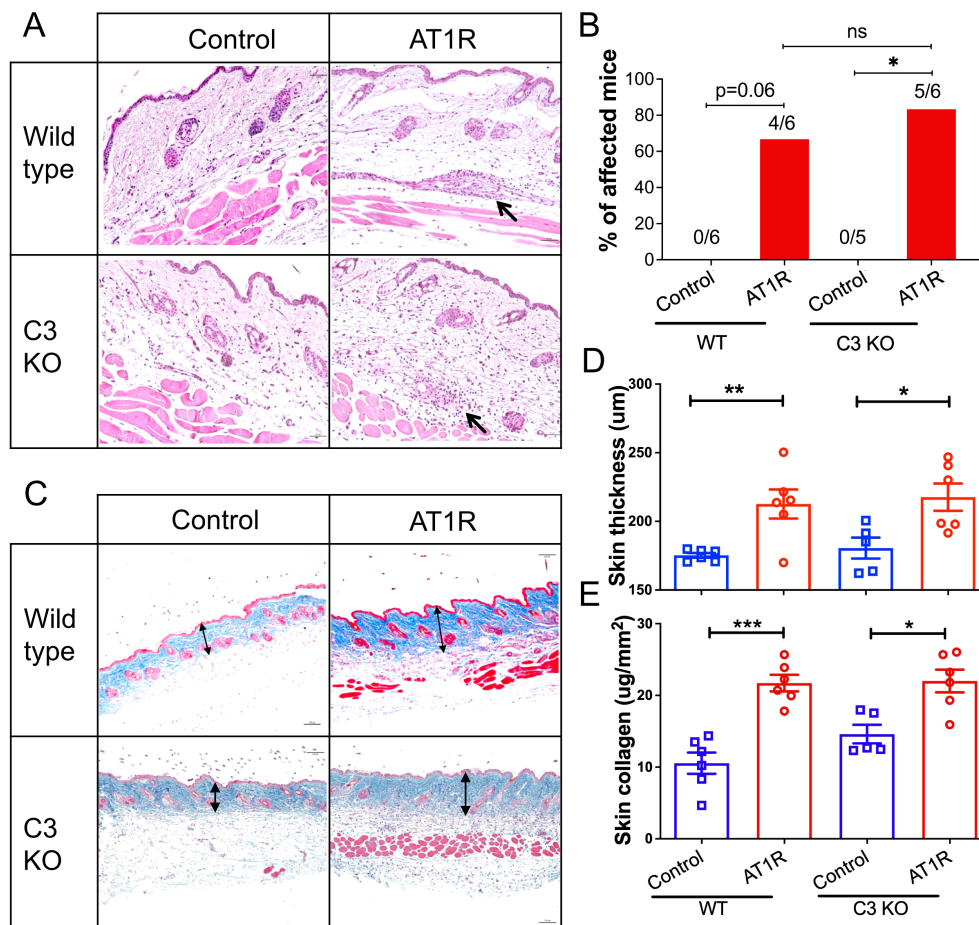


FIGURE 3

C3 deficiency does not affect skin inflammation. Wild type and C3-deficient (C3 KO) mice were immunized with control ME or AT1R ME. (A). Representative micrographs of H&E-stained skin sections of control ME- or AT1R ME-immunized mice. Black arrows indicate inflammatory cell infiltration around blood vessels. Scale bar = 50 μm. (B). Incidence of skin inflammation in mice. The numbers of mice with skin inflammation/all mice are presented on the top of corresponding bars. (C). Representative micrographs of Masson's Trichrome stained skin sections of control ME- or AT1R ME-immunized mice. Double-headed arrows indicate the collagen layer of the skin. Scale bar = 100 μm. (D). Quantitative analysis of skin thickness. Thickness of the skin was defined as the thickness of the collagen layer stained in blue from the Masson's Trichrome staining. (E). Quantitative analysis of collagen content. Collagen content was determined using Sircol collagen detection kit and expressed as μg per mm² of the skin. Statistical analysis was performed by using two-way ANOVA test. *, $p < 0.05$, **, $p < 0.01$ ***, $p < 0.001$.

Figure 4C, a strong correlation was observed between pulmonary cell apoptosis rate and pulmonary inflammation in AT1R-immunized C3-deficient mice ($R^2 = 0.74$, $p = 0.029$), while such correlation was much weaker in the other three groups (data not shown).

Discussion

Clinical studies have shown that SSc is featured by hypocomplementaemia and complement activation (14–17), implicating a role of complement system in the development of the disease which warrants further investigation. To our knowledge, this is the first study to examine the role of complement system in animal models for SSc.

In the present study, C3-deficient mice generated anti-AT1R autoantibodies and developed inflammation in the lung and skin as well as skin fibrosis. Moreover, IgG deposition, but not C3 deposition, was observed in the lung of AT1R-immunized wild

type mice. Therefore, our results demonstrate that complement system is a dispensable factor for the disease development in the AT1R-induced mouse model. This notion is in line with the concept that anti-AT1R antibodies are functional autoantibodies which mediate disease pathology in a Fc-independent manner by a specific binding and stimulation of the AT1R (6, 9).

Previous studies have shown that C3 deficiency can impair the generation of antibodies against various pathogens and that C3 fragments such as C3b and C3d may act as a molecular adjuvant (26–30), suggesting a role for C3 in modulating humoral immunity. Interestingly, in our study, no significant difference in the antibody response against AT1R was observed between C3-deficient mice and wild-type controls. This discrepancy may be attributed to the strong adjuvant effect of CFA, which could potentially compensate for the absence of C3. This notion is supported by the observation that CFA indeed raises antibody responses similar to C3b as an adjuvant (30). Thus, the impact of C3 deficiency on humoral responses may be context-dependent.

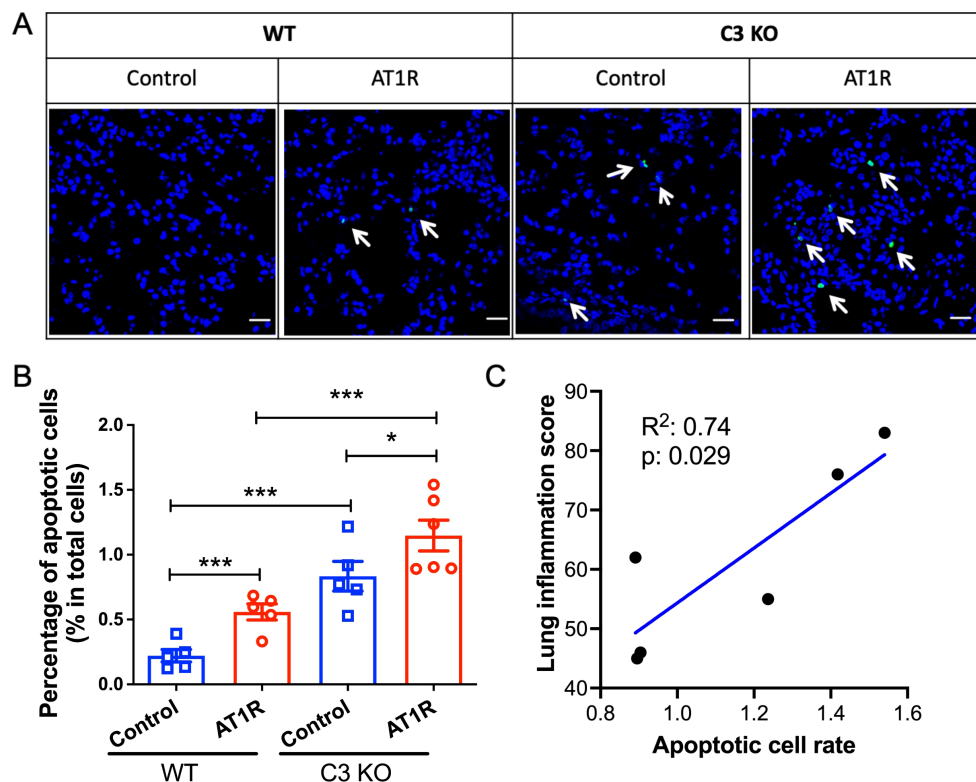


FIGURE 4

Increased apoptosis of lung epithelial cells in C3-deficient mice. (A). Representative micrographs of apoptotic cells in lung cryosections from WT or C3 deficient mice visualized by TUNEL assay. Positive apoptotic cells are labelled in green fluorescence. Scale bar= 25mm. (B). Quantitative analysis of apoptotic cells as determined in the TUNEL assay. The percentage of apoptotic cells in the total cells were presented as mean $\pm$ SD. Statistical analysis was performed by using two-way ANOVA test. *, $p < 0.05$, ***, $p < 0.001$. (C). The correlation of severity of lung inflammation and apoptotic cell rate from the AT1R immunized C3 deficient mice. Blue line shows the simple linear regression line. R square and p values were presented in the plot.

Importantly, C3-deficient mice developed a more severe AT1R-induced pulmonary inflammation than wild type controls, while such difference was not observed in the disease manifestations in the skin. This finding suggests that C3 plays an anti-inflammatory and protective contribution specifically in the development of pulmonary inflammation. This finding suggests that enhancing C3 activity could be a potential therapeutic strategy for managing pulmonary manifestations in SSc. However, this clinical implication requires further evidence. Specifically, it is necessary to determine whether increasing C3 activity can inhibit lung inflammation in experimental models of SSc. Additionally, clinical data linking enhanced C3 activity with improved lung function are needed.

It is noteworthy that C3a, typically considered a proinflammatory mediator, may also exhibit anti-inflammatory properties (31), supporting an anti-inflammatory role for C3. However, since C3 deposition was not observed in the lungs of this model, it is unlikely that the anti-inflammatory effect of C3 in this context is due to C3a. We propose instead that intracellular C3 may play an anti-inflammatory role by regulating pulmonary cell death, a mechanism suggested by previous studies (25, 32). In 2019,

Kulkarni and colleagues reported that airway epithelial cells store intracellular C3 through biosynthesis and/or uptake of exogenous C3 during inflammation (25). Recently, Kulak et al. reported that intracellular C3 protects beta-cells from IL-1 β -induced cytotoxicity via interaction with Fyn-related kinase (32). In line with this notion, recently Sahu et al. reported that complement C3 protects lung epithelial cells from bacteria induced lung inflammation and injury and such protection is through Factor B rather than C3a receptor (33).

One major limitation of the present study is that it employs only a single experimental model of SSc. Given the heterogeneous nature of SSc, each model represents only specific aspects of its pathogenesis. Therefore, the findings of this study should be interpreted with caution and not overgeneralized.

In conclusion, the present study showed that C3-deficient mice exhibited more severe lung inflammation and increased pulmonary cell apoptosis in a mouse model for SSc. These findings reveal an anti-inflammatory and anti-apoptotic effect of C3 in the lung. The unexpected anti-inflammatory role of C3 carries clinical implications, warranting further investigation.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Animal Research Ethics Board of the Ministry of Energy Change, Agriculture, Environment, Nature, and Digitalization, Kiel, Germany (Number 104-8/17). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JY: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. AV: Resources, Writing – original draft, Writing – review & editing. XyY: Investigation, Writing – original draft, Writing – review & editing. TG: Investigation, Methodology, Writing – review & editing. HH: Methodology, Writing – original draft, Writing – review & editing. GR: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. FP: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. XhY: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

HH is the owner and GR is an advisor of the company CellTrend, Germany, which produced the membrane extracts and the tests for the detection of anti-AT1R antibodies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1491324/full#supplementary-material>

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The AMD-associated genetic polymorphism CFH Y402H confers vulnerability to Hydroquinone-induced stress in iPSC-RPE cells

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Introduction: Age-related macular degeneration (AMD), a degenerative disease of the macula, is caused by an interplay of diverse risk factors (genetic predisposition, age and lifestyle habits). One of the main genetic risks includes the Y402H polymorphism in complement Factor H (FH), an inhibitor of complement system activation. There has been, and continues to be, much discussion around the functional consequences of this Y402H polymorphism, whether the soluble FH protein confers its risk association, or if the cells expressing the protein themselves are affected by the genetic alteration. In our study, we examined the cell characteristics of the retinal pigment epithelium (RPE) cells, which play a major role in retinal homeostasis and stability and which are synonymously linked to AMD.

Methods: Here, we employ RPE cells derived from induced pluripotent stem cells (iPSC) generated from donors, carrying either homozygous 402Y (low risk) or 402H (high risk) variants of the *CFH* gene. RPE cells were treated with Hydroquinone (HQ), a component of cigarette smoke, to induce oxidative damage.

Results: Intriguingly, RPE cells carrying high genetic risk proved more vulnerable to oxidative insult when exposed to HQ, as demonstrated by increased cytotoxicity and caspase activation, compared to the low-risk RPE cells. The exposure of RPE cells to RPE conditioned medium, normal human serum (NHS) and inactivated NHS (iNHS) had minimal impact on cell cytotoxicity and caspase activation, nor did the presence of purified soluble FH rescue the observed effects. Considering the known connection of oxidative stress to proteotoxic

stress and degrading processes, we investigated the unfolded protein response (UPR) and autophagy. When exposed to HQ, RPE cells showed an increase in autophagy markers; however, iPSC-RPE cells carrying high genetic risk showed an overall reduced autophagic flux.

Discussion: Our findings suggest that the degree of cellular susceptibility to oxidative stress is not conferred by soluble FH protein and other complement sources, but intercellularly because of the corresponding genetic risk predisposition. Our data support the hypothesis that RPE cells carrying high genetic risk are less resilient to oxidative stress.

KEYWORDS

age-related macular degeneration (AMD), complement factor H (CFH), retinal pigment epithelium (RPE) cells, oxidative stress, autophagy

Introduction

Age-related macular degeneration (AMD) is a complex and progressive disease of the macula, mainly affecting the elderly population, culminating in vision loss. As the number of cases is expected to increase, threatening the patient's quality of life, AMD places a major burden on national health systems (1). From a clinical perspective, late-stage AMD is divided into wet AMD or choroidal neovascularization (CNV) and dry AMD, which accounts for the majority of AMD cases and for which therapeutic options are limited. Dry AMD is defined by geographic atrophy (GA), characterized by degeneration of the photoreceptors (PR) and underlying Retinal Pigment Epithelium (RPE) cells (1). RPE cells hold several functions crucial to maintaining retina visual function and to supporting PR function. As a cellular monolayer interfacing the neuroretina and the choroidal blood supply space, RPE cells are responsible for nutrient supply, fluids, and discard material exchange, including the processing of shed PR outer segments (POS). Moreover, RPE cells possess a variety of antioxidant mechanisms to protect the retina from excessive oxidative stress (2). The exact mechanism by which RPE cells become dysfunctional in AMD is not fully understood and most importantly how the combination of AMD risk factors interacts to contribute to RPE disease phenotypes remains quite complex to understand. Besides aging, which is the primary cause of AMD, unhealthy life-style choices, such as smoking, contribute to disease progression. Moreover, genetic predisposition plays a crucial role in AMD risk (3).

Genome-wide association studies (GWAS) have identified several associations between genetic variance and AMD risk and progression. Several risk loci have been identified as modifying risk for disease, but two main risk loci predominate: one located in chromosome 10 (Chr10q26) around the *ARMS2/HTRA1* genes (4), and the other in chromosome 1 (Chr1q32) known as the Regulators of Complement Activation (RCA) cluster: that contains genes regulating the complement system (5). This RCA cluster includes the Complement Factor H (*CFH*) gene, encoding for the Factor H

(FH) protein and its truncated splicing variant FHL-1 (6). A very common polymorphism, leading to a Y402H amino acid change in the FH/FHL-1 proteins, is a defining feature of the genetic haplotype with the strongest association to AMD risk.

The FH/FHL-1 proteins function as negative regulators of the complement system, by binding to host surfaces and helping deactivate the central protagonist of complement activation, C3b (7). A proteolytic enzyme, complement factor I (FI), can bind and cleave C3b, inactivating it to produce iC3b. However, FI can only achieve this in the presence of a co-factor. Despite several different cell-bound FI co-factors, FH and FHL-1 represent the only soluble FI co-factors that can provide protection to a host's acellular surfaces, such as basement membranes and the glycocalyx (8). The Y402H polymorphism occurs within a major anchoring domain of FH and FHL-1, and is believed to hamper the proteins' ability to bind surfaces such as Bruch's membrane (9). Indeed, markers of complement over-activation are seen in the eyes of human donors carrying genetic risk haplotypes including the Y402H polymorphism (10). Interestingly, markers of complement over-activation can also be found systemically in patients with AMD, although some debate remains around whether this is a systemic phenomenon, or a local, tissue-specific, event that can be detected systemically (11).

However, in recent years a novel concept of non-canonical FH functions has been introduced (12). This includes a number of cellular functions which have been suggested to be regulated by FH, mainly in its intracellular form and most likely independent from its function as complement system inhibitor in the extracellular space (12, 13). Recent studies have highlighted that FH, and the Y402H polymorphism, could contribute directly to RPE pathology. It was shown that iPSC-RPE carrying the Y402H polymorphism presents mitochondria metabolism impairments (14), mitochondria abnormalities (15), and an accumulation of lysosomes and phagosomes (15, 16). As RPE cells and neuroretina coexist in a metabolic balance with each other, these alterations could affect the ability of RPE cells to metabolically support the retina and process

the discard material deriving from the PRs *via* the autophagy-lysosomal flux (17, 18). No less importantly, these altered features could render the RPE cells even more dysfunctional in AMD-like conditions, such as increased oxidative stress. Indeed, several factors contributing to AMD often culminate in excessive oxidative stress, as a consequence of aging or cigarette smoke exposure (19). Accumulation of Reactive Oxygen Species (ROS) can damage cellular homeostasis, leading to the buildup of damaged organelles and proteins by oxidation (20, 21). Healthy RPE is quite resistant to the oxidative damage throughout its lifetime, and can continue to operate degrading pathways, such as autophagy and endoplasmic reticulum-associated degradation (ERAD), to process and expel the damaged material (22). However, RPE cells that present impaired mitochondria, such as iPSC-RPE cells carrying the Y402H polymorphism of FH (15), may be predisposed to a less efficient oxidative stress response and therefore may not be able to survive an excessive oxidative stress environment. In these circumstances, RPE dysfunction will be elevated and pave the way to PR degeneration, ultimately being the cause of vision loss.

In accordance with this hypothesis, in our previous work, we have shown that hTERT-RPE1 cells deprived of FH via silencing, are more vulnerable to oxidative stress insult (H_2O_2), involving alteration in metabolic pathways, as oxidative phosphorylation and glycolysis, mitophagy and inflammation (23).

To move forward and better understand the influence of FH dysregulation in RPE pathology during AMD progression, we employed, in this work, iPSC-RPE cells derived from either low or high genetic risk donors, which closely represent the maturity of RPE cells *in vivo*. To investigate the impact of oxidative stress in mature RPE cells, we employed a more physiological oxidative agent: Hydroquinone (HQ). HQ is a benzene metabolite that has high redox activity and is a well-known substance used to induce oxidative stress (24). HQ is a component of cigarette smoke and indeed elevated plasma levels of HQ have been found in smokers compared to non-smokers (25). In AMD research, both *in vitro* and *in vivo* studies proved that exposure to HQ causes oxidative damage and AMD-like pathology in mice and immortalized RPE cell lines (26, 27).

In this work, we investigated the differential stress response of human iPSC-RPE cells to oxidative stress conferred by Hydroquinone. We analyzed differentiated human RPE, where low-risk (LR) cells are homozygous carriers of the FH 402Y variant, while high-risk cells (HR) carry the FH 402H variant. We demonstrate that RPE cells carrying the FH 402H variant are more vulnerable to HQ-induced oxidative stress in a concentration-dependent manner, affecting autophagy as well as increasing apoptosis independent of extracellular levels of complement.

Methods

Induced pluripotent stem cells and RPE differentiation

Human induced pluripotent stem cells (iPSC) were obtained from different sources (specified in [Supplementary Figure 1A](#)). iPSC

cell lines 1, 2, 4, 5, and 6 were obtained from genetically screened human donors as explained previously (16). iPSC cell lines 3, 7, 8 were generated as previously described (28) and purchased from the Spanish National Stem Cell Bank. iPSCs were grown on hESC-matrigel (354277, Corning) coated plates in E8 medium, medium was changed daily. iPSCs were differentiated to RPE cells according to previously described protocol (29, 30). Briefly, iPSCs were seeded on hESC matrigel (356237, Corning) in differentiation medium (DMEM/F12, 31331, Gibco; N2 supplement, 17502, Gibco; B27 supplement, 17504, Gibco; NEAA, 11140, Gibco; Knock Out SR, 10828, Gibco). Briefly, medium was replaced every two days for 14 days following specific formulations: Nicotinamide 10 mM (Day 0-4, NO636-100G, Sigma-Aldrich), Noggin 50ng/ml (day 0-4, 1967-NG-025/CF, R&D systems), DKK-1 10 ng/ml (day 0-6, 5439-DK-010/CF, R&D Systems), IGF-1 10ng/ml (day 0-6, 1291-G1-200, R&D Systems), bFGF 5 ng/ml (day 2-4, AF-100-18B, Peprotech), activinA 100 ng/ml (day 4-14, 120-14E, Peprotech), SU5402 10 μ M (day 6-14, sc-204308, Santa Cruz).

At day 14, cells were enriched in medium containing rock inhibitor (StemMACS Y27632), and seeded in Geltrex (A1413302, Gibco) or hESC-matrigel coated plates for expansion. Medium was changed twice a week and RPE cells were matured for at least 30 days. RPE cells were passaged using TrypLE protocol according to manufacturer instructions (12604; Thermo Fisher Scientific). Cells were used for experiments from passage 2 to passage 4 in the desired plate format (12 well-culture inserts, 96-well plates, 12-well plates) and were cultured for at least 50 days before experiments. Cell culture treatments were carried for 48hours before analyses and included: Hydroquinone (HQ) 100- 200 μ M (H9003, Sigma-Aldrich), Tunicamycin 5 μ g/ml (SML1287, Sigma-Aldrich), Bafilomycin (50 nM, B1793, Sigma), purified FH 1 μ g/ml (A137, Comptech), Normal human serum 1% (H4522, Sigma-Aldrich), inactivated human serum 1% (H3667, Sigma-Aldrich).

Western blot

Cellular lysates were collected in IP lysis buffer (87787, Thermo Fisher Scientific) with protease and phosphatase inhibitors (1861281, Thermo Fisher Scientific). After 30 minutes incubation at 4°C, followed by centrifugation, Protein concentration was measured according to the Bradford method following manufacturer instructions (500-0006, Biorad). Proteins were resuspended in NuPAGE™ LDS Sample Buffer containing reducing agent (NP0007, Invitrogen, California, USA), separated on 8-16% SDS-PAGE gels (XP08165Box; Thermo Fisher Scientific) and transferred on PVDF membranes (0.45 μ m, 10600023, Cytiva). Membranes were exposed overnight to the primary antibodies (anti-FH, sc-166608, Santa Cruz, Texas, USA; anti-Bip, #3177, Cell Signalling; anti-IRE1a, #3294, Cell Signalling; anti-LC3, 0231-100/LC3-5F10, Nano tools; anti-SQSTM1, BML-PW9860-100, Enzo; anti-Bax, #12105, Cell Signalling; anti-Bcl-2, #2870, Cell Signalling; anti-Sod2, #13141, Cell Signalling; anti-Tubulin, #2148, Cell Signalling) and for 1 hour to HRP-conjugated anti-mouse or anti-rabbit secondary antibody (1:2.000, 7076S, 7074P2, Cell Signaling, Massachusetts, USA). Immunoreactivity was visualized

with PierceTM ECL Western Blotting Substrate (32106, Thermo Fisher Scientific, Massachusetts, USA) and detected with FusionFX instrument (Vilber Lourmat, France).

RNA extraction, cDNA synthesis and qRT-PCR

Cell pellets resolved in Purezol (732-6880, Biorad, Germany), homogenized by inversion, and incubated at room temperature for 5 minutes. After addition of chloroform, samples were vortexed for 15 seconds, incubated for 5 minutes at room temperature, and then centrifuged at 12,000 g for 15 minutes at 4°C. The aqueous phase was collected and mixed with isopropanol for precipitation. Samples were centrifuged at 12,000 g for 15 minutes at 4°C. Pellets were rinsed twice with EtOH 75%, dried, and resuspended in 20 µl of RNase-free water. RNA purity and concentration were measured using Nanodrop. cDNA was synthesized via reverse-transcription of 2–5 µg of RNA using M-MLV Reverse Transcriptase (200 U, M1705, Promega, Wisconsin, USA), random primers (10 ng/µl, C1181, Promega, Wisconsin, USA) and dNTPs (0.5 mM, U1515, Promega) in a total volume of 20 µl. cDNA was used to analyze differences in gene expression by qRT-PCR employing iTaq Universal SYBR Green Supermix (1725122, Biorad, Germany) along with gene-specific forward and reverse primers (250 nM) according to manufacturer instructions. Relative mRNA expression of each gene of interest (GOI) was quantified by using PRPL0 as the housekeeping control gene. Primers are listed below: PINK1 (fwd 5'-GGC TTG GCA AAT GGA AGA AC -3', rev 5'-CTC AGT CCA GCC TCA TCT ACT A -3'), PARKIN (fwd 5'-CCA CAC TAC GCA GAA GAG AAA -3', rev 5'-GAG ACT CAT GCC CTC AGT TAT G -3'), PPARGC1A (fwd 5'-AGA GCG CCG TGT GAT TTA T -3', rev 5'-CTC CAT CAT CCC GCA GAT TTA -3'), CAT (fwd 5'-CTG GAG CAC AGC ATC CAA TA -3', rev 5'-TCA TTC AGC ACG TTC ACA TAG A -3'), GPX1 (fwd 5'-CAT CAG GAG AAC GCC AAG AA -3', rev 5'-GCA CTT CTC GAA GAG CAT GA -3'), CFH (fwd 5'-CTG ATC GCA AGA AAG ACC AGT A -3', rev 5'-TGG TAG CAC TGA ACG GAA TTA G -3'), PRPL0 (fwd 5'-GGA GAA ACT GCT GCC TCA TAT C -3', rev 5'-CAG CAG CTG GCA CCT TAT T -3').

ELISA

Cell culture media from the apical and basal compartments of RPE culture inserts were collected. Cellular debris were removed by centrifugation. PEDF ELISA (RD191114200R, BioVendor) was performed according to manufacturer instructions. Freshly prepared standards, quality controls, and samples were incubated in ELISA plates at room temperature on shaker for 1 hour. After washes, Biotin-labelled antibody was added to each well and incubated at room temperature on shaker for 1 hour. Then, following washes, the Streptavidin-HRP Conjugate was added into each well and incubated as before. Signal reaction was initiated by adding the detection substrate and stopped after 5 minutes with Stop Solution. Absorbance was measured at 405 nm at the Spark multimode microplate reader (Tecan, Switzerland).

TER

TER measurements are performed with a Millicell-ERS-2 meter according to the manufacturer's instructions. The electrodes were applied on the apical and basal sides of iPSC-RPE, the current was passed, and resistance values (Ohms) were noted down for each well. The average of three measures was recorded for each well. Actual resistance (Ohms*cm²) is calculated by multiplying TER to the area of the insert.

Immunostaining

iPSC-RPE monolayers grown on transwells were fixed in 4% PFA at room temperature. Small sections were selected and used for immunostaining. Briefly, samples were washed in PBS for 10 minutes and incubated 5 minutes with 0.3% Triton. After two rinses in PBS, samples were shortly incubated with TrueBlock solution (23007, Biotium). After three rinses in PBS, samples were blocked in NGS (normal goat serum, S26, EMD Millipore Corp., USA) for 1 hour and then exposed overnight at 4°C to the primary antibody (ZO-1, 610966, BD transduction). Following three washes in PBS, samples were incubated with secondary antibody (Goat anti-mouse Alexa 568, A11031, molecular probes). Samples were mounted on coverslips with Fluoromount-G solution (17984-25, Electron Microscopy Sciences, Pennsylvania, USA). Images were obtained using Z-stacks on a Zeiss Axio Imager Z1 ApoTome Microscope.

In vitro assays

Cytotoxicity and caspase activity were assessed using the MultiTox-Fluor Assay (G9201, Promega) and the Caspase-Glo Assay (G8091, Promega). Cytotoxicity, defined by cell membrane damage, was assessed by cell-impermeable bis-AAF-R110 (bis-alanylalanyl-phenylalanyl-rhodamine 110) dye, which is cleaved by dead-cell proteases released in the cell culture supernatants after membranes damages. Fluorescence is read at 485Ex/520Em. After detection, caspase 3/7 reagent was added in the wells and incubated for 10 minutes at room temperature in the dark. Equal volumes were transferred to OptiPlate-96 (6005290, Perkin Elmer) and luminescence signals were measured. Spark multimode microplate reader (Tecan, Switzerland) was used for fluorescence and luminescence measurements.

Levels of H₂O₂ were measured using the ROS-Glo H₂O₂ assay (G8820, Promega) and assay was performed according to manufacturer instructions. Briefly, cells were exposed to the H₂O₂ substrate and incubated in the incubator for 4 hours. At this point, H₂O₂ detection buffer (containing cysteine and signal enhancer) was added, incubated 10 minutes at room temperature in the dark under shaking. Equal volumes were transferred to OptiPlate-96 and luminescence was measured.

CellTiter-Glo assay (G9241, Promega) was used to measure total ATP levels present in the cultures. CellTiter-Glo reagent was added to the cells, and incubated at room temperature for 10

minutes. Equal volumes were transferred to OptiPlate-96 and luminescence was measured.

JC1 dye (T3168, ThermoFisher Scientific) was used to monitor mitochondrial health, as exhibits differential accumulation depending on mitochondria membrane potential. At lower potential JC-1 is present as monomer and it is indicated by green fluorescence, while at higher membrane potential JC-1 aggregates in the mitochondria and emits red fluorescence. Therefore, a JC-1 red/green ratio is indicative of mitochondria polarization. The cells were exposed to the JC-1 dye for 15 minutes in the incubator and rinsed three times in PBS and fluorescence signals were measured in the RPE layer at the Spark multimode microplate reader (Tecan, Switzerland).

Transmission electron microscopy

iPSC-PRE samples were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.1 M sodium cacodylate buffer (pH 7.4, Electron Microscopy Sciences, Munich, Germany) overnight at 4°C. After rinsing in 0.1 M sodium cacodylate buffer, samples were post-fixed in 1% OsO₄ for 1.5 hours at room temperature, washed in cacodylate buffer, and dehydrated with 50% ethanol. Tissues were counterstained with 6% uranyl acetate dissolved in 70% ethanol (Serva, Heidelberg, Germany), followed by graded ethanol concentrations up to 100% and Propyleneoxide. The dehydrated samples were incubated in 2:1, 1:1, and 1:2 mixtures of propylene oxide and Epon resin (Serva) for 1 hour each. Finally, samples were infiltrated with pure Epon for 2 hours. Samples were embedded in fresh resin in block molds and cured for 3 days at 60°C. Semithin sections (500 nm) were cut on a Reichert Ultracut S (Leica Microsystems, Wetzlar, Germany) and stained with Richardson staining solution. Ultrathin sections (50 nm) were cut on the same Ultracut, collected on copper grids, and counterstained with Reynold's lead citrate. Sections were analyzed with a Zeiss EM 900 transmission electron microscope (Zeiss) equipped with a 2k x 2k CCD camera.

Statistical analyses

All data sets were tested for normal distribution (D'Agostino and Pearson test) and were subjected to outliers' identification via ROUT method (Q=1%). *In vitro* assay data obtained from each HQ-treated group were normalized to the respective control untreated group for each cell line (represented as a dotted line in the graphs and set as 1) to avoid artefacts deriving from different cell numbers. The relative effects of HQ in each group were derived with paired Student T-test (#) compared to controls (dotted line). Differences between LR and HR groups were derived with unpaired Student T-test (*). Data showing JC-1 ratio, which is not dependent on number of cells, were analyzed with one-way ANOVA with Tukey's multiple comparisons test. Relative mRNA expression was calculated with the $\Delta\Delta C_t$ method, considering the average of all ΔC_t of all LR RPE cells as controls. Gene expression changes were analyzed with one-way ANOVA with Tukey's multiple

comparisons test. Western Blot images were analyzed for signal quantification using Fiji (ImageJ), and relative protein expression levels were analyzed using one-way ANOVA with Tukey's multiple comparisons test. Analyses were performed using GraphPad Prism 10 software. The different dots in the bar graphs represents multiple biological replicates derived from multiple assessments of iPSC-RPE cells derived from multiple cell lines specifically indicated in the respective figure legends. Data are shown as mean $\pm$ SEM. Individual data points represent biological replicates. Significance level was set as $p \leq 0.05$.

Results

iPSC-RPE carrying high genetic risk (HR) are more vulnerable to Hydroquinone mediated-damage

To assess the impact of genetic risk variants on RPE cells, we used iPSC-RPE lines and used the FH Y402H polymorphism as main criteria for classification into low risk (LR) or high risk (HR), where LR (n=3) are homozygous carriers of the FH 402Y and HR are homozygous carriers of FH 402H (n=5). Both groups present a mixed genotype at Chr10 for ARMS2 and donors were selected independently of their AMD status (detailed in [Supplementary Figure 1A](#)).

The RPE cells were evaluated for maturity based on established criteria. Both LR and HR RPE cells exhibited mature characteristics, including pigmentation ([Figures 1A–D](#)), hexagonal shape confirmed by immunostaining of tight-junction protein ZO-1 ([Figures 1E–H](#)), and monolayer formation with normal apical microvilli ([Figures 1I, J](#)). Strong tight junctions and proper polarization were confirmed by proper trans-epithelial resistance ([Figure 1L](#)), consistent with previous studies. In addition, the cell lines also showed a polarized secretion of PEDF, a characteristic of mature RPE cells, with higher levels in the apical extracellular compartment ([Figure 1K](#)). RPE markers were also validated by gene expression analyses ([Supplementary Figure 1B](#)). Given the hypothesized role of FH in RPE pathology during AMD progression, we confirmed that RPE cells do express *CFH* mRNA ([Supplementary Figure 1C](#)), exhibit FH in protein lysates from both LR and HR iPSC-RPE cells ([Figure 1M](#)) and secretion of C3 ([Supplementary Figure 1D](#)).

Our objective was to investigate how genetic predisposition influences AMD pathology at the RPE level. We hypothesized that RPE cells with high genetic risk (HR) may be more susceptible to damage from adverse conditions such as aging or unhealthy lifestyle factors. One of the key features of these AMD risk factors is increased oxidative stress. As such, we combined genetic risk with HQ, an oxidative agent found in cigarette smoke: smoking is itself a large contributor of AMD-risk ([31](#)). We treated LR and HR iPSC-RPE cells with increasing concentrations of HQ (100 and 200 μ M) for 48 hours and assessed the RPE stress response ([Figure 2A](#)). Initially, we compared membrane damage levels in a cytotoxicity assay by comparing HQ-treated LR and HQ-treated HR iPSC-RPE cells and their respective non HQ-treated controls (dotted line set as 1). Only HR iPSC-RPE cells exhibited cytotoxicity at 200 μ M HQ ([Figure 2B](#)).

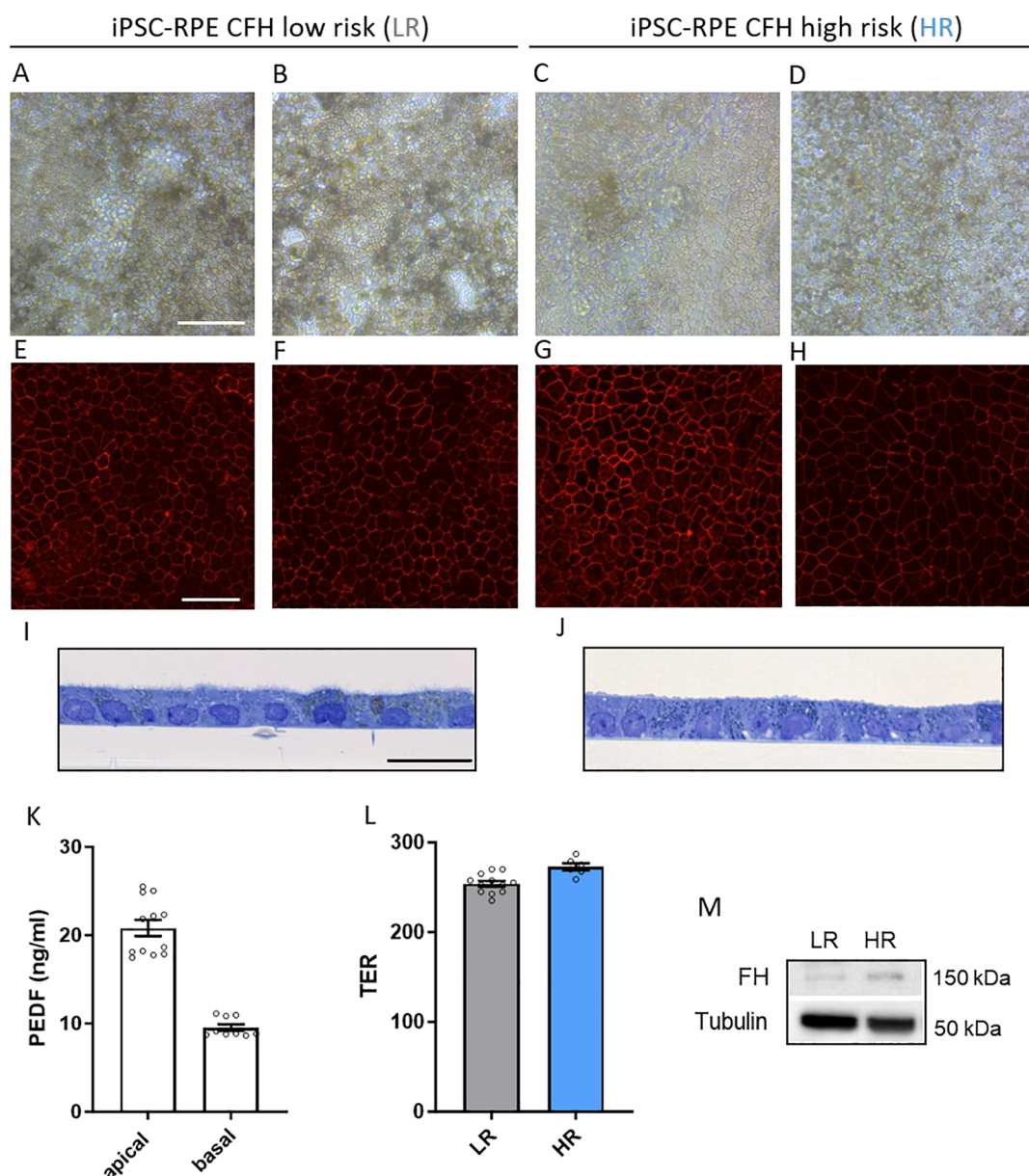


FIGURE 1

Generation of CFH low risk (LR) and high risk (HR) iPSC-RPE. (A–D) Representative light microscopy images of iPSC-RPE cells derived from LR donors iPSC-RPE2 (A), iPSC-PRE3 (B) and HR donors iPSC-RPE5 (C), iPSC-PRE8 (D). Images show proper RPE cuboidal shape and visible pigmentation. Scale bar = 100 μ M. (E–H) Representative immunostaining images of RPE marker ZO-1 in LR donors iPSC-RPE1 (E), iPSC-PRE3 (F), and HR donors iPSC-RPE6 (G), iPSC-PRE7 (H). Scale bar = 50 μ M. (I, J) Transmission electron micrographs semithin sections of LR donors iPSC-RPE3 (I) and HR donors iPSC-RPE8 (J). Images show an RPE monolayer and microvilli typical of RPE cells. Scale bar = 20 μ M. (K) Pigment epithelium-derived factor (PEDF) ELISA of apical and basal supernatant. Data collected from iPSC-RPE3, 5, 6, 7, 8. (L) Trans-epithelial resistance (TER) measured in iPSC-RPE3, 5, 8. (M) Representative WB images of FH levels in iPSC-RPE1 and iPSC-RPE5 cellular lysates. $n=3$. Tubulin was used as housekeeping control.

As apoptosis is a primary cell death response to oxidative stress, we examined apoptosis markers in both LR and HR iPSC-RPE cells. At the lower HQ concentration (100 μ M), both LR and HR cells showed significant caspase 3/7 activation, with a more pronounced response in HR iPSC-RPE cells. At the higher concentration (200 μ M), only HR iPSC-RPE cells exhibited significantly elevated caspase levels compared to untreated HR controls (Figure 2C). Additionally, we

investigated the protein expression levels of Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic). We calculated the Bax/Bcl-2 ratio, which was elevated in HR iPSC-RPE cells towards Bax expression even under untreated conditions, indicating a predisposition to higher apoptotic levels. This ratio remained unchanged in HQ-treated LR iPSC-RPE cells but increased significantly in HR iPSC-RPE cells after 200 μ M HQ exposure (Figures 2D, E).

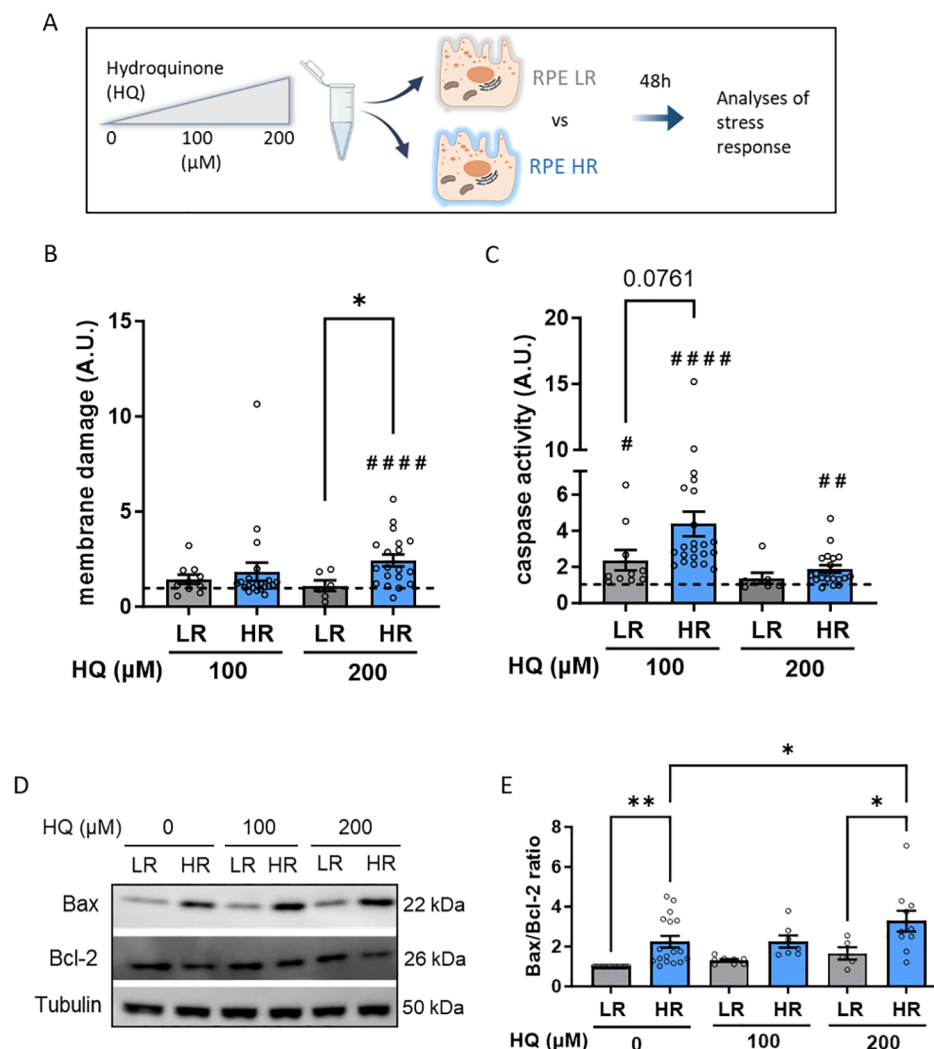


FIGURE 2

Hydroquinone (HQ) induces cellular damage and apoptosis in CFH high risk (HR) iPSC-RPE. **(A)** Schematic representation of the experimental set up. **(B, C)** Membrane damage assessed by cytotoxicity assay GF-AFC **(B)** and Caspase-3 activity **(C)** in LR and HR iPSC-RPE. Data points were collected from LR iPSC-RPE1, 2, 3 ($n=3$ biological replicates) and from HR iPSC-RPE4, 5, 6, 7, 8 ($n=5$ biological replicates). HQ-treated relative values are normalized to the respective untreated controls (dotted line) in each individual experiment for each cell line. HQ effects in each group were assessed with paired Student's *t*-test (#) compared to controls (dotted line). Differences between LR and HR groups were determined with unpaired Student's *t*-test (*). **(D, E)** Representative WB images **(D)** of Bax and Bcl-2 levels LR and HR iPSC-RPE cells treated with HQ. Data points were collected from LR iPSC-RPE1, 2, 3 ($n=3$ biological replicates) and from HR iPSC-RPE4, 5, 6, 7, 8 ($n=5$ biological replicates). Tubulin was used as housekeeping control. Quantification of Bax/Bcl-2 ratio is shown in **(E)** Differences between LR and HR groups were assessed with one-way ANOVA (*). Data are shown as mean $\pm$ SEM. ** $p < 0.01$. ## $p < 0.01$ #### $p < 0.0001$.

iPSC-RPE carrying high genetic risk (HR) show mitochondrial impairment

The ability to maintain metabolic balance even when exposed to oxidative conditions and an adverse environment is crucial for RPE homeostasis. This is the cellular environment during AMD progression. Healthy RPE cells are supposed to cope with stress and minimally vary their metabolic functions. To assess whether the susceptibility to HQ in HR iPSC-RPE cells was reflected at a metabolic level, we used *in vitro* assays to measure ATP levels and mitochondrial polarization. Indeed, we observed that, although both LR and HR cells exhibited reduced ATP levels after HQ exposure, the reduction was more pronounced in HR cells

(Figure 3A). To investigate mitochondrial integrity, we used a JC1 assay, which allows the assessment of mitochondrial membrane potential, and we demonstrated that only the HR cells, when treated with higher concentrations of HQ, showed a decreasing trend in mitochondrial membrane depolarization (Figure 3B). Interestingly, we observed mitochondrial impairment in HR cells even under control conditions, which may predispose these cells to greater damage in response to oxidative insults such as HQ exposure. Using electron microscopy (EM), we observed that mitochondria in LR and HR RPE were localized differentially. Classically, mitochondria are localized in the basal compartments of RPE cells, as was indeed observed in LR RPE cells, whereas HR iPSC-RPE showed a partial localization of mitochondria in the apical compartment

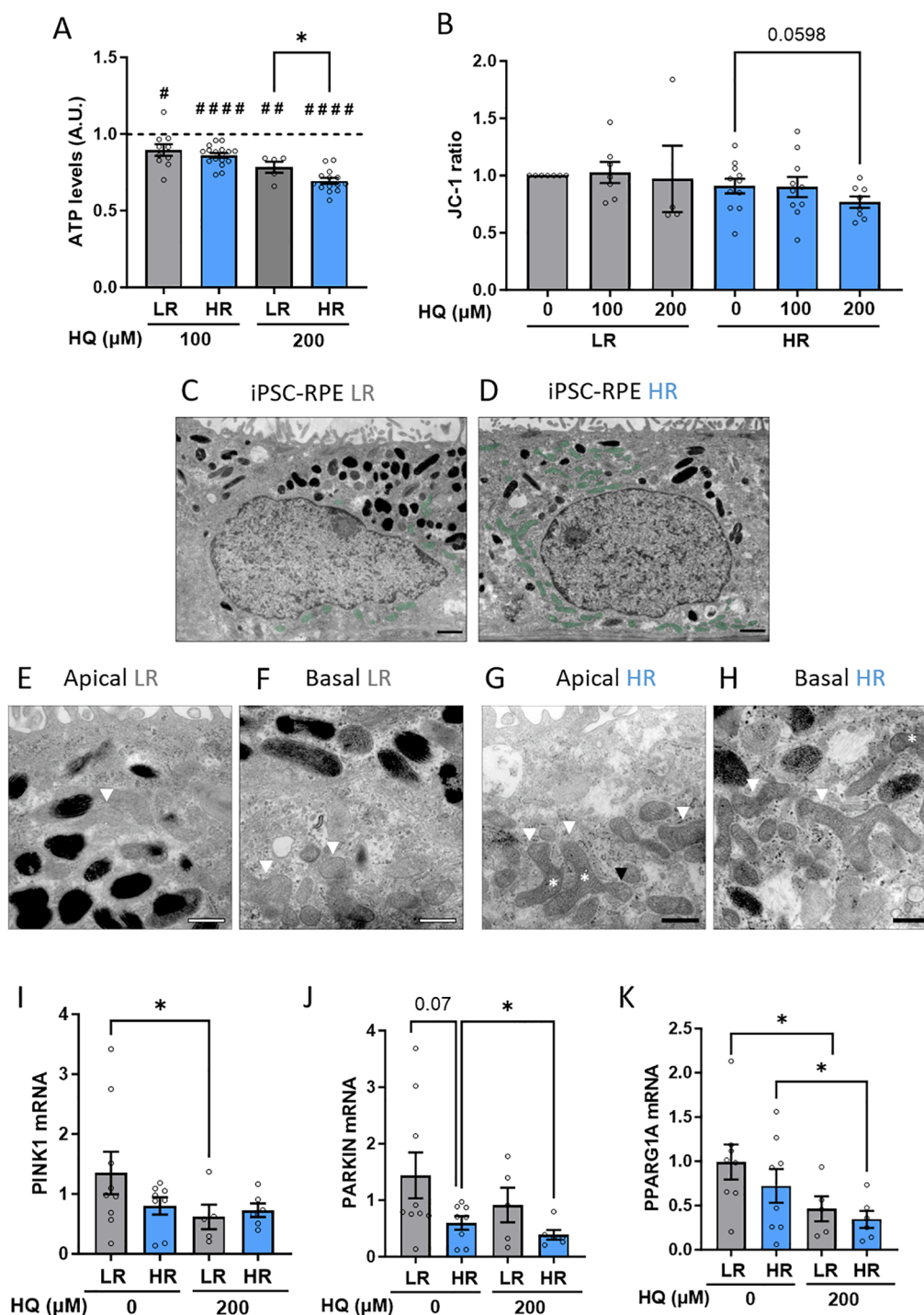


FIGURE 3

CFH high risk (HR) iPSC-RPE cells show impaired mitochondria homeostasis. **(A)** ATP levels assessed by cell titer assay in LR and HR iPSC-RPE. HQ-treated relative values are normalized to the respective untreated controls (dotted line) in each individual experiment for each cell line. HQ effects in each group were determined with paired Student's t-test (#) compared to controls (dotted line). Differences between LR and HR groups were determined with unpaired Student T-test (*). **(B)** JC-1 fluorescence measurements were recorded, and ratio was determined. HQ effects in each group were assessed with paired Student's t-test (#) compared to untreated controls. **(C–H)** Representative EM images of LR iPSC-RPE3 and HR iPSC-RPE8 showing mitochondria abnormalities. **(C, D)** show the mitochondria localization (highlighted in green) in the apical compartment in HR **(D)** compared to basal localization **(C)**. scale bar = 1 μm. **(E–H)** show mitochondria morphology. White arrowheads in **(E, F)** mark healthy mitochondria in LR iPSC-RPE cells. In **(G, H)** white arrowheads mark polymorphic mitochondria, black arrowheads mark swollen mitochondria, asterisks mark bent mitochondria. Scale bar = 500 nm. **(I, J)** Gene expression levels of PINK1 **(I)**, PARKIN **(J)** and PPARG1A **(K)** analyzed via RT-qPCR in LR and HR iPSC-RPE treated with HQ. Significance was determined with one-way ANOVA (*). Data are shown as mean ± SEM. Data points were collected from LR iPSC-RPE1, 2, 3 (n=3 biological replicates) and from HR iPSC-RPE4, 5, 6, 7, 8 (n=5 biological replicates). ## p < 0.01 #### p < 0.0001.

(Figures 3C, D, mitochondria highlighted in green). Furthermore, the mitochondria are altered in HR RPE cells, as previously shown (15). Mitochondria in LR RPE cells appear to be organized, with a double membrane and clear cristae, irrespective of basal or apical localization (Figures 3E, F, mitochondria marked with white arrowheads). On the other hand, morphology of mitochondria in HR RPE cells appears polymorphic, varying in shape and size (Figures 3G, H, polymorphic mitochondria marked with white arrowheads). Mitochondria in HR RPE cells have vacuoles, indicating swollen mitochondria; the inner cristae structure is damaged (Figures 3G, H, swollen mitochondria marked with black arrowheads). Another feature in HR RPE cells was the presence of bent mitochondria (Figures 3G, H, bent mitochondria marked with asterisk), which has been previously observed in photoreceptor mitochondria of older mice and is associated with mitochondrial motility (32). Moreover, in both groups it was possible to detect pinched mitochondria, which are an indication of fission and fusion, and we found those features were present in higher number in the HR RPE cells, indicating altered mitochondrial dynamics. Therefore, we comparably investigated a possible involvement of mitophagy in the differential response to stress. Although no major differences in the level of the main mitophagy markers PINK and PARKIN (33) were observed in the untreated condition, LR and HR RPE cells differentially regulated the expression of those genes in response to HQ. In LR cells we found PINK1 downregulated while it remained unchanged in HR cells. HR downregulated PARKIN in response to HQ, while LR did not. In parallel, the levels of PGC1 α , a transcription factor regulating mitochondrial biogenesis (34) was reduced in both LR and HR after HQ treatment (Figures 3I–K).

HQ-mediated damage in iPSC-RPE carrying high genetic risk (HR) is not dependent on extracellular FH or complement sources

Recent research has proven the existence of non-canonical functions of FH, which are distinct from its well-established role as a complement system inhibitor in the extracellular space. These non-canonical functions suggest that FH has a role in influencing cellular homeostasis within the intracellular environment. Our study investigated whether the increased susceptibility of HR iPSC-RPE cells to Hydroquinone (HQ)-induced damage could be linked to these non-canonical functions of FH.

We first exposed HR iPSC-RPE cells to HQ in the presence of plasma-purified soluble FH to determine if FH could attenuate the HR phenotype (Figure 4A). Notably, HR iPSC-RPE cells exposed to purified FH exhibited FH in their cellular lysates, indicating either cellular uptake or binding to the cellular membrane (Figure 4B). Purified FH appeared to have a higher molecular weight, likely due to differential glycosylation, as observed and hypothesized in other models (35, 36). However, no rescue effect on cytotoxicity or caspase activation was observed in HR iPSC-RPE cells exposed to HQ and purified FH (Figures 4C, D).

We then treated HR iPSC-RPE cells with HQ in combination with conditioned medium collected from either LR or HR iPSC-RPE cells (Figure 4E). The use of conditioned medium provided mixed results, with reduced cytotoxicity in HR iPSC-RPE cells exposed to both LR and HR conditioned media (Figure 4F), yet caspase activation remained elevated in HR iPSC-RPE treated with HQ (Figure 4G). This suggests that neither secreted FH variant is able to rescue our observed HQ-induced elevation of caspase activity; rather, the susceptibility appears to be intrinsically linked to the endogenous expression of the HR variant in iPSC-RPE cells.

Given FH's canonical role as a complement inhibitor, we assessed whether complement activation contributes to the observed damage in HR iPSC-RPE cells. We performed HQ treatment experiments in the presence of normal human serum (NHS), in which complement activation is possible, and inactivated NHS (iNHS), in which complement activation is limited (Figure 4H). Our findings indicate that increased cytotoxicity and caspase activation in HQ-exposed HR iPSC-RPE cells are independent of complement system activation, as no differences were observed between treatments with NHS or iNHS (Figures 4I, J).

Autophagy flux is impaired in iPSC-RPE carrying high genetic risk (HR)

To further elucidate the mechanisms underlying the increased vulnerability of HR iPSC-RPE cells to hydroquinone (HQ)-induced stress, we investigated the oxidative stress response. Given that HQ is an oxidative agent, we first assessed oxidative stress by measuring hydrogen peroxide (H₂O₂) levels. As expected, H₂O₂ levels increased in both LR and HR iPSC-RPE cells in a concentration-dependent manner; however, no significant differences were found between LR and HR cells (Supplementary Figure 2A). We also analyzed the expression of key antioxidant components, including catalase (CAT, Supplementary Figure 2B), glutathione peroxidase 1 (GPX1, Supplementary Figure 2C), and superoxide dismutase 2 (SOD2, Supplementary Figures 2D, E). CAT and GPX1 gene expression levels were similar between LR and HR iPSC-RPE cells. However, in both the untreated and HQ-treated conditions, a modest but significant reduction in SOD2 protein levels was observed in HR iPSC-RPE cells compared to LR cells.

Under conditions of oxidative stress, the endoplasmic reticulum (ER), which is crucial for protein synthesis, folding, and regulation, may become dysfunctional under oxidative stress conditions. To explore this possibility, we assessed the ER stress response and the unfolded protein response (UPR) by examining the expression of the chaperone Binding immunoglobulin protein (BiP), an initial sensor of unfolded proteins, and inositol-requiring enzyme 1 α (IRE1 α), a major UPR effector (Supplementary Figures 3A–C). Although there was a significant increase in BiP in LR cells after treatment with HQ, no significant differences were noted between HR and LR iPSC-RPE cells. To ensure that HR iPSC-RPE cells do not have an inherently impaired ER stress response, we treated both cell groups with Tunicamycin (TM), a known ER stress inducer. HR

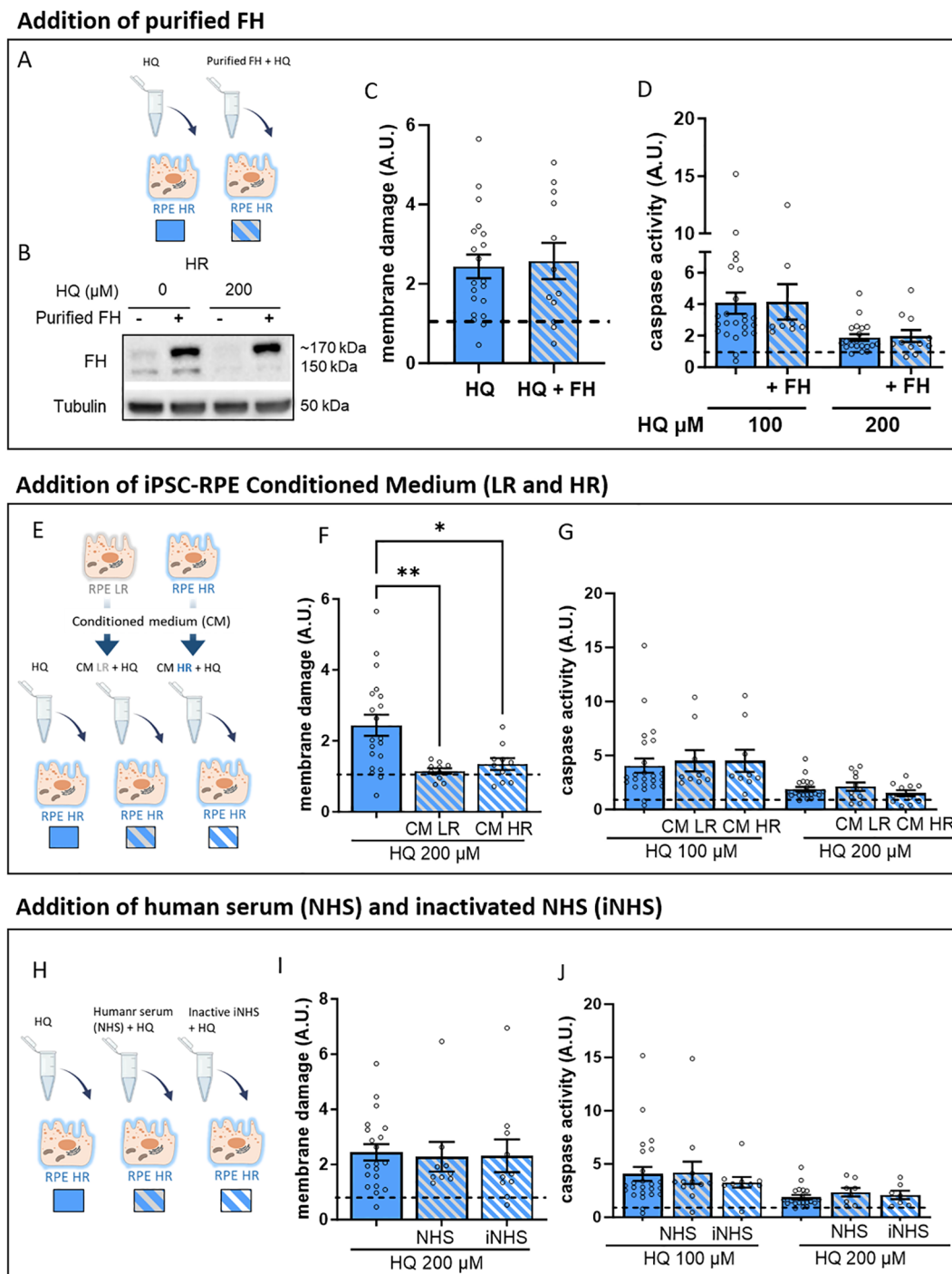


FIGURE 4

Addition of extracellular FH and complement sources does not affect the vulnerability to HQ-mediated stress in HR iPSC-RPE. **(A)** Schematic representation of the experimental set up of FH supplementation. **(B, C)** Membrane damage assessed by cytotoxicity assay GF-AFC **(B)** and Caspase-3 activity **(C)** in HQ-treated HR iPSC-RPE with supplementation of purified FH. Values are normalized to the respective controls (dotted line) in each individual experiment for each cell line. Data are shown as mean $\pm$ SEM. **(D)** Schematic representation of the LR and HR iPSC-RPE conditioned medium supplementation experimental set up. **(E, F)** Membrane damage assessed by cytotoxicity assay GF-AFC **(E)** and caspase-3 activity **(F)** in HQ-treated HR iPSC-RPE with supplementation of LR and HR conditioned medium. Values are normalized to the respective controls (dotted line) in each individual experiment for each cell line. Significance was derived with 2-way ANOVA. **(H)** Schematic representation of the experimental set up of HQ-treated HR iPSC-RPE supplemented with NHS and iNHS. **(I, J)** Membrane damage assessed by cytotoxicity assay GF-AFC **(I)** and caspase-3 activity **(J)** in HQ-treated HR iPSC-RPE with supplementation of LR and HR conditioned medium. Values are normalized to the respective controls (dotted line) in each individual experiment for each cell line. Data points were collected from HR iPSC-RPE5, 6, 7, 8 ($n=4$ biological replicates). Data are shown as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$.

iPSC-RPE cells showed a small, but significant elevated cytotoxicity and both LR and HR iPSC-RPE cells showed caspase activation in response to TM (Supplementary Figures 3D, E). The Bax/Bcl-2 ratio was, however, higher in TM-treated HR iPSC-RPE cells compared to TM-treated LR cells, reflecting the differences observed in basal conditions (Supplementary Figures 3F, G). Both cell groups demonstrated a similar UPR activation in response to TM, with significant upregulation of BiP and IRE1 α (Supplementary Figures 3H–J).

Lastly, we examined the impact of oxidative/proteotoxic stress on the autophagic flux. Both LR and HR iPSC-RPE cells exhibited accumulation of LC3-II and p62 in response to HQ treatment, indicating increased autophagy (Figures 5A–C). However, detailed analysis using Bafilomycin A1 (BafA1), a commonly used autophagy inhibitor, revealed that LR iPSC-RPE cells had a higher capacity for autophagy compared to HR iPSC-RPE cells, as evidenced by a significant reduction in LC3-II levels in BafA1-treated HR iPSC-RPE cells (Figures 5D, E).

Discussion

AMD leads to a devastating loss of visual acuity and central vision, as a result of a complex combination of risk factors,

including genetic predisposition, age and unhealthy life-style habits (37, 38). Smoking has been identified as a major risk that synergizes with genetic risks accelerating the onset and progression of the disease (37). However, it remains enigmatic so far as to how, where and when the combination of genetic and life-style-associated risk contributes to the disease. About 50% of AMD patients present polymorphisms in the complement factor H (*CFH*) gene, an inhibitory component of the complement pathway. Risk variants in the *CFH* gene lead to reduced inhibitory function of the Factor H protein (FH) (7). Until recently, FH risk variants were thought to confer susceptibility to AMD solely through over-activation of the complement system, and clinical trials were designed to inhibit this process.

Two recent studies we could show that local loss of *CFH* in RPE results in pathophysiological consequences not linked to the complement pathway. Loss of *CFH* in RPE cells results in an increase of inflammatory cytokines and chemokines not directly linked to the complement pathway (39). Searching for the causative molecular mechanisms, we identified the NF- κ B pathway as the major pathway involved in those changes. We could further show that *CFH* loss in RPE cells has severe consequences on energy metabolism, resulting in a reduction of both glycolysis and mitochondria respiration (23). Using hTERT-RPE1 cells we could show, that loss of FH expression in these cells results in a higher

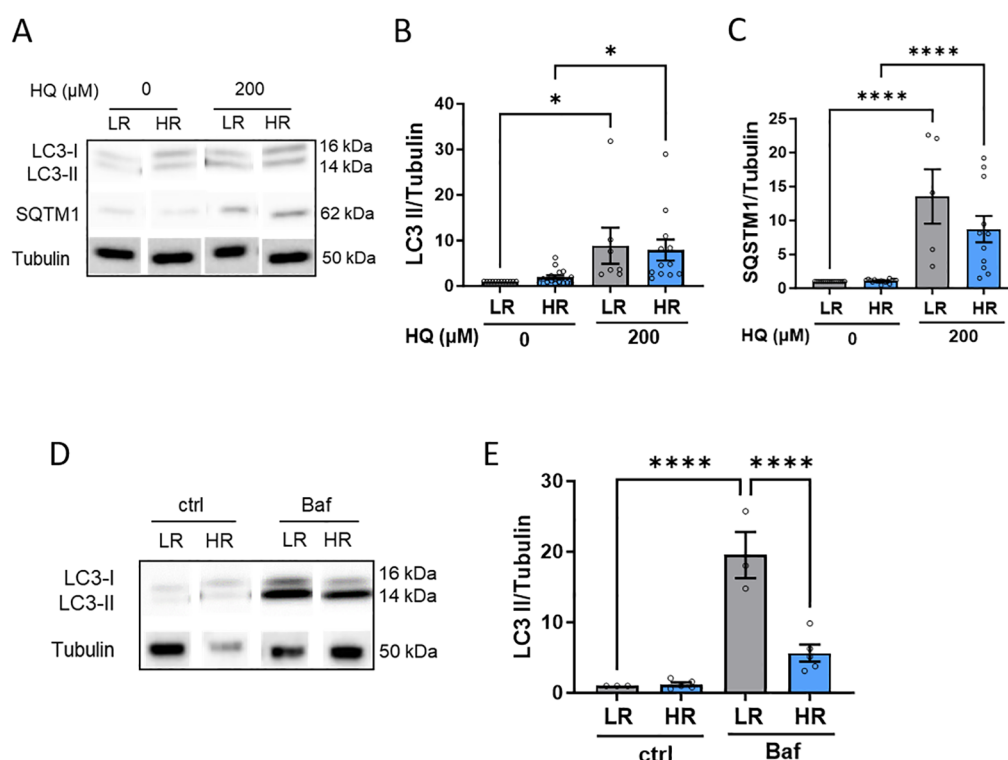


FIGURE 5

HR iPSC-RPE shows impaired autophagic flux. (A–C) Representative WB images (A) of LC3 and SQSTM1 in LR and HR iPSC-RPE cells treated with HQ. Tubulin was used as housekeeping. Quantification is shown for LC3 (B) and SQSTM1 (C). Data points were collected from LR iPSC-RPE1, 2, 3 (n=3 biological replicates) and from HR iPSC-RPE4, 5, 6, 7, 8 (n=5 biological replicates). (D, E) Representative WB images (D) of LC3 LR and HR iPSC-RPE cells treated with Bafilomycin. Tubulin was used as housekeeping. Quantification are shown for LC3 (E). Data points were collected from LR iPSC-RPE1, 3 (n=2 biological replicates) and from HR iPSC-RPE6, 8 (n=2 biological replicates). Differences between groups were determined with one-way ANOVA (*). Data are shown as mean $\pm$ SEM. **** $p < 0.0001$.

vulnerability to H_2O_2 and an impairment in mitochondrial metabolism (23). These studies, however, were performed by *CFH* knockout within RPE cells. A variety of studies with ARPE19 cells, found a higher cell damage in response to a multiplicity of oxidative stimuli (H_2O_2 , tBH, 4-HNE, HQ, CSE) (26, 40–42).

To get closer to human pathology, we employed RPE cells derived from induced pluripotent stem cells (iPSC) generated from donors, carrying either homozygous 402Y (low risk) or 402H (high risk) variant of the *CFH* gene. RPE cells derived thereof were treated with Hydroquinone (HQ), a component of cigarette smoke, to induce oxidative damage. RPE cells carrying the high genetic risk in *CFH* proved more vulnerable to oxidative insult when exposed to HQ, as demonstrated by increased cytotoxicity and caspase activation, compared to the low risk RPE cells. Considering the known connection of oxidative stress to proteotoxic stress and degrading processes, we investigated the unfolded protein response (UPR) and autophagy. When exposed to HQ, RPE cells showed an increase in autophagy markers, however HR RPE cells showed an overall reduced autophagic flux. The degree of cellular susceptibility to oxidative stress turned out to be solely conferred by cell-endogenously produced intracellular FH, as soluble FH protein and other complement sources did not influence the response to HQ. Moreover, having both, high-risk and low-risk genotypes for Chr10q26 (*ARMS2/Htra1*) included, we can show, that the degree of cellular susceptibility to oxidative stress solely associates to the risk haplotype on chromosome Chr1q32, but not to chromosome 10q26.

The risk loci on Chr1q32 includes the protective I62V polymorphism and a risk Y402H polymorphism in FH, and a FHR deletion region, all of which contribute to the genetic haplotype with the highest association with AMD (43). However, most of the research to date has focused on the FH Y402H polymorphism, and properly genotyped iPSC-RPE with fully defined haplotypes would be needed to fully elucidate the contribution of each of the individual risk polymorphisms on Chr1q32 to RPE homeostasis. It has been shown that FH Y402H leads to excessive activation of the alternative pathway of the complement system and products of complement activation may accumulate at a systemic level or at the Bruch's-choroid interface, or locally around the RPE cells themselves (44). However, knowledge gained in recent years has revealed novel non-canonical functions of the complement system (13, 45). These non-canonical cellular functions include T-cell activation and metabolism (46) and regulation of differential transcriptional activity in cancer cells (47). Autophagy and lysosomal capacity have been found to be controlled intracellularly by C3 in pancreatic β -cells (48). These are only some of the recent discoveries that point out a prominent role of the complosome (active intracellular complement components) in tissue homeostasis and disease pathology (45). Some of those functions have been found to depend on intracellular complement system activation, while others simply depend on the intracellular activity of complement proteins, exerting non-canonical functions.

In our study, HR RPE cells showed increased cytotoxicity in response to HQ in a dose-dependent manner. In response to HQ, we observed an increase in H_2O_2 in both groups of cells (LR and HR), confirming that the degree of cell damage is not determined by the levels of ROS per se, but rather by the differences in the

oxidative stress response. HQ-induced cell damage by mediating apoptosis was confirmed by caspase-3 activation and an increase in the Bax-Bcl2 ratio in HR RPE cells. The addition of exogenous FH or conditioned medium containing FH did not rescue either cytotoxicity or caspase activation in a risk variant-dependent manner. These results are consistent with previous observations in colorectal cancer line - ccCRC cells, endothelial cells, and immortalized fibroblasts (47, 49). In these cell types, reduced levels of intracellular FH were associated with reduced viability whereas the addition of FH had no effect (47). On the contrary, exogenous FH was able to reverse the H_2O_2 mediated damage in ARPE19 cells and the 4-HNE damage in iPSC-RPE cells (40, 50) most likely by reducing the pro-inflammatory status in response to the oxidative stimuli. However, in our study, no differences in inflammation were observed when comparing iPSC-RPE LR and HR, either under physiological conditions or when stressed with HQ (data not shown). This was confirmed in a parallel study where IL-6 levels were similar when comparing iPSC-RPE cells derived from donors with different disease states and genotypes (14).

iPSC-RPE cells and primary RPE cells carrying the Y402H polymorphism were shown to accumulate swollen lysosomes, show reduced lysosomal activity, damaged mitochondria and impaired autophagy: all changes that affect the metabolic balance between the RPE and the neuroretina (14–16, 51). However, whether these features are regulated by complement inside the RPE cells or mediated by complement dysregulation outside the cells remained to be clarified in these studies. Consistent with these findings, we also observed mitochondrial abnormalities and reduced autophagic flux in HR iPSC-RPE cells. In particular, we observed reduced autophagic flux and imbalanced mitochondrial dynamics in HR RPE cells, which may be the manifestation of reduced autophagic and lysosomal function and the cause of mitochondrial damage, as previously suggested (51). Taken together, these studies point to a severe physiological imbalance of RPE cells carrying a risk haplotype on Chr1q32 in response to exogenous lifestyle-related stressors.

Our data suggest that endogenously expressed FH protects the RPE. It remains to be shown whether and how this protection of the RPE contributes to protect the neuroretina. Recent work has shown that a dysfunctional RPE triggers photoreceptor degeneration and, in particular, rod cell loss in the parafovea of AMD patients (52). In this study using a complement inhibitor, Pegcetacoplan directed against C3 conversion, drug-treated eyes showed a significantly slower GA lesion progression rate compared with sham-treated controls. To date, inhibition of the complement system has been the main therapeutic target for dry AMD. However, its efficacy may be highly dependent on the patient's genotype and stage of the disease. Individuals with AMD may be responsive to complement inhibition in the early stages of the disease, when the influence of life-style factors and aging is less. Consequently the susceptibility to stress due to the intracellular FH Y402H variant has a limited effect. This is supported by the fact that both LR and HR iPSC-RPE cells were damaged by the extracellular complement system in the absence of additional stress (16). As the disease progresses or in the presence of combined higher risk, the effect of intracellular FH on RPE physiology, which is most likely independent of complement activation, may no longer respond to complement inhibitory

therapies. This hypothesis reinforces the need to consider different avenues for AMD treatment, such as patient stratification or combination therapy, combining complement inhibition with approaches that protect RPE physiology.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethik-Kommission an der Medizinischen Fakultät der Universität und am Universitätsklinikum Tübingen. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from Banco Nacional de Lineas Celulares, Spain or previously generated and reported (NEI). Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

AA: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. IS: Investigation, Methodology, Validation, Writing – review & editing. A-CA-G: Investigation, Methodology, Writing – review & editing. MS: Investigation, Methodology, Writing – review & editing. SB: Investigation, Methodology, Writing – review & editing. BA-G: Data curation, Funding acquisition, Investigation, Methodology, Supervision, Visualization, Writing – review & editing. EK: Data curation, Project administration, Visualization, Writing – original draft. RS: Investigation, Methodology, Writing – review & editing. KB: Funding acquisition, Resources, Writing – review & editing. RF-G: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing. BC: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing. SC: Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Visualization, Writing – review & editing. MU: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1527018/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Generation of iPSC-RPE cells (A) Summary table with the information relative to genetics and origin of the iPSC lines. Spanish National Stem Cell Bank (BNLC). (B) Gene expression levels of RPE marker BEST1 analyzed via RT-qPCR in LR and HR iPSC-RPE. Data are normalized to Best1 expression in hTERT-RPE1 cells. (C) Gene expression levels of CFH analyzed via RT-qPCR in LR and HR iPSC-RPE. (D) C3/C3b levels analyzed by ELISA in cell culture supernatants in LR and HR iPSC-RPE. Data are shown as mean $\pm$ SEM.

SUPPLEMENTARY FIGURE 2

Hydroquinone (HQ) impact on oxidative stress in LR and HR iPSC-RPE. (A) H2O2 levels assay in LR and HR iPSC-RPE. HQ-treated relative values are normalized to the respective controls (dotted line) in each individual experiment for each cell line. HQ effects in each group were assessed with paired Student's t-test (#) compared to controls (dotted line). (B, C) Gene expression levels of CAT (B) and GPX1 (C) analyzed via RT-qPCR in LR and HR iPSC-RPE treated with HQ. (D, E) Representative WB images (D) of Sod2 levels LR and HR iPSC-RPE. Tubulin was used as housekeeping control. Quantification of Sod2 levels is shown in E. Differences between LR and HR groups were determined with one-way ANOVA (*). Data points were collected from LR iPSC-RPE1, 2, 3 (n=3 biological replicates) and from HR iPSC-RPE4, 5, 6, 7, 8 (n=5 biological replicates). Data are shown as mean $\pm$ SEM.

SUPPLEMENTARY FIGURE 3

Impact of HQ and TM on the UPR response of LR and HR iPSC-RPE. **(A–C)** Representative WB images **(A)** of Bip and IRE1 α levels in LR and HR iPSC-RPE cells treated with HQ. Tubulin was used as housekeeping control. Quantification of Bip **(B)** and IRE1 α **(C)** levels are shown. Differences between LR and HR groups were assessed with one-way ANOVA (*). Data are shown as mean $\pm$ SEM. **(D, E)** Membrane damage assessed by cytotoxicity assay GF-AFC **(D)** and caspase3 activity **(E)**. TM-treated relative values are normalized to the respective controls (dotted line) in each individual

experiment for each cell line. TM effects in each group were determined with paired Student's t-test (#) compared to controls (dotted line). **(H–J)** Representative WB images **(H)** of Bip and IRE1 α levels in LR and HR iPSC-RPE cells treated with TM. Tubulin was used as housekeeping control. Quantification of Bip **(I)** and IRE1 α **(J)** levels are shown. Differences between LR and HR groups were assessed with one-way ANOVA (*). Data are shown as mean $\pm$ SEM. Data points were collected from LR iPSC-RPE1, 2, 3 (n=3 biological replicates) and from HR iPSC-RPE4, 5, 6, 7, 8 (n=5 biological replicates).

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Bradykinin B1 receptor signaling triggers complement activation on endothelial cells

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Introduction: The complement and kallikrein-kinin systems (KKS) are both activated during vascular inflammation, and there are many known interactions between the two systems. This study investigated if KKS activation induced complement activation on endothelial cells, and if activation was dependent on bradykinin B1 receptor (B1R) signaling.

Methods: KKS was activated in normal human serum by kaolin or activated factor XII (FXIIa). ADP-preactivated primary glomerular endothelial cells (PGECs) were incubated with serum, with or without kaolin or FXIIa, and with or without the B1R antagonist (R715) or the inositol triphosphate receptor (IP3R) inhibitor 2-aminoethoxydiphenyl borate (2-APB). Complement factors C3a, factor Ba and C5b-9 were evaluated by ELISA or immunoblotting. B1/B2 receptor double knock-out and wild-type mice were injected with lipopolysaccharide from *E. coli* B5:O55, to induce KKS activation.

Results: Supernatants from PGECs incubated with serum exposed to kaolin or FXIIa exhibited higher levels of Ba and C5b-9, which were significantly reduced in the presence of the B1R antagonist. Complement activation induced by FXIIa was also reduced in the presence of the IP3R inhibitor. Likewise, cell lysates showed higher levels of C3a and C5b-9 in the presence of kaolin and FXIIa, and complement activation was significantly reduced in the presence of the B1R antagonist. B1/B2 receptor double knock-out mice exhibited less C3 and C5b-9 deposition in glomeruli compared to wild-type mice.

Conclusion: This study demonstrates that KKS activation contributes to complement activation on the endothelium by B1R signaling. Blocking the B1R may have a role in reducing complement deposition and its effects on the endothelium.

KEYWORDS

bradykinin, complement, kallikrein-kinin system, glomerular endothelial cells, kidney, mice

Introduction

The kallikrein–kinin system (KKS) is activated during vascular inflammation such as vasculitis (1), in response to trauma (2), sepsis (3, 4), and myocardial infarction (5). In patients with hereditary angioedema, uncontrolled KKS activation and bradykinin (BK) release lead to vascular leakage and the development of angioedema (6). Activation of the KKS can occur by different routes, in a soluble or cell-bound manner. Circulating factor XII (FXII) is autoactivated to factor XIIa (FXIIa) on negatively charged surfaces (7), by lipopolysaccharide (LPS) (8, 9), collagen (10), or misfolded proteins (11) or artificially by kaolin (12). FXIIa activates prekallikrein to kallikrein, which in turn activates more FXII. An alternative pathway for FXII activation occurs on endothelial cells whereby FXII is autoactivated after binding to its complex receptor composed of cytokeratin 1 (CK1) and the globular head of the C1q receptor or urokinase plasminogen activator receptor (13). Prekallikrein bound to high molecular weight kininogen (HK) on endothelial cells is activated to kallikrein by prolylcarboxypeptidase (14). Kallikrein cleaves HK to release bradykinin, which is rapidly processed to des-Arg⁹-bradykinin (DABK) in plasma. BK binds to the constitutively expressed bradykinin B2 receptor (B2R) and DABK exerts its effects by binding to the inducible bradykinin B1 receptor (B1R). Expression of the B1R is induced during chronic inflammation.

B1R and B2R are G-protein coupled receptors (GPCRs), and both induce an increase in intracellular Ca²⁺ via the inositol 1,4,5 triphosphate (IP3)-receptor (15, 16). Ligand binding to kinin receptors activates the endothelial cell and causes conformational changes to the cell membrane (17), prostaglandin release (18), and nitric oxide production (19). In addition, B1R has been shown to induce neutrophil chemotaxis (20). These alterations to the cell and its surroundings lead to increased vascular permeability, vasodilation, and an inflammatory response.

The complement system is also activated during vascular inflammation. All three pathways of complement activation, the classical, lectin, or the alternative pathway, lead to the formation of a C3 convertase, which cleaves C3 into the opsonin C3b and the anaphylatoxin C3a. C3b forms the C3 convertase with factor Bb and can bind to formed C3 convertase, thus generating the C5 convertase. This initiates the terminal pathway whereby C5 convertase cleaves C5 to yield C5a and C5b. C5b forms a complex with C6, C7, C8 and multiple C9s (21). The membrane attack complex (MAC or C5b-9) is formed and can lead to pathogen elimination, or, in sublytical concentrations, to cell activation (22).

There are many known interactions between the complement system and the KKS. The KKS and the classical pathway of complement share the same inhibitor, C1-inhibitor (7). Moreover, kallikrein can cleave C3 and factor B (23, 24) and FXIIa can activate the classical pathway of complement by activating C1r and, to a lesser extent, C1s (25). Our group has shown that complement activation occurs on endothelial extracellular vesicles released from glomerular endothelial cells when vasculitis plasma was perfused over the cells. Complement deposition on the extracellular vesicles was reduced by B1R and B2R antagonists or by the C1 inhibitor,

suggesting a possible interaction between the KKS and complement on glomerular endothelial vesicles. Furthermore, using a mouse model of glomerulonephritis, we reported that a B1R antagonist reduced glomerular C3 deposition (26).

The aim of the current study was to assess the interactions between the KKS and complement on glomerular endothelial cells and the intracellular pathway by which this activation occurs. The KKS was activated on glomerular endothelial cells by kaolin or FXIIa and cell supernatants and lysates assessed for complement C3a, Ba, and C5b-9. Experiments were performed in the presence of a B1R antagonist as well as an IP3 receptor inhibitor. KKS activation was induced *in vivo* in mice by LPS to assess glomerular C3 and C5b-9 deposition in wild-type as well as B1R/B2R double knock-out mice.

Methods

Serum samples

Whole blood was obtained from healthy adult controls without ongoing medications (n=6, one female) in vacutainer serum tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Generally, the alternative pathway is more easily activated in samples from male donors (27). The tubes were left for 45 min at rt, allowing the blood to clot. The clot was removed by centrifugation at 1,500 × g for 10 min at 4°C, and the remaining serum was aliquoted and stored at −80°C until used. Serum samples were used in cell experiments. The study was conducted according to the Declaration of Helsinki with the approval of the Swedish Ethical Review Authority. Informed written consent was obtained from all participants.

Primary glomerular endothelial cells

Primary glomerular endothelial cells (PGECs, Cell Systems, Kirkland, WA, USA) were used in all cell experiments and grown to confluency in a 12-well plate, as previously described (20). These cells express the kinin B1R (28). PGECs were preactivated by incubation with adenosine diphosphate (ADP, 1 mM, Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Cells were washed and, in some experiments, preincubated with the B1R antagonist R715 (1 μM, Tocris Bioscience, Bristol, UK) or 2-aminoethoxydiphenyl borate (2-APB, 100 μM, Sigma-Aldrich, in dimethyl sulfoxide (DMSO)) to block the intracellular IP3 receptor, for 30 min at 37°C. Normal serum (1:4) was added with or without kaolin (0.1 mg/mL, Sigma-Aldrich) or with or without factor XIIa (FXIIa, Enzyme Research Laboratories, South Bend, IN, USA, 1.38 μg/mL for experiments with the B1R antagonist, 13.8 μg/mL for experiments with 2-APB) for an additional 30 min. The supernatant was removed, centrifuged at 10,000 × g for 2 min and stored at −80°C until analyzed. Cells were washed twice with PBS and lysed by adding radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology, Dallas, TX, USA) followed by sequential freezing and thawing. Protein concentrations in lysates were assessed in using the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates were stored at −20°C until analyzed.

Kallikrein activity assay

Kallikrein activity in cell supernatants exposed to serum and stimulated with kaolin or FXIIa was analyzed using the chromogenic substrate PNAPEP 1902 (Cryoep, Montpellier, France). Samples were mixed 1:2 with PNAPEP 1902, and absorbance was measured at 405 nm at 60 min using a GloMax Discovery System (Promega, Madison, WI). Both kaolin (Figure 1A) and FXIIa (Figure 1B) were potent kallikrein activators, as expected. The B1R antagonist R715, in the presence or absence of kallikrein activators (Supplementary Figures 1A, B), and 2-APB in the presence of FXIIa, did not affect the assay (Supplementary Figure 1C).

High molecular weight kininogen and C3a detected by immunoblotting

High molecular weight kininogen (HK) in cell supernatants and C3a in cell lysates were detected by immunoblotting. Samples were diluted 1:500 (HK) or to the same protein concentration (C3a) in sample buffer (0.14 M Tris buffer, pH 6.8 containing 4% (w/v) sodium dodecyl sulfate (SDS), 10% glycerol, (all from Sigma-Aldrich), 0.3% bromophenol blue (LKB Products, Bromma, Sweden)) with 10% 2-mercaptoethanol (Sigma-Aldrich) followed by boiling for 5 min. Samples were run on 4%–20% Mini-PROTEAN TGX gel (Bio-Rad Laboratories, Hercules, CA, USA) followed by transfer to a 0.2- μ m polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories). Membranes were blocked with 10% casein (Vector Laboratories, Oxfordshire, UK) followed by 1-h incubation with sheep anti-human HK antiserum AS88 (1:5000) (29) or rabbit-anti human C3a (1:2000, Complement Technology, Tyler, TX, USA). After three washes in PBS-Tween (Medicago, Uppsala, Sweden), membranes were incubated with secondary antibody donkey anti-sheep horseradish peroxidase (HRP) (1:2,000, Dako, Glostrup, Denmark) or goat anti-rabbit HRP (1:1,000, Dako, Glostrup, Denmark) for 1 h at rt. After additional washing, the signal was detected by chemiluminescence using PierceTM ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA). Cell supernatants exhibited cleavage of HK induced by kaolin (Figure 1C) or by FXIIa (Figure 1D), as expected. The latter was not affected by the presence of the B1R antagonist R715 (Supplementary Figure 2). For C3a, the pixel intensity of the C3a band was measured and differences in C3a staining presented as fold change. Cells incubated with normal serum alone were defined as 1.

C3a, Ba, and C5b-9 detection by ELISA

The presence of C3a, Ba, and C5b-9 in cell supernatants was analyzed by ELISA (Quidel Corporations, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, supernatants were diluted 1:200 (C3a), 1:2,000 (Ba), and 1:20 (C5b-9) in diluent

media provided by the manufacturer and incubated for 1 h at rt. Sequential washing was performed, and conjugate was added to the wells and incubated for 1 h (C3a, Ba) or 30 min (C5b-9). Plates were washed five times and incubated with the substrate for 15 min, after which reactions were stopped and read at 450 nm. To analyze C5b-9 levels in cell lysates, samples were diluted to the same protein concentration and assessed as described above.

As a control C3 (24 μ g/mL) and C5b-9 (1 μ g/mL, both from Complement Technology) were incubated with FXIIa (1.38 μ g/mL) for 30 min at 37°C in the absence of cells and serum and C3a as well as C5b-9 analyzed by ELISA, as described above. In addition, kaolin (0.1 mg/mL) and FXIIa (1.38 μ g/mL) were incubated with normal serum for 30 min at 37°C in the absence of cells, and C3a detected by ELISA as described above. As kallikrein has been shown to cleave C3 in a pure system (23) and zymosan activates the complement system in serum (30), those were included as positive controls in experiments analyzing the presence of C3a.

B1/B2 receptor knockout mice injected with lipopolysaccharide

C57BL/6N B1/B2 receptor knockout (31) and corresponding wild-type mice were bred in the animal facilities at Lund University. Female and male mice were used at 8–10 weeks of age. Double knockout mice were used as B1R-deficient mice overexpress the B2R (32). Mice were treated with 2 mg/kg lipopolysaccharide (LPS) from *Escherichia coli* B5:O55 (InvivoGen, San Diego, CA, USA) or PBS vehicle i.p. Mice were observed and sacrificed within 24 h when clinical signs of illness occurred, such as ruffled fur, decreased activity, hunched posture, squinting, or neurological defects (for the disease score, see Supplementary Table 1). All remaining mice were sacrificed at 24 h. Blood samples were taken while mice were under isoflurane anesthesia via heart puncture, into syringes filled with 100 μ l of EDTA. Samples were centrifuged at 2,000 $\times$ g for 10 min and the plasma stored at –80°C until analyzed. Kidneys were fixed in 4% paraformaldehyde (PFA, Histolab Products AB, Askim, Sweden), embedded in paraffin and sectioned (4 μ m) onto glass slides.

Animal experiments were approved by the regional Animal Ethics Committee (approval 17452-20) and performed in accordance with regulations of the Swedish Board of Agriculture and the European Directive on the protection of animals used for scientific purposes.

Residual prekallikrein in murine plasma

Mouse plasma was analyzed using the kallikrein chromogenic assay described above. As kallikrein has a very short half-life in plasma, an alternative approach was applied measuring residual prekallikrein (33). To this end, plasma samples were diluted 1:4 in serum-free cell media (Lonza Group AG, Basel, Switzerland) and incubated with or without kaolin (0.1 mg/mL) for 10 min at 37°C.

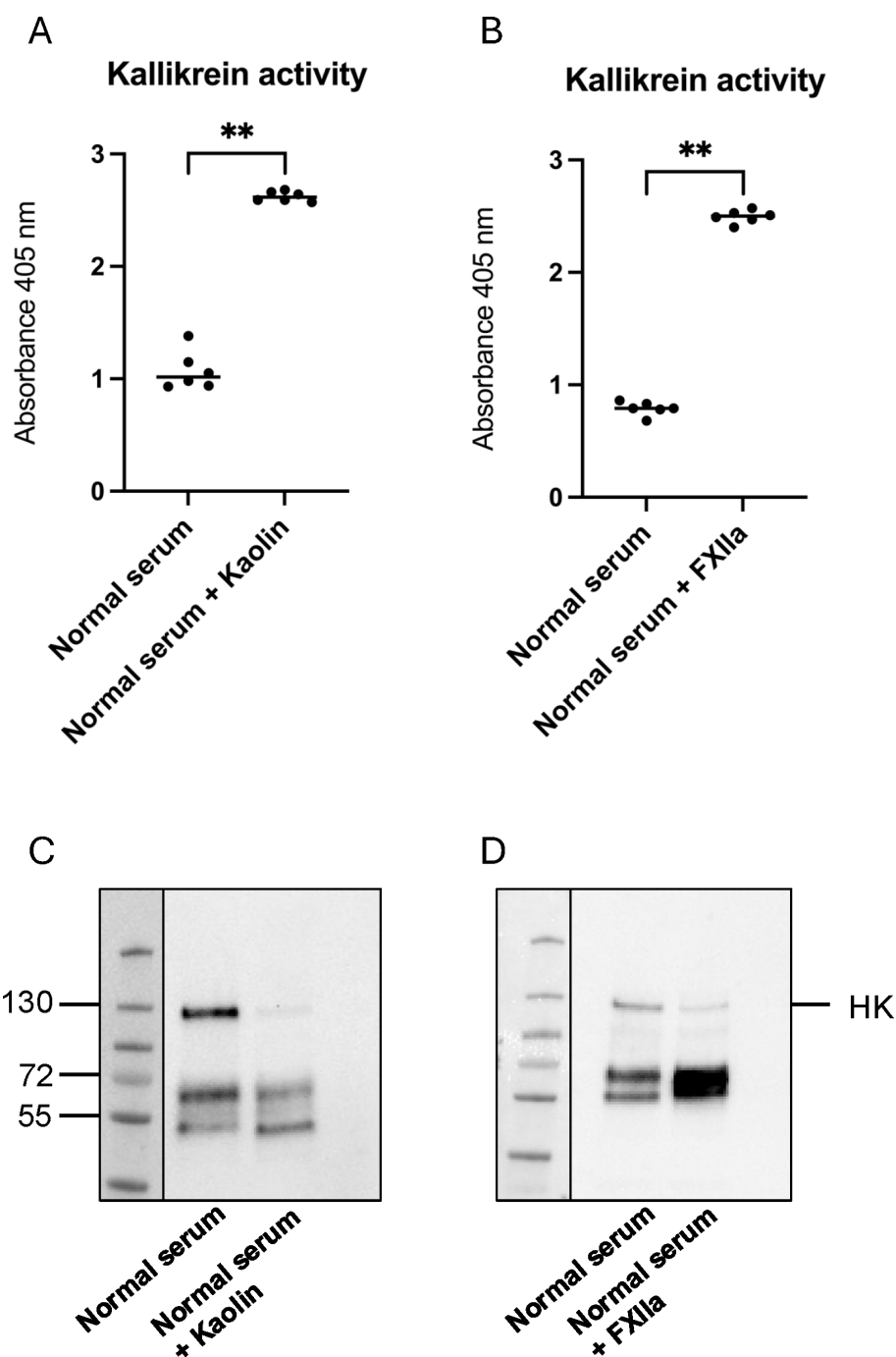


FIGURE 1

Activation of the kallikrein–kinin system in supernatants from cells treated with kaolin and FXIIa. (A) Kallikrein activity in supernatants from cells stimulated with kaolin (n=6) compared with controls (n=6). (B) Kallikrein activity in supernatants from cells stimulated with factor XIIa (FXIIa) (n=6) compared with controls (n=6). (C) Representative image of high molecular weight kininogen (HK) and its degradation products in supernatants from cells stimulated with kaolin (n=6). (D) Representative image of HK and its degradation products in supernatants from cells stimulated with FXIIa (n=6). **P < 0.01.

Samples were centrifuged at $10,000 \times g$ for 2 min to discard kaolin. The supernatants were combined with PNAPEP 1:2, and absorbance was measured at 405 nm at 0 min and 60 min. Residual prekallikrein was calculated as the difference between samples incubated with kaolin at 60 min and the samples without kaolin at 0 min. A lower value indicates prekallikrein consumption in plasma.

Urea levels in murine plasma

Urea was measured in EDTA plasma using a QuantiChrom Urea Assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. All samples were run as duplicates and detection carried out using the GloMax Discover System.

Kidney tissues

Kidney sections were deparaffinized and stained with hematoxylin–eosin to evaluate histopathological changes. Tissues were visualized using a Nikon Eclipse Ti-E microscope with a Nikon color camera using NIS Elements AR software v.5.11.01 (Nikon Instruments Inc., Tokyo, Japan) and assessed in a blinded manner.

Immunofluorescence for C3 and C5b-9 was carried out as previously described (26). Sections were incubated with rabbit anti-C3 (4 µg/mL, Hycult Biotech, Uden, Netherlands) or rabbit anti-C5b-9 (5 µg/mL, a kind gift from Professor Paul Morgan, Cardiff University, UK) at 4°C overnight, or negative control rabbit IgG (Dako) at the same concentrations. Tissues were assessed in a blinded fashion. Fluorescence in each glomerulus was scored from 0 to 3 using a template presented in [Supplementary Figure 3](#). Each score was multiplied by the number of glomeruli with that score in the entire kidney section. The sum of all fluorescence was calculated and divided by the total number of glomeruli in that section, to establish the mean fluorescence/glomerulus.

Statistics

Statistical analysis was performed using GraphPad prism software (GraphPad Software, Version 9, La Jolla, Ca). The non-parametric Mann–Whitney U test was used for two group comparisons. Multiple group comparisons were performed using the Kruskal–Wallis test followed by Dunn’s multiple comparisons test. Cell experiments performed on the same day with the same serum donor, stimulated with and without kaolin or FXIIa and in the presence and absence of R715 or 2-APB, were not considered independent, and thus data were analyzed as paired using the non-parametric Wilcoxon signed rank test. A P value ≤0.05 was considered significant.

Results

B1 receptor on glomerular endothelial cells triggers complement activation

Supernatants from PGEs incubated with kaolin or FXIIa, in the presence or absence of the B1R-antagonist R715, were analyzed for C3a, Ba, and C5b-9. C3a in supernatants was increased when cells were activated with kaolin, and the B1R antagonist did not lower concentrations of C3a ([Figure 2A](#)). Levels of Ba and C5b9 were elevated in supernatants incubated with kaolin ([Figures 2B, C](#)) and significantly reduced in the presence of the B1R-antagonist. Cell lysates activated with kaolin showed higher levels of both C3a ([Figure 2D](#)) and C5b-9 ([Figure 2E](#)), and these levels were significantly reduced in the presence of the B1R-antagonist. Levels of Ba were not assayed in cell lysates because Ba is not cell-bound.

Supernatants incubated with FXIIa were likewise analyzed for C3a, Ba, and C5b-9. Concentrations of Ba ([Figure 3A](#)) and C5b-9 ([Figure 3B](#)) were elevated when incubated with FXIIa and

significantly lowered in the presence of the B1R antagonist. Levels of C3a in supernatants were not elevated when activated with FXIIa, and no reduction was seen in the presence of the B1R antagonist ([Supplementary Figure 4](#)). Cell lysates showed higher levels of C3a ([Figure 3C](#)) and C5b-9 ([Figure 3D](#)) in the presence of FXIIa, which were significantly reduced in the presence of the B1R antagonist.

Control experiments demonstrated that FXIIa did not cleave C3 to C3a ([Supplementary Figure 5A](#)) or affect levels of C5b-9 in a pure system ([Supplementary Figure 5B](#)). In addition, normal human serum incubated with kaolin or FXIIa in the absence of cells did not generate C3a ([Supplementary Figure 5C](#), zymosan was used as the positive control).

Kinin receptor-mediated complement activation was reduced by an inositol 1,4,5-trisphosphate receptor antagonist

2-APB is an IP₃ receptor antagonist. The following experiments were performed to investigate if 2-APB affected FXIIa-induced complement activation, by blocking one of the intracellular signaling pathways of GPCRs. Supernatants and cell lysates from PGEs incubated with FXIIa in the presence or absence of 2-APB were analyzed for complement activation. Ba ([Figure 4A](#)) and C5b-9 ([Figure 4B](#)) were increased in supernatants of cells incubated with FXIIa and lowered in the presence of 2-APB. C3a levels in cell supernatants were not tested in the presence of 2-APB because these were not elevated by incubation with FXIIa, as described above. The effect of 2-APB on cell lysates could not be tested as the DMSO vehicle in itself decreased complement levels in lysates but did not affect complement measurements in supernatants. 2-APB did not affect kallikrein activity ([Supplementary Figure 1C](#)).

LPS injection in mice decreased prekallikrein

B1/B2 receptor knockout mice (n=21) and wild-type mice (n=21) were treated with LPS to induce kallikrein–kinin system activation or injected with the PBS vehicle, 12 treated mice and 9 controls in each group. Mice were sacrificed within 14 h–24 h. All mice injected with *E. coli* O55:B5 LPS exhibited weight loss compared with controls ([Figure 5A](#)), and most of them exhibited clinical signs of disease, regardless of genotype ([Figure 5B](#); for the disease score, see [Supplementary Table 1](#)). Prekallikrein was consumed in the plasma of LPS-treated mice ([Figure 5C](#)), indicating kallikrein activation. Urea levels were slightly higher in LPS-treated mice versus untreated mice, but this did not achieve statistical significance ([Figure 5D](#)).

LPS-treated mice exhibited histopathological changes in the kidneys

LPS-injected mice exhibited histopathological changes in kidney sections not present in PBS-control mice ([Table 1](#)). The

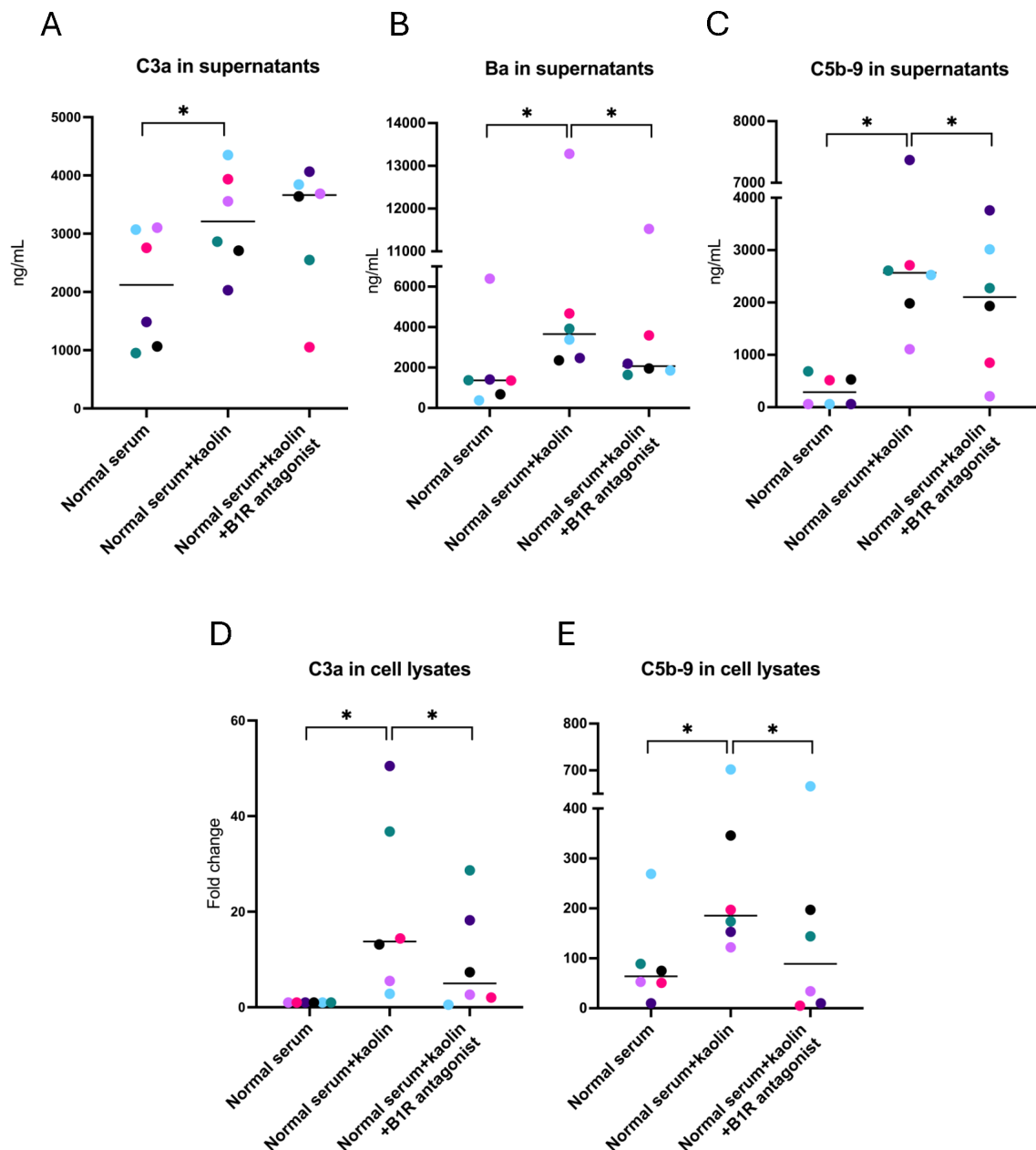


FIGURE 2

Complement activation in glomerular endothelial cells incubated with kaolin and the effect of the B1R antagonist. (A) C3a in glomerular endothelial cell supernatants. (B) Factor Ba in glomerular endothelial cell supernatants. (C) C5b-9 in glomerular endothelial cell supernatants. (D) C3a in glomerular endothelial cell lysates. Differences in C3a staining are presented as fold change, in which cells incubated with normal serum alone (left lane) were defined as 1. (E) C5b-9 in glomerular endothelial cell lysates. The Wilcoxon signed rank test for paired samples was used in which each color represents one experiment. * $P < 0.05$. B1R, bradykinin receptor 1.

changes observed were vacuolization of tubular cells (Figure 5E) and interstitial widening suggestive of interstitial edema, as well as infiltrates (Figure 5F). Control mice exhibited no kidney pathology (Figure 5G). Pathology was more pronounced in mice taken after 24 h compared with 14 h. LPS-treated wild-type mice exhibited significantly more epithelial desquamation, compared with LPS-treated B1/B2 receptor knockout mice. Edema and epithelial vacuolization were slightly more prominent in LPS-treated wild-type mice compared with B1/B2 receptor knockout mice, but this

did not achieve statistical significance (Table 1). No pathological changes were seen in glomeruli.

Mice treated with LPS displayed complement deposition in kidneys

Kidney sections from LPS-treated mice were stained for C3 and C5b-9 and compared with mice treated with the PBS vehicle. LPS-

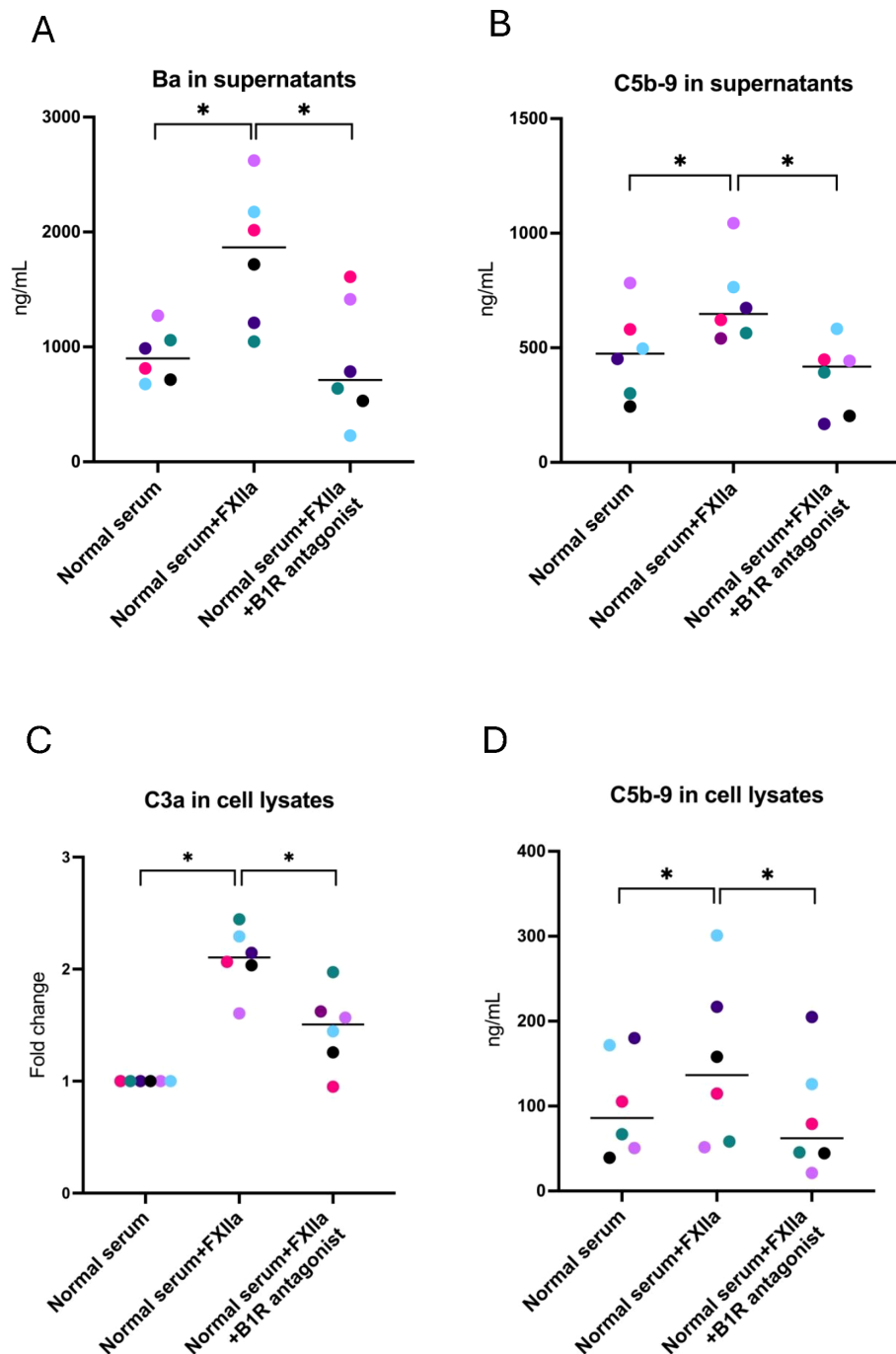


FIGURE 3

Complement activation in glomerular endothelial cells incubated with FXIIa, and the effect of the B1R antagonist. (A) Factor Ba in glomerular endothelial cell supernatants. (B) C5b-9 in glomerular endothelial cell supernatants. (C) C3a in glomerular endothelial cell lysates. Differences in C3a staining are presented as fold change, in which cells incubated with normal serum alone (left lane) were defined as 1. (D) C5b-9 in glomerular endothelial cell lysates. The Wilcoxon signed rank test for paired samples was used, in which each color represents one experiment. * $P < 0.05$. B1R, bradykinin receptor 1.

treated wild-type mice exhibited significantly more C3 staining in glomeruli (Figure 6A) compared with the B1/B2 receptor knockout mice (Figure 6B) and PBS controls. The latter showed minimal glomerular staining (Figure 6C). Likewise, LPS-treated wild-type mice showed more C5b-9 staining (Figure 6D) than B1/B2 receptor knockout mice (Figure 6E) and controls (Figure 6F). Complement

deposition in glomeruli was scored in most mice, and fluorescence levels/glomerulus are presented for C3 in Figure 6G and for C5b-9 in Figure 6H, showing that LPS-treated wild-type mice had significantly more complement deposition than B1/B2 receptor knockout mice whereas the PBS controls had minimal glomerular complement staining.

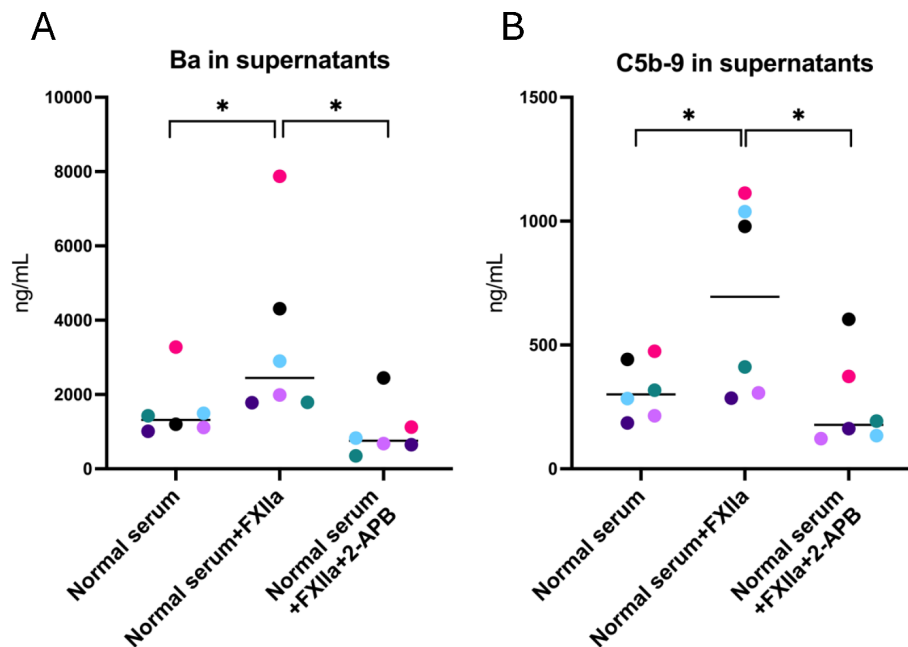


FIGURE 4

Complement activation of glomerular endothelial cells stimulated with FXIIa and the effect of IP3-receptor inhibition. (A) Factor Ba in glomerular endothelial cell supernatants. (B) C5b-9 in glomerular endothelial cell supernatants. Wilcoxon's signed rank test was used for statistical comparisons and experiments are sorted by color. *P < 0.05. 2-APB, 2-aminoethoxydiphenyl borate.

Discussion

FXIIa stimulates kallikrein activation leading to HK cleavage and the release of bradykinin and DABK, which bind the two kinin receptors B2R and B1R, respectively. Here, we show that kaolin and FXIIa induce complement activation on the glomerular endothelium in a B1R-dependent manner. Complement activation was detected by an increase in C3a and C5b-9 in cell supernatants and lysates. Increased C5b-9 suggests that even the terminal complement pathway was activated potentially leading to endothelial cell injury. Additionally, increases of the factor B cleavage product, Ba, in cell supernatants suggests that FXIIa-induced complement activation occurs via the alternative pathway. Complement release and deposition was, at least in part, triggered by B1R signaling as the B1R antagonist as well as the IP3R antagonist, which blocks the intracellular signaling pathway of GPCRs, both reduced complement activation. In wild-type mice, LPS stimulation led to kallikrein activation, as well as complement deposition in glomeruli. Although kallikrein activation was similar, complement activation was reduced in B1R/B2R double knockout mice. Taken together, the *in vitro* and *in vivo* data suggest that signaling via bradykinin receptors, and particularly B1R, contributes to complement activation on the glomerular endothelium. As complement C3a is an anaphylatoxin and C5b-9 is associated with cell activation or membrane injury, the presence of these complement proteins on the cell could contribute to vascular inflammation.

LPS can induce complement activation (34) particularly by activating the alternative pathway (35). In the current study, LPS was used to stimulate kallikrein activity, as bacterial endotoxins

have been shown to activate FXII *in vitro* (36) and induce the KKS *in vivo* (37). As LPS can induce activation of both complement and the KKS, we cannot rule out that C3 and C5b-9 deposition in glomeruli of wild-type mice was due to direct activation of the alternative pathway of complement. However, the B1R/B2R receptor double-knockout mice exhibited significantly less C3 and C5b-9 deposition, indicating that complement deposition was partly associated with signaling via these kinin receptors.

Kallikrein was previously demonstrated to cleave C3 (23). Thus, kaolin or FXIIa could activate kallikrein and induce complement activation extracellularly. The inhibitory effects of the B1R antagonist R715 and the IP3R inhibitor 2-APB suggest, however, that intracellular activation is occurring as well. Bradykinin receptor signaling occurs by activation of phospholipase C and IP3-associated release of calcium into the cytoplasm (38). By blocking the B1R and IP3R *in vitro*, and by studying the effects of LPS in B1R/B2R knockout mice, we show that complement deposition is associated with cellular signaling via kinin receptors.

We have previously demonstrated that vasculitis plasma perfused over glomerular endothelial cells led to release of complement-coated endothelial extracellular vesicles. B1R and B2R antagonists reduced complement deposition on the vesicles (26). Complement deposition can be harmful for cellular integrity, particularly formation of C5b-9 in the membrane; thus, the cell may rid itself of harmful substances by releasing extracellular vesicles. Here, we show that complement is also deposited on the glomerular endothelial cells themselves, in response to activation of the KKS. Thus, the cells cannot rid themselves entirely of complement by shedding vesicles and blocking the kinin receptors may further reduce complement deposition.

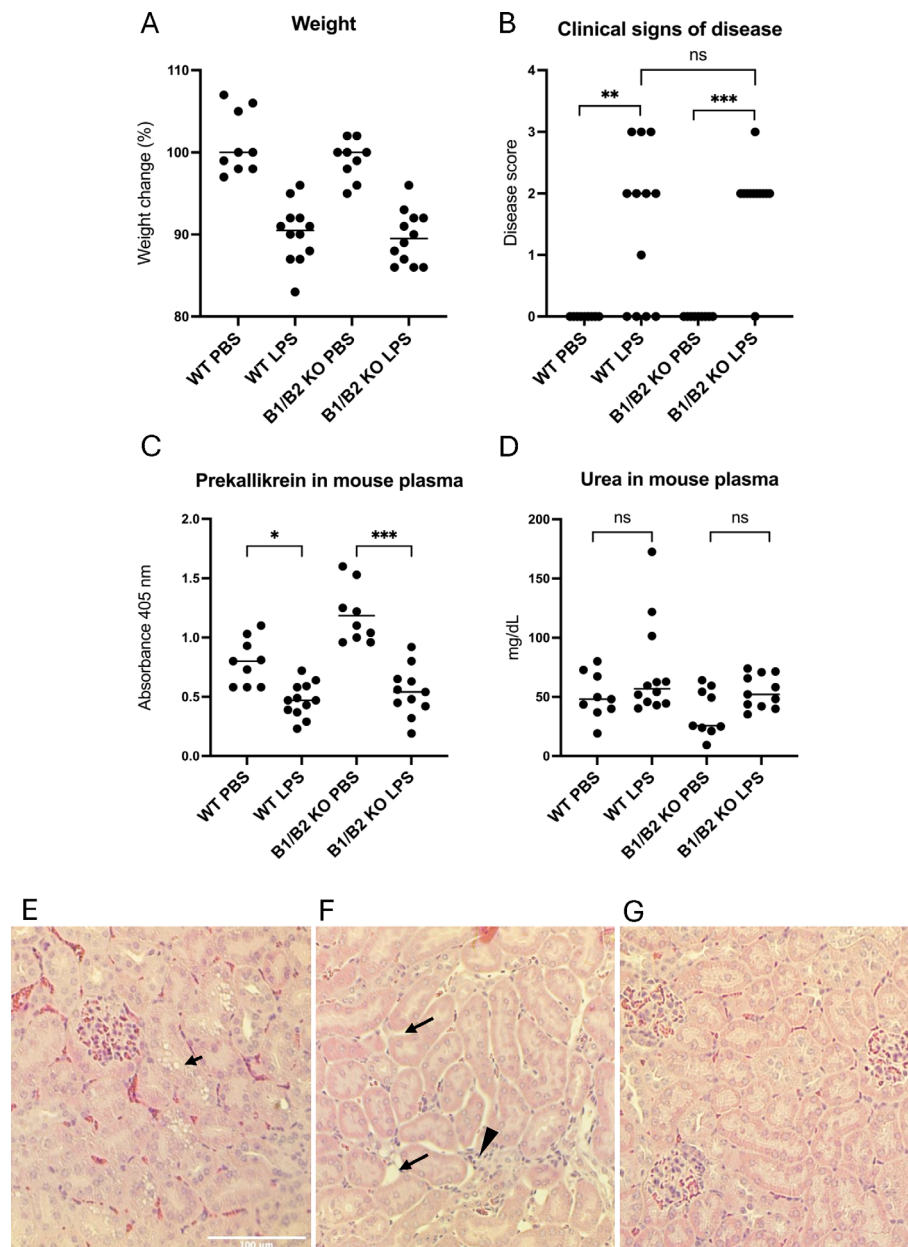


FIGURE 5

Wild-type and B1/B2 receptor knockout mice injected with LPS. **(A)** Body weight changes in mice treated with LPS (wild-type $n=12$, B1/B2 receptor knockout $n=12$) or PBS (wild-type $n=9$, B1/B2 receptor knockout $n=9$). Each dot represents one mouse, and the bar represents the median.

(B) Clinical signs of disease scored in the same mice. A score of 0–3 was given to each mouse at the end of the experiment, as per [Supplementary Table 1](#). **(C)** Prekallikrein in plasma samples from the same mice. **(D)** Plasma urea concentrations of the mice.

(E) A representative kidney section from an LPS-treated wild-type mouse. Arrow indicates epithelial vacuolization. **(F)** A representative kidney section from an LPS-treated wild-type mouse. Arrow indicates interstitial widening, suggesting edema. Arrowhead indicates infiltrative cells. **(G)** A representative kidney section from a wild-type mouse given PBS, showing normal mouse kidney histology. Kruskal–Wallis test followed by Dunn’s multiple comparison used for statistical comparisons in panels A–C. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns: not significant. LPS, lipopolysaccharide, WT, wild-type, B1/B2, B1/B2 receptor, KO, knockout.

Endothelial cells were stimulated with kaolin or FXIIa, and the B1R antagonist decreased complement C3a and C5b-9 deposition on cell lysates. The B1R antagonist did not, however, reduce C3a in supernatants, most probably because the effect is most prominent on cell membranes when C3a is bound to its receptor. We previously showed that a B1R antagonist decreased glomerular C3 deposition in a glomerulonephritis mouse model (26), which is in line with the

results presented herein, showing the importance of both the kinin receptors for mediating complement activation in glomeruli *in vivo*. These results collectively suggest that inhibitors of the KKS and its receptor signaling can decrease complement activation on the glomerular endothelium during vascular inflammation.

Conditions with profound vascular inflammation in the kidney may potentially benefit from KKS inhibition. For example, patients

TABLE 1 Kidney histopathology in LPS-treated mice.

Histopathological finding	LPS-treated (n=14)		Controls (n=6)	
	WT (n=7)	B1R/B2R KO (n=7)	WT (n=3)	B1R/B2R KO (n=3)
Tubular vacuolization	1 (1-3) ^a	1 (0-2)	0	0
Tubular epithelial desquamation	1 (0-2)*	0 (0-1)	0	0
Interstitial edema	1 (0-2)	1 (0-1)	0	0

^aScores are presented as median; range is presented in parentheses. An entire kidney section was analyzed from each mouse. Histopathological changes were scored in a blinded fashion as 0 (absent), 1 (mild), 2 (moderate), and 3 (severe). Statistical comparisons were performed using Kruskal–Wallis test followed by Dunn’s multiple comparison, comparing LPS-treated wild-type to LPS-treated B1/B2 receptor knockout mice. *P < 0.05. LPS, lipopolysaccharide; WT, wild type; B1R/B2R, B1/B2 receptor; KO, knockout.

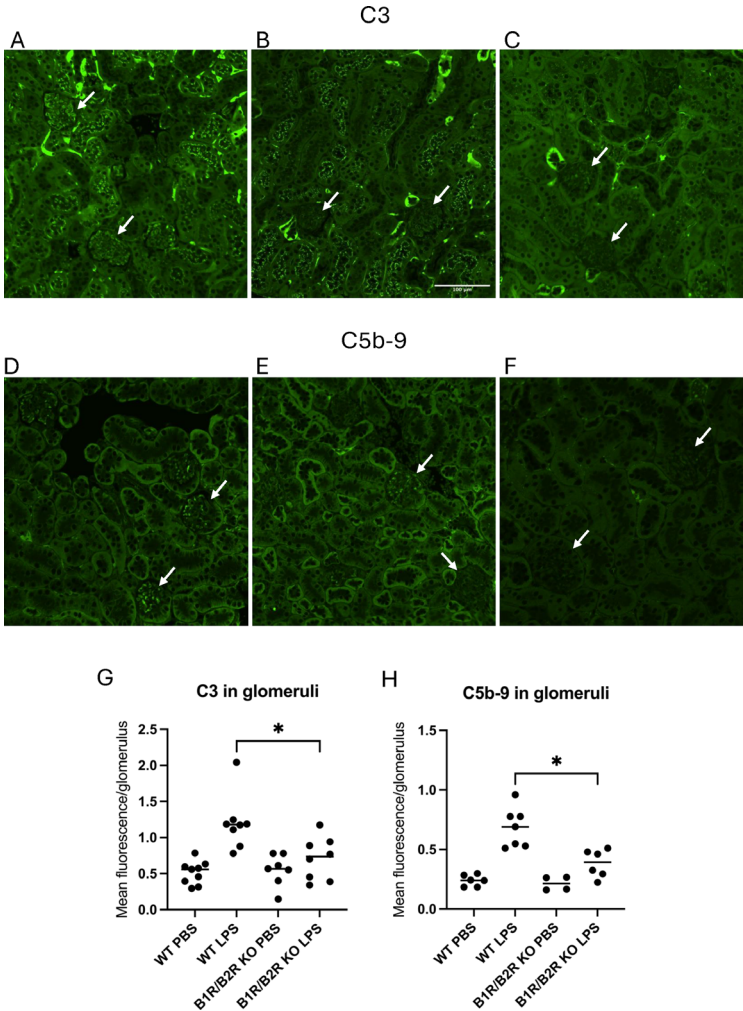


FIGURE 6 C3 and C5b-9 in kidneys in LPS-treated wild-type and B1/B2 receptor knock out mice. **(A)** C3 in a representative kidney section from an LPS-treated wild-type mouse. Arrows indicate glomeruli scored as 1 (lower glomerulus) and 3 (upper), respectively. **(B)** C3 staining in a representative kidney section from an LPS-treated B1/B2 receptor knockout mouse. Arrows indicate two glomeruli scored as 0. **(C)** C3 staining of a representative kidney section from a PBS vehicle-treated wild-type mouse exhibiting mild staining in tubules but not in glomeruli. Arrows indicate two glomeruli scored as 0. **(D)** C5b-9 in a representative kidney section from an LPS-treated wild-type mouse. Arrows indicate glomeruli scored as 1 (upper) and 2 (lower), respectively. **(E)** C5b-9 in a representative kidney section from an LPS-treated B1/B2 receptor knockout mouse. Arrows indicate glomeruli scored as 0 (lower) and 1 (upper), respectively. **(F)** C5b-9 in a representative kidney section from a PBS vehicle-treated wild-type mouse injected with PBS stained for C5b-9. Arrows indicate two glomeruli scored as 0. **(G)** Kidney sections from wild-type mice injected with PBS (n=9), LPS (n=8), B1/B2 receptor knockout mice injected with PBS (n=7), or LPS (n=8) were stained for C3, and the intensity was calculated and presented here as mean fluorescence/glomerulus. **(H)** Kidney sections from wild-type mice injected with PBS (n=6) or LPS (n=7) and B1/B2 receptor knockout mice injected with PBS (n=4) or LPS (n=6) and stained for C5b-9; the intensity was calculated and presented here as mean fluorescence/glomerulus. Kruskal–Wallis test followed by Dunn’s multiple comparison was used for statistical comparisons. *P<0.05. LPS, lipopolysaccharide; WT, wild type; B1/B2, B1/B2 receptor; KO, knockout.

with diabetes nephropathy have complement deposits in the kidney (39) and are often treated with angiotensin-converting enzyme (ACE) inhibitors to reduce proteinuria. ACE inhibitors increase bradykinin concentrations by blocking its degradation (40). Thus, conditions in which both complement and kinin proteins or peptides may negatively affect the kidney could benefit from blockade of kinin receptors.

There are currently several approved pharmaceutical agents that can effectively block activation of the KKS, and more are under development. Most of them are used as treatment or prophylaxis in hereditary angioedema. These treatments include the C1 inhibitor, a monoclonal antibody targeting FXII, garadacimab, and kallikrein inhibitors (41). All these inhibitors will inhibit the generation of kinin peptides, and hence their signaling through B1R and B2R. Icatibant is the only approved kinin receptor antagonist, targeting the B2R (42). To date, there is no commercially available inhibitor of the B1R.

The B1R is induced by inflammatory stimuli, and upon ligand binding, it does not desensitize and thus generates prolonged intracellular signaling (16). Therefore, the B1R is of importance during chronic inflammation. In a mouse model of late-stage sepsis, treatment with a B1R antagonist improved survival rates and reduced kidney injury and lung lesions (4). The results of the current study suggest that KKS inhibition by a B1R antagonist may have a beneficial effect in vascular inflammation, which should be addressed in clinical trials.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving humans were approved by Swedish Ethical Review Authority. The studies were conducted in accordance with the national legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Animal Ethics Committee Lund University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ILF: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. AG: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. MW: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. IA: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. AT: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. A-CK: Formal

analysis, Investigation, Methodology, Visualization, Writing – original draft. FQ: Conceptualization, Investigation, Methodology, Writing – original draft. MB: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft. DK: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1527065/full#supplementary-material>

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Urine complement analysis implies complement activation is involved in membranous nephropathy

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Background: The onset and progression of membranous nephropathy (MN) have been associated with complement activation, yet the overall characteristics of this activation in the kidneys remain unclear. In our study, we utilized urine proteomic data to investigate the features of complement activation. We examined the relationship between urine complement components and both clinicopathological features and clinical outcomes in patients with MN.

Methods: Differential expression proteins (DEPs) analysis was performed using proteomic data from urine samples collected from 50 patients with MN, 50 patients with IgA nephropathies (IgAN), and 72 healthy controls (HC). Then, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses were carried out on the DEPs identified in MN. We further investigated the differentially expressed urinary complement proteins in MN patients, exploring their relationships with clinicopathological features and clinical remission. Next, 11 representative complements were selected for validation. Immunohistochemistry and immunofluorescence techniques were employed to compare the expression of CD59 and C5b-9 in renal tissues from MN patients, with analyses conducted on both the clinical remission group and the no remission group ($n = 6$ in each group).

Results: Total 1,427 differentially expressed proteins were identified between the MN and HC groups. KEGG pathway analysis showed significant enrichment of these DEPs in the complement-activated pathway within the MN group. Additionally, a correlation was found between proteinuria and the levels of 27 urinary complement proteins. Notably, Collectin12 (*collec12*) and C1s were positively correlated with tubular atrophy/interstitial fibrosis (TIF) and monocyte infiltration. Furthermore, urine CD59 emerged as a predictor of clinical remission. Lower deposition of C5b-9 in renal tissue and higher expression of CD59 were detected in clinical remission group than non-remission group.

Conclusion: In patients with MN, abnormal levels of complement components in urine are commonly observed. Currently, the use of complement inhibitors has brought new hope for the treatment of MN. The factor B inhibitor LNP023 and the factor D inhibitor BCX9930 are undergoing clinical trials for the treatment of MN. Our study indicates that complement abnormalities could serve as clinical biomarkers for tracking the progression of MN, predicting clinical remission, and guiding targeted complement therapy for those affected.

KEYWORDS

membranous nephropathy, complement, proteomics, histopathology, clinical remission

Introduction

In recent years, membranous nephropathy (MN) has emerged as the leading cause of nephrotic syndrome in adult patients, with approximately 40% of cases progressing to end-stage renal disease within 5 to 15 years (1). In primary MN, endogenous glomerular antigens, such as the M-type phospholipase A2 receptor (PLA2R) and the 7A-containing platelet-responsive protein type 1 structural domain (THSD7A), bind to antibodies, forming immune complexes in the glomerulus. These immune complexes trigger the complement cascade, primarily activating the lectin-complement pathway, which is one of the three pathways of complement activation. It is activated by recognition of non-self or altered self glycan structures by mannose-binding lectin (MBL), collectin-10, collectin-11 or ficolins (ficolin-1, -2, -3). Activation of complement leads to the formation of the membrane attack complex (MAC, C5b-9), which is the end product of complement activation that inserts directly into the podocyte membrane (2). Lead to podocyte damage, disruption of the glomerular filtration barrier, and ultimately to proteinuria in patients with MN (3). Notably, components from all three complement pathways can be detected in renal biopsies of patients diagnosed with MN.

Although the presence of activated complements in the injured glomeruli of patients with MN is well-established, the overall characteristics, initiating pathways, and pathogenic roles of complement activation in this condition remain unclear. Various studies have demonstrated that complement activation in renal tissues of MN is closely associated with renal injury and poor outcomes. Glomerular C3 staining intensity has been found to correlate with proteinuria (4, 5) and extensive glomerular C3 deposition can serve as a predictor of renal failure in patients with MN (6). Additionally, C5b-9 deposition has been linked to lower renal survival in MN (7), as well as the presence of MBL and C4d deposits is associated with increased renal interstitial fibrosis and no remission in patients with MN (8–10). Recent studies utilizing laser microdissection and mass spectrometry (MS/MS) have detected abundant complement proteins such as C3, C4, C5, C6-9, complement factor H (CFH), vitronectin (VTN), and clusterin (CLU) in the renal tissues of MN patients (11, 12). However, it is not possible to repeatedly obtain and use renal tissue for tracking complement activation.

Previous studies have shown that activated complement can be found in both blood and urine, and its levels correlate with clinical and pathological features, as well as disease progression in patients with MN. The presence of circulating MBL-associated serine proteases-1/2 (MASP-1/2), MBL, C3, C4, C5a, CFH, and complement factor B (CFB) has been found to be linked with disease progression according to several studies (13–18). Urinary levels of MBL, C4d, and C5a have shown positive correlations with urinary protein (19). Additionally, Studies have demonstrated that high levels of urinary C3dg and C5b-9 are significantly associated with MN, and patients with high urinary C5b-9 have exhibited an unstable clinical course (20). It has also been noted that persistent C5b-9 excretion is linked to poor clinical outcomes (21, 22). Several clinical trials targeting the

complement system are currently underway in patients with MN, following these findings.

The overall expression profile of complement components in the blood and urine of patients with MN has not been thoroughly explored in previous studies, which mainly focus on individual complement proteins. This study aims to analyze the complement component profile in the urine of MN patients using proteomic data obtained from liquid chromatography–tandem mass spectrometry (LC–MS/MS). Additionally, we will examine the relationship between urinary complement levels, clinicopathological features, and clinical remission in MN patients. The findings from this study could offer valuable insights for monitoring complement activation, evaluating complements in clinical prognosis, and developing complement-targeted therapies for MN patients.

Materials and methods

Participants and study design

A total of 248 participants who visited Sichuan Provincial People's Hospital from January 1, 2018 to December 31, 2023 were included in this study. We divided 248 participants into a discovery cohort and a validation cohort. The discovery cohort consisted of 80 patients with MN, 50 patients with IgA nephropathy (IgAN), and 68 healthy controls (HC) to explore the overall characterization of urinary proteins by LC–MS/MS detection of urinary proteomic data. The validation cohort included 80 patients with MN and was used to validate the association of urinary complement proteins with clinicopathologic features and clinical remission. A total of 248 urine samples from subjects with MN ($n = 130$), IgAN ($n = 50$), and HC ($n = 68$) were collected in tubes according to standard operating procedures. Inclusion criteria: (1) MN was diagnosed by renal biopsy. (2) Clinical and pathological data were accessible. (3) Urine specimens were collected in the early morning of renal biopsy according to standard procedures. (4) Patients agreed to use urine specimens and renal pathological specimens remaining for experiments. Exclusion criteria: (1) Secondary MN such as secondary to autoimmune disease, diabetes mellitus, tumors, infections, medications, etc. (2) Treatment with hormones, immunosuppressants, or biologics, etc. prior to the renal biopsy. (3) Age less than 18 years old. (4) Pregnancy. (5) Patient did not agree to participate in the study. The study adhered to the principles of the Declaration of Helsinki and obtained approval from the Medical Ethics Committee of Sichuan Provincial People's Hospital. The written informed consent was obtained from all participants.

The standard procedure for urine collection and mass spectrometric analysis

The standard procedure for urine collection is as follows: a 50 mL centrifuge tube is used to collect the clean midstream urine from the patient in the morning for renal biopsy, in a volume of approximately

30 mL. Care is taken to avoid contaminants such as feces, menstrual blood, vaginal secretions, and prostatic fluids from mixing with the specimen. After collection, the urine was rapidly centrifuged for 20 min (1,000 g, 4°C) to remove the sediment. After centrifugation, the supernatant urine was dispensed into 1.5 mL EP tubes and continued to the next step of the experiment or frozen at −80°C. The urinary protein digestion, mass spectrometric analysis, and spectral establishment used in this study were described in our previous report (23). Urinary peptide analysis was analyzed by LC-MS/MS and performed using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, United States) (23). The urine proteins were expressed by normalization of the abundance of one protein/all proteins in each patient.

The screening process and enrichment analysis of differential expression proteins

We define log2 (fold change) absolute values >1.5 and *p* values <0.05 (in MN vs. HC and MN vs. IgAN, respectively) to identify differential expression proteins (DEPs). Subsequently, all identified DEPs underwent Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses by using the DAVID database.¹ Then, the DEPs of complement components were further analyzed. And their correlations with clinicopathological features and clinical remission were investigated.

Clinical and pathological data collection

The baseline clinical parameters, laboratory data, and renal pathological parameters of all patients while receiving renal biopsy were collected. We also collect treatment regimens and follow-up data from all patients.

According to the 2012 KDIGO guidelines, complete remission, partial remission, and no remission are defined as follows: (1) Complete remission (CR): urinary protein excretion <0.3 g/d, accompanied by normalization of serum albumin concentration and normalization of serum creatinine. (2) Partial remission (PR): urinary protein excretion <3.5 g/d, 50% or more reduction from peak, accompanied by improvement or normalization of serum albumin concentration and stable serum creatinine. (3) No remission (NR): patients did not achieve clinical remission after treatment (24).

Definitions of the various pathologic stages of MN. Stage I: normal glomerular capillaries under light microscopy, and scattered or regularly distributed small deposits of immune complex-like electron-dense material in the region of the interstitial space between the glomerular basement membrane and epithelial cells under electron microscopy. Stage II: uneven thickening of glomerular capillaries to form “spikes” under light microscopy. Electron microscopy reveals the presence of electron dense material deposits under the epithelial cells. Stage III: light microscopy shows newly formed basement membrane-like material encircling the deposits, forming a reticular or chain-like appearance. Electron dense material

is deposited within the basement membrane or under the epithelium under electron microscopy. Stage IV: significant thickening of the basement membrane under light microscopy.

Validation by enzyme-linked immunosorbent assay

We selected 11 differential complement-related proteins for further validation assay in urine samples by using ELISA kits manufactured by Shanghai Zhuo Cai, Inc. The criteria for screening urinary complement proteins for validation are as follows: (1) There should be a significant correlation with clinical or pathological parameters observed in a discovery cohort of patients with MN; (2) The proteins could represent distinct complement activation pathways; (3) Previous studies have confirmed their important role in MN; or, (4) There are complement inhibitors/antibodies available for utility. The 11 complement-related proteins were C4a, C9, CD59, CFB, CFH, CFD, MASP2, VTN, CLU, serpincl1, and colecl2. All assays were conducted according to the manufacturer's instructions. The frozen samples were rapidly thawed at 37°C and promptly transferred to the ice to prevent complement activation before dilution. After dilution, the samples were promptly loaded into microanalytical wells. Each urine sample underwent only one freeze–thaw cycle before analysis. The concentration of urinary complement analyzed by ELISA was normalized to the urinary protein-to-creatinine ratio.

Immunohistochemistry and immunofluorescence in renal tissue

The kidney tissue embedded in paraffin was sliced into sections with a thickness of 2 μm. After deparaffinization and hydration, autoclaved antigen repair was performed. Endogenous peroxidase was blocked with 3% peroxide-methanol and incubated with goat serum working solution for 15 min at room temperature. For immunohistochemistry, primary anti-CD59 antibody at a dilution of 1:200 and primary anti-C5b-9 antibody at a dilution of 1:500 was added and incubated overnight at 4°C, followed by incubation with secondary antibodies at 37°C for 1 h. Diaminobenzidine was then added, ensuring that each sample was color developed for 3 min. Finally, the nuclei were stained with hematoxylin for 10 min. For immunofluorescence, primary anti-CD59 or anti-C5b-9 antibody diluted 1:100 was added first and incubated overnight at room temperature, followed by incubation with the corresponding fluorescein isothiocyanate (FITC)-coupled secondary antibodies for 1 h at 37°C, and finally staining of nuclei with DAPI for 5 min. Primary anti-CD59 antibody, goat anti-mouse IgG H&L (FITC), and goat anti-rabbit IgG H&L (TRITC) were obtained from Zenbio under item numbers 220,768, 511,101 and 511,202, respectively. Primary anti-C5b-9 antibody was obtained from Abcam under item number ab55811.

Statistical analysis

The correlations between urinary complement levels and other variables were analyzed by using correlation analysis with Spearman.

¹ <https://david.ncicrf.gov/>

TABLE 1 Baseline demographic, clinical, and pathological characteristics of patients with MN.

Characteristics	Discovery cohort (n = 50)	Validation cohort (n = 80)	p value
Gender (male, %)	25 (50%) ^a	51 (64%)	0.123
Age (year)	50.7 ± 13.0 ^b	51.5 (35.5, 59.0)	0.620
Hypertension (mmHg)	13 (26%)	39 (49%)	0.020
Serum creatinine (μmol/L)	65.30 (53.40, 75.30)	74.40 (61.60, 90.20)	0.007
eGFR (mL/min/1.73 m2)	100.04 ± 20.94	98.60 (82.04, 108.50)	0.055
Proteinuria (g/24 h)	3.97 (2.09, 7.71) ^c	5.29 (2.39, 8.04)	0.409
Serum albumin (g/L)	26.01 ± 6.80	26.19 ± 6.38	0.884
Serum C3 (g/L)	1.18 (0.97, 1.32)	1.06 (0.95, 1.19)	0.037
Serum C4 (g/L)	0.24 (0.20, 0.32)	0.29 ± 0.09	0.123
Basement membrane thickening			
Percentage of all patients	49 (98%)	77 (96%)	0.576
Monocyte infiltration			
Percentage of all patients	28 (56%)	55 (69%)	0.118
Per patient (%)	5 (0, 5)	5 (0, 5)	
Tubular atrophy/interstitial fibrosis			
Percentage of all patients	27 (54%)	52 (65%)	0.137
Per patient (%)	5 (0, 5)	5 (0, 5)	

MN, membranous nephropathy; eGFR, estimated glomerular filtration rate.

^aData are expressed as frequency and ratio.

^bData are expressed as means ± standard deviations.

^cData are expressed as medians and interquartile ranges (IQRs).

Student's *t*-test and non-parametric tests were used to assess differences between groups. Statistical significance was determined at a two-tailed *p* value (*p* < 0.05). All statistical analyses were conducted using SPSS Statistics version 26.0 (SPSS Inc., Chicago, IL, United States).

Results

Comparison of baseline characteristics of MN patients in the discovery and validation cohorts

There were 50 patients with MN in the discovery cohort and 80 patients in the validation cohort. There were no statistically significant differences in age, gender, 24 h proteinuria, eGFR, serum albumin, monocyte infiltration, and tubular atrophy/interstitial fibrosis (TIF) between the two cohorts. The validation cohort showed a notably higher serum creatinine level [74.40 (61.60, 90.20) μmol/L vs. 65.30 (53.40, 75.30) μmol/L, *p* = 0.007] and proportion of hypertension [39 (49%) vs. 13 (26%)] and lower serum C3 level [1.18 (0.97, 1.32) g/L vs. 1.06 (0.95, 1.19) g/L, *p* = 0.037] than the discovery cohort. However, there was no statistically significant difference in eGFR and renal C3 deposition levels between the two groups. Although the validation cohort had a significantly higher proportion of patients with hypertension than the discovery cohort, none of the hypertensive patients had hypertensive nephropathy (Table 1).

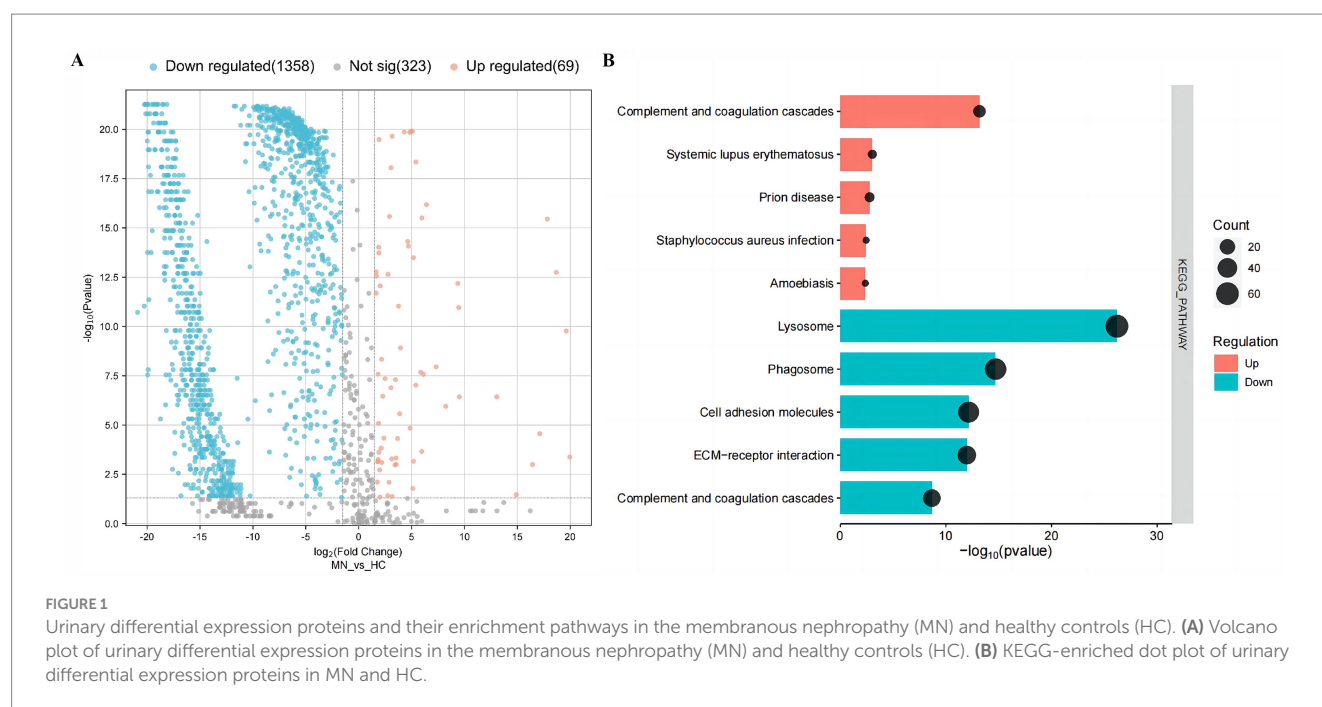
A total of 85 patients with MN were included in the follow-up study. This study comprised two groups: the discovery cohort, which

consisted of 24 patients, and the validation cohort, which included 61 patients. Statistical analysis revealed no significant differences in baseline eGFR, proteinuria, tubular atrophy/interstitial fibrosis, or lymphocytic infiltration between the two cohorts. However, the validation cohort exhibited a higher serum creatinine level (61.7 (46.55, 74.2) μmol/L vs. 74.05 (61.08, 87.80) μmol/L, *p* = 0.014) (Supplementary Table S1).

GO and KEGG analysis of urinary differential expression proteins

A total of 1750 proteins were identified in the urine of patients with MN, IgAN, and HC using LC-MS/MS. Notably, the abundance levels of 1,427 proteins exhibited significant differences between the MN and HC groups. Specifically, 69 proteins demonstrated elevated levels, whereas 1,358 proteins were found to have reduced levels (Figure 1).

To gain deep insights into the characteristics of urinary proteins in MN patients, the 1,427 DEPs were subjected to Gene Ontology (GO) analysis. In the biological process (BP) category, the DEPs were predominantly associated with mechanisms of cell adhesion and proteolysis. In the cellular component (CC) category, these proteins were primarily linked to the extracellular exosome and extracellular region. Furthermore, within the molecular function (MF) category, the proteins were chiefly associated with calcium ion binding and protein binding activities. Additionally, the KEGG enrichment analysis indicated that the up-regulated DEPs were predominantly associated with the complement and coagulation cascades. In contrast, the



down-regulated DEPs were primarily linked to lysosomal pathways, as well as the complement and coagulation cascades (Figure 1) (Supplementary Figure S1).

Correlation analysis of urinary complement proteins and both clinical and pathological features in MN patients

Based on the KEGG analysis, the results prompted a focused examination of complement-related proteins. Among the quantified proteins, we identified a total of 44 complement components. Table 2 presents the comparative analysis of urinary complement proteins across the groups of MN, IgAN, and HC. The results revealed 40 complement proteins with differential expression between the MN and HC groups, of which 16 were up-regulated and 24 down-regulated. Furthermore, a comparison between patients with MN and those with IgAN identified 14 complement proteins that exhibited significant differences in abundance ($p < 0.05$), with 1 protein being up-regulated and 13 down-regulated (Figure 2).

Then we conducted a comprehensive analysis to investigate the correlation between urinary complement proteins and a range of clinical and pathological parameters. Our results indicate a significant positive correlation between proteinuria and the levels of 21 urinary complement proteins. Notably, we observed strong associations with C4a ($r = 0.712$, $p < 0.05$), C3 ($r = 0.615$, $p < 0.05$), and C9 ($r = 0.685$, $p < 0.05$). In contrast, we identified a significant negative correlation between 24 h proteinuria and the abundance of six urinary complement proteins, including CD55 ($r = -0.543$, $p < 0.05$) and CD59 ($r = -0.451$, $p < 0.05$). We also observed a notable positive correlation between serum albumin levels and the abundance of four urinary complement proteins, specifically CD55 ($r = 0.465$, $p < 0.05$). Conversely,

we identified a significant negative correlation with the abundance of 18 urinary complement proteins, including C9 ($r = -0.520$, $p < 0.05$), C4a ($r = -0.503$, $p < 0.05$), and complement factor B (CFB) ($r = -0.493$, $p < 0.05$). Serum creatinine (Scr) demonstrated a significant positive correlation with nine urinary complement proteins, notably C4a ($r = 0.451$, $p < 0.05$) and complement factor H (CFH) ($r = 0.362$, $p < 0.05$) (Figure 3) (Supplementary Table S2).

Additionally, there was a positive correlation found between the pathologic stage and the abundance of 7 urinary complement-associated proteins, including CPN1 ($r = 0.419$, $p < 0.05$), CFD ($r = 0.345$, $p < 0.05$), and serpincl1 ($r = 0.358$, $p < 0.05$). Renal tubular atrophy/interstitial fibrosis (TIF) and monocyte infiltration were positively correlated with the abundance of colecl2 ($r = 0.384$, $p < 0.05$; $r = 0.373$, $p < 0.05$) and C1s ($r = 0.292$, $p < 0.05$; $r = 0.289$, $p < 0.05$) (Figure 3) (Supplementary Table S2). These findings underscore the potential role of urinary complement proteins as biomarkers for assessing proteinuria and related renal pathologies.

Analysis of differences in urinary complement activation at different stages of progression and levels of damage

We also compared the differences in complement activation between stage II and stage I of MN. Several urinary complements were higher in patients with stage II MN than in stage I, including CFD, CLU, C6, and C4a (Supplementary Table S3). Urinary MASP2 and COLEC12 were higher in patients with tubular atrophy/interstitial fibrosis than in those without (Supplementary Table S4). Urinary CD55 was significantly higher in patients without renal tissue C3 deposition than in those with deposition (Supplementary Table S5).

TABLE 2 Comparisons of urinary complement proteins between MN patients and HC or IgAN patients, respectively.

Urine complements	<i>p</i> value	<i>p</i> value	Urine complements	<i>p</i> value	<i>p</i> value
	MN vs. HC	MN vs. IgAN		MN vs. HC	MN vs. IgAN
COLEC12	2.90×10^{-20}	0.002	CPN1	2.67×10^{-6}	0.785
C1R	0.001	0.992	CPN2	1.03×10^{-17}	2.84×10^{-4}
C1S	3.26×10^{-13}	0.564	CLU	2.86×10^{-16}	0.001
C2	0.008	0.066	VTN	8.55×10^{-11}	0.123
C4A	0.038	0.053	CFHR1	1.26×10^{-16}	0.529
C4B	7.53×10^{-8}	6.59×10^{-5}	CFHR2	1.86×10^{-5}	0.326
MASP2	9.79×10^{-17}	0.148	CFHR3	0.157	0.322
CFD	4.98×10^{-5}	0.661	C3AR1	7.00×10^{-3}	0.322
CFB	0.006	0.039	SERPINF1	0.001	0.053
CFI	9.98×10^{-16}	0.004	SERPINC1	2.62×10^{-16}	0.85
CFH	1.48×10^{-4}	0.058	SERPINA1	1.23×10^{-20}	1.80×10^{-5}
C3	4.79×10^{-5}	0.041	SERPINA3	$9.49\text{E} \times 10^{-15}$	0.944
C5	0.877	0.827	SERPINA5	8.05×10^{-21}	0.001
C6	2.27×10^{-10}	0.488	SERPING1	8.27×10^{-20}	1.61×10^{-8}
C7	7.90×10^{-19}	0.156	SERPINB3	2.61×10^{-16}	0.175
C8A	0.001	0.416	SERPINA4	8.67×10^{-18}	0.085
C8B	2.76×10^{-5}	0.303	SERPINF2	2.31×10^{-12}	0.03
C8G	0.591	0.069	SERPINA6	8.72×10^{-13}	0.015
C9	0.001	0.833	SERPINA7	0.941	0.933
CD55	3.08×10^{-20}	0.006	SERPIND1	4.41×10^{-10}	0.067
CD59	1.49×10^{-12}	0.008	SERPINB6	1.04×10^{-4}	0.322
CD93	2.15×10^{-18}	0.159	SERPINB5	9.56×10^{-8}	0.159

MN, membranous nephropathy; IgAN, immunoglobulin A nephropathy; HC, health controls.

Analysis of urinary complement proteins and clinical remission of MN

In the discovery cohort, a total of 24 out of 50 patients with MN were followed up. Among these, 19 cases (79.2%) received immunosuppressive therapy (combination of steroids and cyclophosphamide; and calcineurin inhibitors, either alone or in conjunction with steroids). Meanwhile, 5 cases were managed with non-immunosuppressive supportive treatment [angiotensin-converting enzyme inhibitors (ACEI) or angiotensin II receptor blockers (ARB)].

We conducted an analysis of the relationship between clinical remission and urinary complements in patients undergoing treatment with immunosuppressants. After 3 months of immunosuppressive therapy, 10 patients (52.6%) achieved clinical remission, while 8 patients (42.1%) did not reach remission, and 1 patient (5.3%) was lost to follow-up before reaching remission. The ratio of CD55/total protein ($1.10 \times 10^{-4} \pm 1.12 \times 10^{-4}$ vs. 0, $p = 0.006$) and CD59/total protein [5.27×10^{-4} (4.43×10^{-4} , 8.09×10^{-4}) vs. 2.05×10^{-4} (2.63×10^{-5} , 4.72×10^{-4}), $p = 0.012$] were significantly higher in the group with clinical remission than in those without remission. While there were no statistically significant differences in the other complement components between the clinical remission and non-remission groups (Table 3).

After 6 months of immunosuppressive therapy, 16 (84.2%) achieved clinical remission, 2 (10.5%) did not reach remission, and 1 (5.3%) was lost to follow-up. Only the ratio of CD59/all protein [5.07×10^{-4} (3.13×10^{-4} , 6.31×10^{-4}) vs. 0, $p = 0.013$] were significantly higher in the group with clinical remission than in the group with no remission (Table 3).

Correlation between urinary complement proteins and clinical and pathologic parameters in the validation cohort of MN patients

A total of 11 complement proteins, including Colec12, MASP2, CFB, CFH, CFD, C4A, C9, VTN, CLU, and Serpinc1, were further validated in a cohort of 80 patients with MN using ELISA. Our results revealed that most urine complement to urine creatinine ratios exhibited a negative correlation with serum uric acid levels, which aligns with the findings from the discovery cohort. This may be due to the fact that the complement and coagulation cascade pathways are major functional pathways involved in the development of gout in hyperuricemic patients. Additionally, we observed no significant correlation between urinary complement proteins and the levels of anti-phospholipase A2 receptor antibody IgG (anti-PLA2R-IgG). But

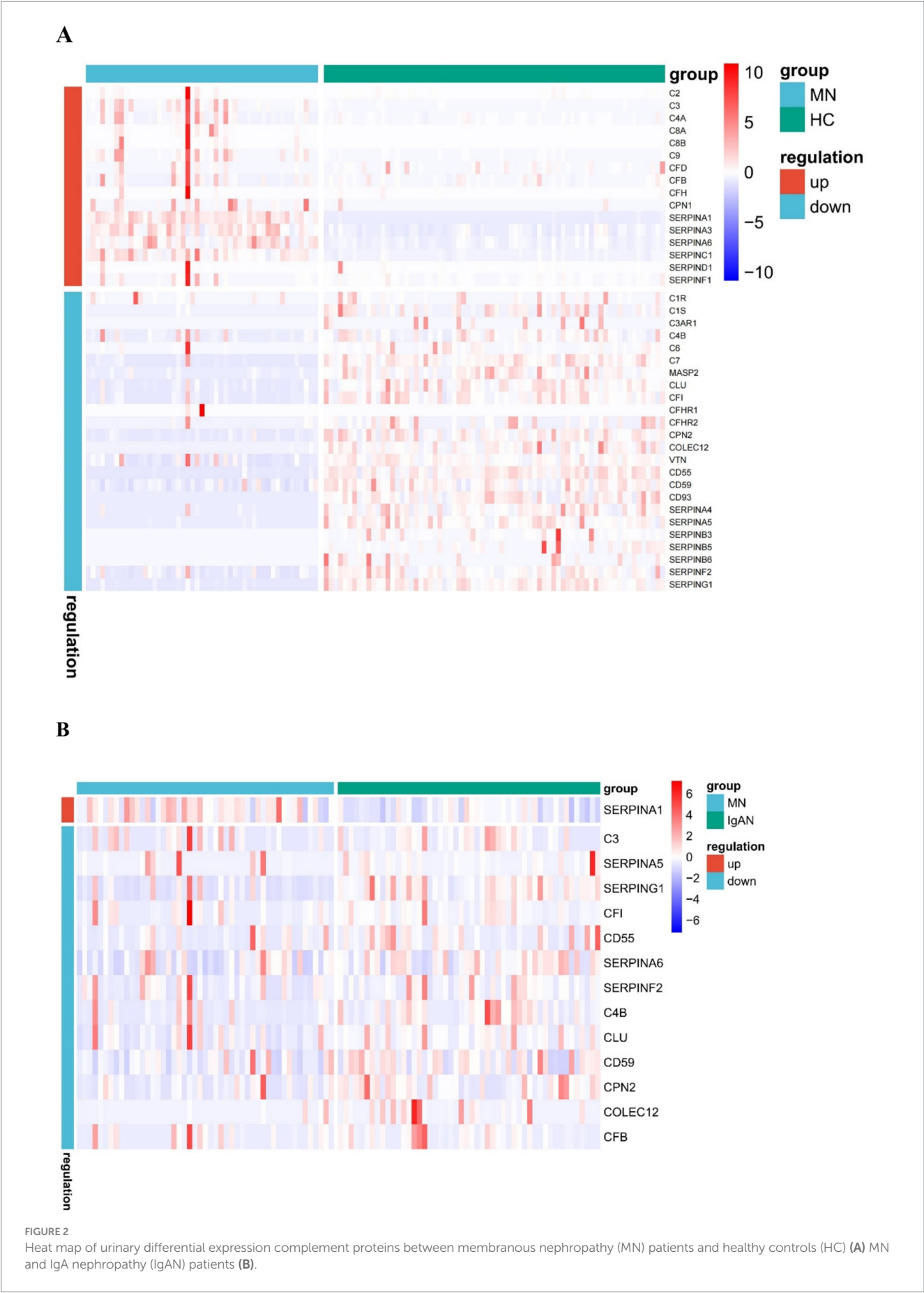


TABLE 3 Comparisons of urinary complement protein levels and clinical parameters between the group with no remission and the group with clinical remission in the discovery cohorts after immunosuppressive therapy.

Characteristics	In 3 months of therapy	No remission (<i>n</i> = 8)	<i>p</i> value	In 6 months of therapy	No remission (<i>n</i> = 2)	<i>p</i> value
	Clinical remission (<i>n</i> = 10)			Clinical remission (<i>n</i> = 16)		
Age, years	48 ± 11 ^a	53 ± 17	0.501	49 ± 14	58 ± 8	0.402
Serum creatinine (μmol/L)	65.07 ± 18.06	67.40 (48.23, 75.55)	0.829	61.70 (47.88, 76.30)	72.90 ± 4.43	0.47
eGFR (mL/min/1.73 m ²)	107.11 (99.77, 115.23) ^b	96.49 ± 22.37	0.203	104.46 (94.95, 113.35)	97.46 ± 8.21	0.549
Proteinuria (g/24 h)	3.73 (2.04, 6.38)	7.07 ± 5.58	0.408	4.18 (2.26, 7.68)	11.39 ± 11.07	0.392
Serum albumin (g/L)	25.5 ± 5.77	25.15 ± 8.09	0.916	25.16 ± 6.21	26.85 ± 13.22	0.746
CD55/all protein	1.10 × 10 ⁻⁴ ± 1.12 × 10 ⁻⁴	0 (0, 0)	0.006	2.50 × 10 ⁻⁴ (1.12 × 10 ⁻⁶ , 1.51 × 10 ⁻⁴)	0 ± 0	0.118
CD59/all protein	5.27 × 10 ⁻⁴ (4.43 × 10 ⁻⁴ , 8.09 × 10 ⁻⁴)	2.05 × 10 ⁻⁴ (2.63 × 10 ⁻⁵ , 4.72 × 10 ⁻⁴)	0.012	5.07 × 10 ⁻⁴ (3.13 × 10 ⁻⁴ , 6.31 × 10 ⁻⁴)	0 ± 0	0.013
C3/all protein	8.52 × 10 ⁻⁴ (2.06 × 10 ⁻⁴ , 2.29 × 10 ⁻³)	4.56 × 10 ⁻³ (3.62 × 10 ⁻⁴ , 1.55 × 10 ⁻²)	0.173	2.40 × 10 ⁻⁴ (0, 6.51 × 10 ⁻⁴)	1.22 × 10 ⁻² ± 5.38 × 10 ⁻³	0.078
C9/all protein	1.38 × 10 ⁻⁴ (5.74 × 10 ⁻⁵ , 2.50 × 10 ⁻⁴)	5.37 × 10 ⁻⁴ (2.13 × 10 ⁻⁵ , 1.58 × 10 ⁻³)	0.46	6.29 × 10 ⁻⁵ (0, 1.48 × 10 ⁻⁴)	3.55 × 10 ⁻⁴ ± 5.02 × 10 ⁻⁴	0.732
CFB/all protein	6.37 × 10 ⁻⁵ (1.28 × 10 ⁻⁵ , 2.76 × 10 ⁻⁴)	3.58 × 10 ⁻⁴ (9.14 × 10 ⁻⁶ , 1.05 × 10 ⁻⁴)	0.633	1.23 × 10 ⁻⁵ (0, 6.54 × 10 ⁻⁵)	1.39 × 10 ⁻³ ± 1.96 × 10 ⁻³	1
CFH/all protein	1.77 × 10 ⁻⁶ (0, 1.73 × 10 ⁻⁵)	0 (0, 4.43 × 10 ⁻⁵)	0.897	0 (0, 2.71 × 10 ⁻⁵)	3.40 × 10 ⁻⁶ ± 4.81 × 10 ⁻⁶	0.941
CLU/all protein	4.23 × 10 ⁻⁴ (1.70 × 10 ⁻⁴ , 6.27 × 10 ⁻⁴)	6.84 × 10 ⁻⁴ (2.03 × 10 ⁻⁴ , 1.46 × 10 ⁻³)	0.408	2.03 × 10 ⁻⁴ (0, 4.42 × 10 ⁻⁴)	2.29 × 10 ⁻³ ± 3.24 × 10 ⁻³	1
MASP2/all protein	1.72 × 10 ⁻⁴ (4.77 × 10 ⁻⁵ , 5.89 × 10 ⁻⁴)	1.26 × 10 ⁻⁴ (4.62 × 10 ⁻⁶ , 9.64 × 10 ⁻⁴)	0.633	1.39 × 10 ⁻⁴ (4.38 × 10 ⁻⁵ , 5.16 × 10 ⁻⁴)	6.25 × 10 ⁻⁴ ± 8.58 × 10 ⁻⁴	0.941

^aData are expressed as means ± standard deviations.

^bData are expressed as medians and interquartile ranges (IQRs).

correlations with their clinicopathological characteristics. These findings suggest that the activation of the complement system may play a crucial role in the pathophysiological processes associated with MN.

The complement system, first identified by Jules Bordet in 1901, is regarded as one of the oldest components of the immune system (25). The system comprises nearly 60 proteins, including those involved in the classical, lectin, and alternative pathways, along with multiple regulatory factors and receptors (26, 27). Complement activation has long been recognized as a key characteristic of MN, primarily occurring through the lectin and alternative pathways, but recent studies, however, have emphasized the classical pathway as an important contributor to its progression (28). Our results indicate that urinary complement proteins of abnormal abundance are involved in the three pathways of complement activation, with significant enrichment in the alternative and terminal pathways.

According to previous researches, serum CFH and CFB are suggested as potential indicators of disease progression (15, 16). The factor B inhibitor LNP023 and the factor D inhibitor BCX9930 are currently in phase II clinical trials for MN. Our research reveals elevated levels of CFB and CFH in urine, with both urine CFB and urine CFH showing a positive correlation with proteinuria and Scr, and negatively correlated with serum albumin. These findings further highlight the significance of CFB and CFH in chronic kidney injury and suggest their potential as biomarkers for chronic damage. Previous studies have found that the intensity of glomerular C3 staining correlates with proteinuria (4, 5) and is a predictor of renal failure in patients with MN (6). Other studies have also demonstrated an association between glomerular C5b-9 and renal dysfunction in MN (7). APL-2, a C3 inhibitor, is currently undergoing phase II clinical trials for the treatment of MN. Our findings revealed significantly elevated levels of urine C3 and C9 in MN patients compared to healthy controls, and these levels exhibited a positive

TABLE 4 Correlation analysis of 11 urinary complement proteins with clinical and pathological parameters in patients with MN.

Characteristics	CLU/Cr	CFB/Cr	C9/Cr	MASP2/Cr	C4a/Cr	colec12/Cr	VTN/Cr	Serpinc1/Cr	CFD/Cr	CFH/Cr	CD59/Cr
Serum creatinine (μmol/l)	$r = -0.167$	$r = -0.314^*$	$r = -0.191$	$r = -0.245^*$	$r = -0.211$	$r = -0.22$	$r = -0.204$	$r = -0.185$	$r = -0.239^*$	$r = -0.249^*$	$r = -0.211$
Serum uric acid (mmol/l)	$r = -0.375^*$	$r = -0.305^*$	$r = -0.324^*$	$r = -0.297^*$	$r = -0.334^*$	$r = -0.328^*$	$r = -0.411^*$	$r = -0.282^*$	$r = -0.299^*$	$r = -0.307^*$	$r = -0.278^*$
Serum Albumin (g/L)	$r = 0.014$	$r = 0.108$	$r = 0.042$	$r = 0.024$	$r = -0.022$	$r = -0.022$	$r = -0.002$	$r = -0.122$	$r = 0.008$	$r = -0.017$	$r = -0.042$
Serum C3 (g/L)	$r = 0.021$	$r = -0.054$	$r = 0.034$	$r = 0.014$	$r = -0.047$	$r = -0.008$	$r = 0.013$	$r = 0.052$	$r = 0.047$	$r = 0.108$	$r = 0.018$
Proteinuria (g/24 h)	$r = -0.152$	$r = -0.248^*$	$r = -0.156$	$r = -0.139$	$r = -0.122$	$r = -0.129$	$r = -0.166$	$r = -0.073$	$r = -0.157$	$r = -0.117$	$r = -0.08$
anti-PLA2R-IgG (RU/ml)	$r = 0.086$	$r = -0.247$	$r = 0.096$	$r = -0.021$	$r = 0.093$	$r = 0.13$	$r = 0.049$	$r = -0.008$	$r = 0.066$	$r = 0.053$	$r = 0.012$
TIF (%)	$r = 0.022$	$r = -0.04$	$r = -0.003$	$r = -0.016$	$r = -0.021$	$r = -0.016$	$r = 0.026$	$r = 0.063$	$r = 0.001$	$r = -0.01$	$r = -0.01$
Monocyte infiltration (%)	$r = 0.16$	$r = 0.062$	$r = 0.151$	$r = 0.079$	$r = 0.067$	$r = 0.127$	$r = 0.188$	$r = 0.222$	$r = 0.093$	$r = 0.09$	$r = 0.13$
Pathological staging	$r = 0.005$	$r = -0.026$	$r = 0.012$	$r = -0.044$	$r = -0.072$	$r = 0.004$	$r = -0.027$	$r = -0.059$	$r = -0.034$	$r = -0.106$	$r = -0.041$

TIF, tubular atrophy/interstitial fibrosis; MN, membranous nephropathy; anti-PLA2R-IgG, anti-phospholipase A2 receptor antibody IgG; * $p < 0.05$.

correlation with proteinuria, consistent with previous studies (20–22). It suggests that end-pathway activation plays a critical role in MN. Additionally, a correlation of C1s with TIF and monocyte infiltration was observed, further supporting the potential involvement of the classical pathway in the pathogenesis of MN. However, the initiation pathways and pathogenic relevance of complement activation in MN are not fully understood. In the future, we will explore the pathways of complement activation to gain a more comprehensive understanding of complement activation in MN.

We compared glomerular complements of MN patients detected by MS/MS (11, 12) with urine complements in MN detected by LC-MS/MS. Of the twenty-nine complement proteins detected in glomeruli, 21 (72.4%) were also detectable in urine. C3 was the complement with the highest spectral number in both glomeruli and urine. Glomerular C3 deposition leads to progression of MN to renal failure (6). Urine C3 correlates positively with proteinuria and cystatin C and negatively with serum albumin. CFHR2, CFHR3, and C1S, which are less abundant in glomeruli, are also less abundant in urine. This suggests that urine complements may be a better response to complement activation in glomeruli.

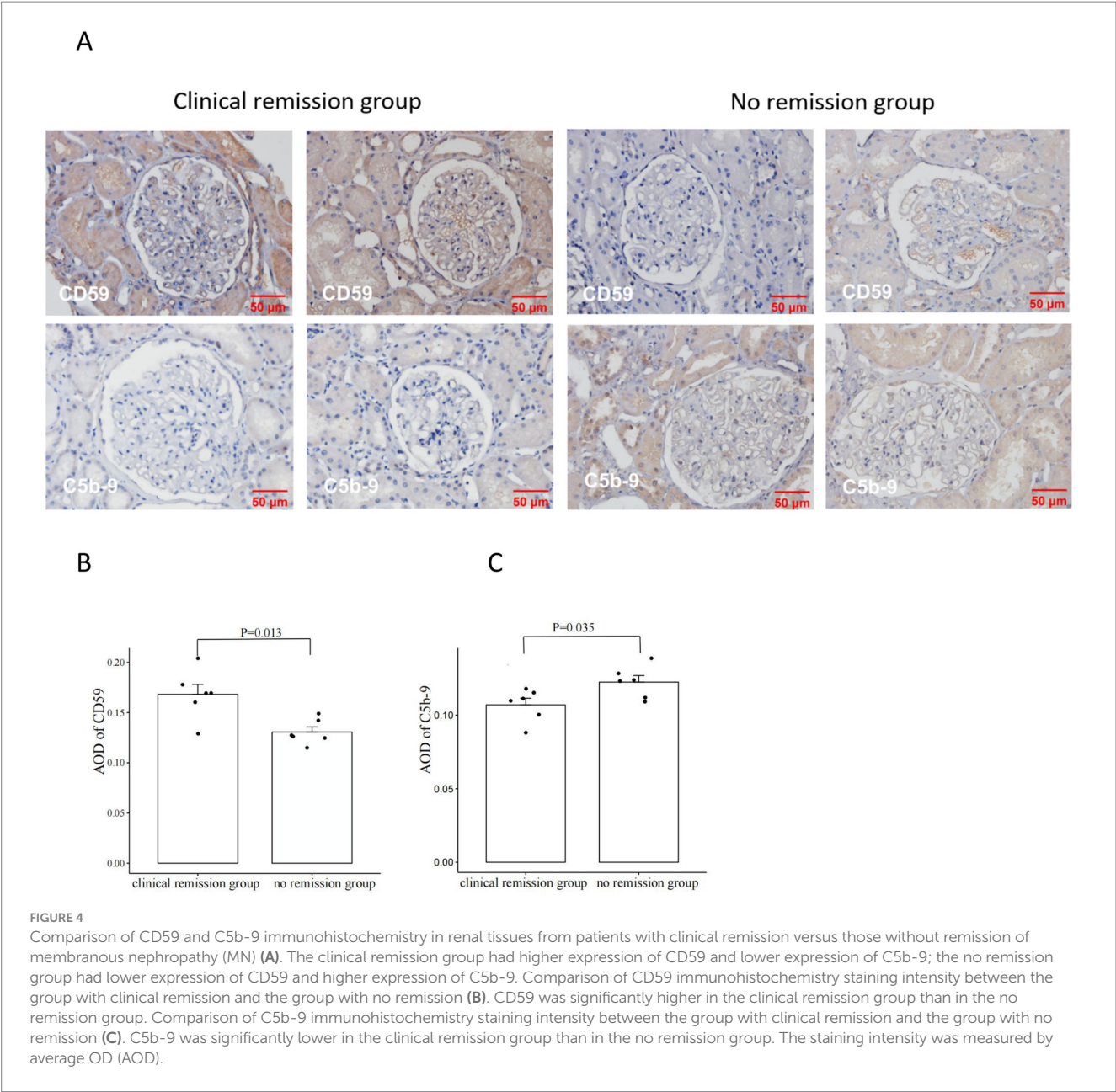
In our previous works, we concentrated on examining the factors associated with the progression and clinical remission of MN (29–31). Our investigation revealed that factors such as anti-PLA2R-IgG titer, inflammatory cell infiltration, and C3 deposition on immunofluorescence were correlated with an elevated risk of treatment failure in patients with PMN (32). In this study, we included measurements of urine complements and found that in the discovery cohort, the ratio of CD59/all protein was significantly higher in the group with clinical remission than in the group with no remission at months 3 and 6 of treatment. Showing the same in the validation cohort, the CD59/Cr was significantly higher in the group with clinical remission than in the group with no remission at month 6 of treatment. However, at month 3 of treatment, although the CD59/Cr was higher in the group with clinical remission than in the group with no remission, the difference was not statistically significant. It may be due to the fact that the validation cohort had higher baseline proteinuria and poorer renal function compared to the discovery cohort, and therefore required prolonged treatment to achieve clinical remission. Thus, we found a strong association between CD59 and clinical remission.

The process of complement activation ultimately leads to the formation of membrane attack complexes (MAC), resulting in cellular destruction. CD59, also known as homology restriction factor-20 (HRF-20), serves as the sole membrane-bound inhibitor that impedes the assembly of MAC and is present in various renal cell types, including glomerular endothelium, tubular epithelium, thylakoid cells, and podocytes (33, 34). Previous animal studies have demonstrated the significant protective effects of CD59 on the kidneys (35–38). Targeting CD59 for treating tubulointerstitial injury in rats has shown promising results (39). It has been observed that CD59 expression in the glomeruli of patients with MN is decreased compared to HC, and urine CD59 excretion is increased in comparison with HC (40). Our study revealed that urine CD59 was significantly lower in MN patients compared to both HC and IgAN patients. Additionally, CD59 exhibited a negative correlation with proteinuria and was associated with the clinical remission of MN patients. The group with clinical remission exhibited elevated levels of CD59 in both renal tissue

TABLE 5 Comparisons of urinary complement protein levels and clinical parameters between the group with no remission and the group with clinical remission in the validation cohorts after immunosuppressive therapy.

Characteristics	In 3 months of therapy	No remission (<i>n</i> = 29)	<i>p</i> value	In 6 months of therapy	No remission (<i>n</i> = 13)	<i>p</i> value
	Clinical remission (<i>n</i> = 20)			Clinical remission (<i>n</i> = 33)		
Age, years	52 ± 13 ^a	47 ± 17	0.366	53 (48, 62)	45 ± 17	0.143
Serum creatinine (μmol/L)	75.85 ± 19.62	76.79 ± 23.00	0.882	74.99 ± 21.63	83.44 ± 18.81	0.238
eGFR (mL/min/1.73 m2)	93.25 ± 18.05	98.84 (75.04, 115.26)	0.771	2.04 ± 19.74	93.06 ± 23.01	0.885
Proteinuria (g/24 h)	4.37 (2.65, 7.94) ^b	5.29 (2.16, 8.37)	0.07	4.14 (2.39, 7.00)	8.44 ± 5.37	0.07
Serum albumin (g/L)	26.02 ± 5.84	24.20 (19.50, 27.10)	0.207	26.50 ± 5.67	24.00 (18.68, 27.00)	0.207
CD59/Cr (ng/mg)	114.34 (56.20, 270.76)	101.51 (52.70, 276.30)	0.984	147.73 (84.45, 300.18)	63.45 (499.18, 136.95)	0.021

^aData are expressed as means ± standard deviations.
^bData are expressed as medians and interquartile ranges (IQRs).



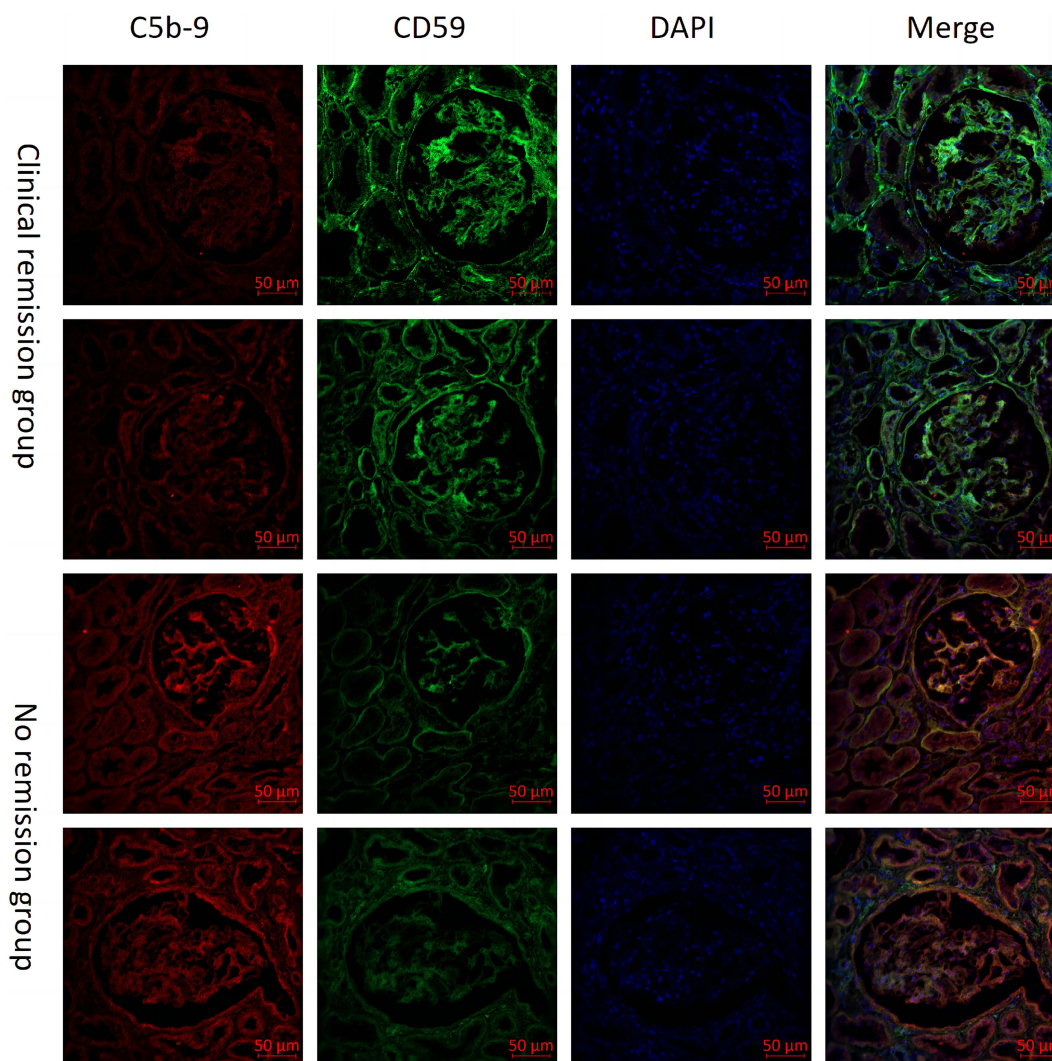


FIGURE 5

Comparison of CD59 and C5b-9 immunofluorescence immunohistochemistry in renal tissues from patients with clinical remission versus those without remission of membranous nephropathy (MN).

and urine, whereas patients in the group with no remission exhibited the opposite pattern. Furthermore, patients in the group with no remission had lower renal tissue CD59 and higher C5b-9 compared to patients in the group with clinical remission. Immunofluorescence co-localization indicated that CD59 and C5b-9 were co-localized in glomeruli and tubules. We hypothesized that MN patients are more susceptible to renal attack due to the deficiency of CD59 in renal tissues, which diminishes its inhibition of C5b-9. We will focus on the precise mechanisms by which these complement proteins influence disease progression and remission in MN and explore their utility in clinical practice.

The activated components in the complement system vary at different stages of progression and degrees of damage in MN. We found that urinary complement activation levels in stage II of MN were generally higher than those in stage I. There were several differentially expressed urinary complements such as CFD, CLU, C6, and C4a, which were derived from the alternative

pathway, the lectin pathway, and the common pathway, respectively. Urinary MASP2, and COLEC12 were higher in patients with tubular atrophy/interstitial fibrosis than in those without. Urinary CD55 was significantly higher in patients without renal tissue C3 deposition than in those with deposition. CD55 acts as a complement inhibitory factor, inhibiting the formation and activity of C3 converting enzyme and C5 converting enzyme, and preventing overactivation of the complement system. From this, we hypothesized that high CD55 expression has a stronger inhibitory effect on the complement system, resulting in less renal C3 deposition. In summary, urinary complement activation levels vary at different stages of progression and degree of injury in MN. Whether the degree of injury and progression of MN can be inferred by monitoring urinary complement levels requires more in-depth study. We will take it into consideration seriously in the future.

Our study uses targeted mass spectrometry for comprehensive quantification of urinary complement proteins. It demonstrates

the profile of urinary complement proteins in patients with MN. The relationship between urinary complement proteins and clinicopathological features and clinical remission was analyzed. However, this study has important limitations. We did not monitor the dynamics of complement changes in MN patients after different drug treatments. Longitudinal studies are needed to monitor complement activation over longer periods of time and could provide additional insights into how chronic complement activation leads to long-term kidney injury and whether it predicts relapse or remission. We did not analyze renal PLA2R and IgG1-4 expression in relation to urinary complement characteristics. The relationship between complement activation and antigenic and IgG subtypes in renal tissue needs to be explored further. Urine specimens were not collected at exactly the same time. The impact of timing on urine protein and complement activation could be explored further. In addition, the origin of the complement proteins detected in urine is unknown. We will focus on identifying the source of these proteins, whether they are secreted by the kidneys, or are being filtered from the bloodstream due to glomerular damage.

In summary, the study showed significant abnormalities in urine complement abundance in patients with MN. The urine levels of complement components exhibited a significant correlation with clinical parameters and the clinical remission of MN patients. Consequently, these components hold potential as biomarkers for predicting MN progression and clinical remission. Our study implies for the use of complement-targeted therapy in MN.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository and accession number(s) can be found at <http://www.proteomexchange.org/> [PXD060494].

Ethics statement

The studies involving humans were approved by Medical Ethics Committee of Sichuan Provincial People's Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

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Author contributions

YX: Validation, Writing – original draft. YL: Writing – review & editing. YZ: Writing – review & editing. GL: Project administration, Supervision, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmed.2025.1515928/full#supplementary-material>

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Roles of MASP-1 and MASP-3 in the development of retinal degeneration in a murine model of dry age-related macular degeneration

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Complement is activated through the three different pathways, which are the classical (CP), lectin (LP), and alternative pathways (AP). Complement activation functions to eliminate invading pathogens, whereas dysregulation of complement activation can induce inflammatory disorders such as age-related macular degeneration (AMD). In retinal degeneration induced by sodium iodate (NaIO₃), a murine model of dry AMD (also called atrophic AMD), it has been suggested that the AP and CP are involved in the disease development. On the other hand, the role of the LP in the development of AMD remains unclear. In the current study, we generated murine dry AMD model with NaIO₃ using mice deficient for mannose-binding lectin-associated serine protease (MASP)-1 and/or MASP-3, which are required for the LP and AP activation, respectively. Wild-type (WT) C57BL/6J mice showed retinal degeneration, including depigmentation and disruption of the retinal pigment epithelium (RPE), atrophy of the photoreceptor layer (PL), and thinning of the outer nuclear layer (ONL) after NaIO₃ injection. In contrast, those pathological changes after NaIO₃ injection were significantly attenuated in MASP-1-deficient (MASP-1^{-/-}), MASP-3-deficient (MASP-3^{-/-}), and MASP-1/3-double deficient (MASP-1/3^{-/-}) mice. These results indicate that both MASP-1 and MASP-3 play a role in photoreceptor degeneration in the NaIO₃-induced murine dry AMD model. In addition, photoreceptor cell death and retinal C3 activation were observed in NaIO₃-injected WT mice, whereas those pathological changes were significantly attenuated in NaIO₃-injected MASP-3^{-/-} and MASP-1/3^{-/-} mice. On the other hand, those pathological changes in NaIO₃-injected MASP-1^{-/-} mice were comparable to those in NaIO₃-injected WT mice. Taken together, our results indicate that MASP-3 plays a pivotal role in C3 activation in the retina most likely via activation of the AP leading to the development of retinal degeneration in the NaIO₃-induced murine dry AMD model. Our results also indicate that MASP-1 plays a role in the development of NaIO₃-induced retinal degeneration in this murine model, although it remains unclear whether its role in the retinal degeneration is through the LP activation.

KEYWORDS

complement, lectin pathway, alternative pathway, MASP-1, MASP-3, age-related macular degeneration

1 Introduction

The complement system is composed of more than 30 proteins and functions in eliminating invading pathogens and apoptotic cells, thereby maintaining host defense and homeostasis (1, 2). Complement activation initiates through three different pathways: the classical (CP), the lectin (LP), and the alternative pathways (AP). The AP is activated at low levels by spontaneous hydrolysis of C3 to C3(H₂O). Factor B (FB) associates with C3(H₂O) and is cleaved by an active form factor D (FD), which is produced by mannose-binding lectin-associated serine protease (MASP)-3, to form a fluid-phase C3 convertase C3(H₂O)Bb (3, 4). C3(H₂O)Bb then cleaves C3 into C3a and C3b. C3a is an anaphylatoxin, while C3b binds to nearby surface of pathogens and associates with FB, resulting in FB cleavage by active FD followed by generation of surface-bound (i.e., solid-phase) C3 convertase C3bBb. Thus, at C3b-binding sites, C3 is successively activated via the amplification loop. Activation of the LP and CP is initiated by binding of the pathway-specific pattern-recognition molecules (PRMs) to their targets. Activation of the LP is initiated by binding of the LP PRMs, such as mannose-binding lectin (MBL), ficolins (ficolin-1 (M-ficolin), ficolin-2 (L-ficolin), and ficolin-3 (H-ficolin)), collectin-10 (CL-10 or CL-L1), collectin-11 (CL-11 or CL-K1) and a heteromeric complex of CL-10 and CL-11 (CL-LK), to the surface of pathogens or to apoptotic/necrotic cells (5). Following this binding, MASP-1, complexed with the LP PRMs, is self-activated, and then MASP-2, also complexed with the LP PRMs, is activated by active MASP-1. MASP-2 cleaves both C4 and C2 to form the C3 convertase C4b2b, whereas MASP-1 cleaves only C2. Activation of the CP is initiated by binding of C1q, the CP PRM, to antigen-antibody complexes. Following this binding, serine protease C1r, complexed with C1q, is self-activated, and then C1s, also complexed with C1q, is activated by active C1r. Similarly to MASP-2 in the LP, C1s cleaves C4 and C2 to form the C3 convertase C4b2b. In this way, all three pathways converge at C3 activation, connect to C5 activation and lead to formation of membrane attack complex (C5b-9), causing membrane destabilization.

On the other hand, on the host cells, complement activation is tightly regulated by regulatory proteins such as decay-accelerating factor (DAF or CD55), membrane cofactor protein (MCP or CD46), and factor H (FH), a soluble inhibitor of the AP C3 convertases (1, 2). FH binds to C3b and acts as a cofactor for factor I (FI), which cleaves surface-bound C3b into inactive iC3b that cannot form the AP C3 convertase C3bBb. Dysfunction of complement regulatory proteins, due to aging or gene mutations, leads to dysregulation of complement activation, which is associated with many inflammatory diseases, including age-related macular degeneration (AMD) (6).

AMD is one of the causes of visual impairment in the elderly people. In the early stage of AMD, drusen, which are extracellular deposits of waste product, are observed under the retinal pigment epithelium (RPE), and in the later stage, it progresses to neovascular AMD (also called exudative or wet AMD) with choroidal neovascularization (CNV) or dry AMD (also called atrophic AMD) without CNV. Dry AMD is characterized by loss of RPE, photoreceptors, and choriocapillaris in the macular. Although the

pathological mechanism of AMD is not yet fully understood, oxidative stress and persistent inflammation play important role in the development of AMD (7). To date, there is accumulating evidence that the complement system plays a significant role in the pathogenesis of AMD. The association between the Y402H gene polymorphism of FH, a major soluble regulator of the AP, and AMD has been reported (8). This polymorphism has been shown to prevent FH from removing cellular debris, resulting in overwhelming complement activation (9–11). Deposition of C3 and FB on the drusen of AMD patients has been reported (12). These reports suggest that activation of the AP is involved in the development of AMD. On the other hand, Murinello et al. observed deposition of IgG and C1q within the choriocapillaris of AMD patients and deposition of C5b-9 within the drusen and around choriocapillaris, suggesting that the CP is also implicated in the development of AMD (13).

It has also been suggested that the AP and CP are involved in the development of dry AMD-like disease in mice induced by intravenous injection of the oxidizing agent sodium iodate (NaIO₃). In this model, RPE cells are damaged first, followed by damage to photoreceptor cells maintained by RPE cells, and C1q, C3 and C4 deposition was observed on the damaged photoreceptor outer segments (14). Furthermore, administration of anti-FD antibody into C4-deficient mice protected against NaIO₃-induced retinal degeneration, suggesting that combined blockade of the CP/LP and AP is required to optimally prevent dry AMD-like disease in mice.

The involvement of the LP in the development of AMD remains largely unclear. In the isolated samples from human donor eyes including RPE, choroid, and neural retina, gene expression levels of LP components were very low as determined by real-time quantitative polymerase chain reaction (RT-qPCR) (15, 16). However, compared to normal donors, increased gene expression levels of ficolin-1 (*FCN1*) in the macular retina of late AMD donors and ficolin-3 (*FCN3*) in the choroidal endothelial cells of early AMD donors have been reported (16). CL-11 was also detected in human retina by RT-PCR (17) and in the neural retina and RPE cells of postmortem human eyes by immunohistochemical staining (18).

We recently reported the levels of C3 and C4 and their activation products C3a and C4a in the aqueous humor of patients with neovascular AMD or cataract (controls) (19). We found that the aqueous humor levels of C3 and C4 were significantly lower in the neovascular AMD eyes compared to the controls. In contrast, the aqueous humor levels of C3a and C4a, as well as the C3a/C3 and C4a/C4 ratios, were significantly higher in the neovascular AMD eyes compared to the controls. We also found that the aqueous humor levels of MASP-2 were significantly lower in the neovascular AMD eyes compared to the controls, possibly due to its consumption. These results suggest that the CP and/or LP are implicated in the development of AMD.

MASP-1 and MASP-3 are splice variants transcribed from the common *Masp1* gene and have identical heavy chains but distinct serine protease domains. We recently generated mice mono-specifically deficient for MASP-1 or MASP-3 by genome editing

of the *Masp1* gene using the CRISPR/Cas9 system (4). We found that sera of MASP-1-deficient mice lacked LP activity, whereas sera of MASP-3-deficient mice lacked AP activity. Thus, MASP-1 and MASP-3 are essential complement factors for the physiological activation of the LP and AP, respectively. To clarify whether the LP is involved in the pathogenesis of dry AMD and further investigate the involvement of the AP, we performed histopathological analysis of the retina of a NaIO₃-induced murine dry AMD model lacking MASP-1 and/or MASP-3.

2 Materials and methods

2.1 Animals

C57BL/6J mice deficient for MASP-1 (MASP-1^{-/-}), MASP-3 (MASP-3^{-/-}) (4), or both (MASP-1/3^{-/-}) (20) were bred in-house for use in the current study. Wild-type (WT) littermates obtained by mating were used. All experiments were performed using 7- to 12-week-old male mice. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement and were approved by the Institutional Animal Care and Use Committee Fukushima Medical University (approval no. 2019069, 2021048 and 2023006).

2.2 Sodium iodate-induced retinal degeneration

The NaIO₃ (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS), and the mice were injected with 20 mg/kg of NaIO₃ through a tail vein. For the non-induced control group, WT mice were injected with the same volume of PBS.

2.3 Histological analyses

Histological analyses were performed on retinal cross sections stained with hematoxylin and eosin (H&E). After cervical spine dislocation under anesthetized by isoflurane, the eyes were enucleated and immersed in a fixative solution containing superfix (Kurabo, Osaka, Japan) for at least 2 h at room temperature. Paraffin-embedded sections of 5 μm cut through the optic disc of each eye were processed standard manner and stained with H&E. Images of the stained sections were taken with a whole slide scanner (NanoZoomer-SQ; Hamamatsu Photonics, Hamamatsu, Japan) and an Image viewing software (NDP.view2; Hamamatsu Photonics).

2.4 RPE flat mount

After removing the excess tissue, the enucleated eye was fixed in 4% paraformaldehyde (PFA) for 20 min on ice, and then washed three times with PBS. The cornea, lens and retina were removed

from the eye ball, and four radial cuts were made on the isolated eyecups. The RPE-eyecups were flat mounted and then blocked with Blocking one histo (Nacalai tesque, Kyoto, Japan) containing 0.3% Triton X-100 for 1 h at room temperature for immunofluorochemistry. They were then incubated overnight at 4°C with a rabbit anti-zonula occludens-1 (ZO-1) polyclonal primary antibody (1:100; Proteintech, Rosemont, IL, USA). After washing the RPE flat mounts, they were incubated for 1 h with an Alexa Flour 488-conjugated donkey anti-rabbit IgG secondary antibody (1:250; BioLegend, San Diego, CA, USA). The RPE flat mounts were washed and mounted in Fluoro-keeper antifade reagent non-hardening type with DAPI (Nacalai tesque) and examined with a microscope (BZ-9000; Keyence, Osaka, Japan). Images of the entire flat mounts were acquired at low magnification, and images of the central, middle, and periphery of the flat mounts were acquired at high magnification. Using an Image J software (version 1.53k; NIH, Bethesda, MD, USA), the percentage of necrotic RPE area, in which the cell shape is unrecognizable, to the total RPE area was calculated.

2.5 TUNEL staining

TUNEL staining was performed using *In Situ* Cell Death Detection kit (Roche Biochemicals, Mannheim, Germany) to detect apoptotic cells, according to the manufacturer's instructions. Eyes enucleated were fixed in 4% PFA for 24 h at 4°C. The eyes were then immersed in 25% sucrose for at least 48 h at 4°C, then embedded in optical cutting temperature (OCT) compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and snap-frozen in liquid nitrogen. Cryosections of 10 μm thickness cut on glass slides (FRONTIER COAT; Matsunami Glass Ind, Osaka, Japan) were dried at room temperature and then stored at -80°C until use. After thawing at room temperature, sections were rinsed in PBS and incubated in 0.1% sodium citrate solution containing 0.1% Triton X-100 for 2 min on ice. They were then placed in the TUNEL reaction mixture at 37°C for 1 h. After washing with PBS, sections were mounted in Fluoro-keeper antifade reagent non-hardening type with DAPI (Nacalai tesque) and photographed using the fluorescent microscope (Nikon, Tokyo, Japan). The number of TUNEL-positive cells in the retinal outer nuclear layer (ONL) was counted within a compartment 400–700 μm from the optic disc.

2.6 Immunofluorochemistry

Retinal sections fixed in PFA were thawed at room temperature and rinsed in PBS. After blocked with blocking reagent containing 10% normal goat serum (Nichirei Biosciences, Tokyo, Japan) in PBS containing 0.3% Triton X-100, sections were incubated overnight with FITC-conjugated goat anti-mouse C3 polyclonal antibody (1:100; MP Biomedicals, Santa Ana, CA, USA). Stained sections were then incubated with TrueBlack (Biotium, Fremont, CA, USA) according to the manufacturer's instructions to quench lipofuscin

autofluorescence. The sections were mounted in Fluoro-keeper antifade reagent non-hardening type with DAPI (Nacalai tesque) and photographed using the fluorescent microscope (Nikon). Mean fluorescence intensity (MFI) of C3 staining in the photoreceptor layer (PL), including photoreceptor inner and outer segments, was obtained by measuring the integrated density and area of 500–700 μm from the optic disc using Image J software (NIH). Background fluorescence intensity was subtracted by the average of three readings in areas of the slide that had no tissue.

For MBL-A and C4 staining of mouse retina sections, eyes were enucleated and embedded in OCT compound and frozen in liquid nitrogen. Cryosections of 10 μm thickness were cut, placed on glass slides, dried at room temperature, fixed with cold acetone, and stored at -80°C until use. Frozen sections were thawed at room temperature before staining. The retinal sections were blocked using 3% Bovine serum albumin (Sigma-Aldrich) in PBS or 10% normal goat serum (Sigma-Aldrich) in PBS containing 0.3% Triton X-100 and then incubated with each primary antibody for MBL-A (1:100; Hycult Biotech, Wayne, PA, USA) and C4 (1:250; Hycult Biotech) overnight at 4°C . After washing, sections were incubated in a secondary antibody for 1 h at room temperature with FITC-conjugated goat anti-rat IgG (1:500; Beckman Coulter, Brea, CA, USA). Stained sections were mounted in Fluoro-keeper antifade reagent non-hardening type with DAPI (Nacalai tesque) and photographed using the fluorescent microscope (Nikon). MFI of MBL-A or C4 staining in the PL was obtained by measuring the integrated density and area of the presented image using Image J software (NIH). Background fluorescence intensity was subtracted by the average of three readings in areas of the slide that had no tissue.

2.7 Western blot analysis

Protein extraction from the retinal tissue was carried out using MT-cell buffer (Sigma Aldrich) supplemented with protease inhibitor cocktail set V (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Protein samples were subjected to SDS-PAGE under reducing conditions and electroblotted onto a PVDF membrane. The membrane was blocked with Blocking One (Nacalai tesque) and then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse C3 polyclonal antibody (MP Biomedicals, LLC-Cappel products, Santa Ana, CA, USA) for 1 h at room temperature. Detection was performed using an ECL Prime Western Blotting Detection Reagent (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Objective protein bands were visualized by chemiluminescence detection using an Amersham Imager 600 (GE healthcare, Buckinghamshire, UK), and band intensities of C3 and iC3b were obtained using an ImageQuant TL software (GE healthcare). After detection of C3 and iC3b, the membranes were incubated with stripping solution (Fujifilm Wako Pure Chemical Corporation) and then subjected to detection of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using rabbit anti-human GAPDH polyclonal primary antibody (1:5000; GeneTex, Irvine, CA, USA) and HRP-conjugated anti-

rabbit IgG secondary antibody (Agilent Technologies, Palo Alto, CA, USA). The band intensity of iC3b and C3 was divided by the band intensity of GAPDH to normalize the total protein amount between samples.

2.8 Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). Data are expressed as means and standard error of the mean (SEM). Significance of differences in values was determined by Dunnett's multiple comparisons test. A P value < 0.05 was considered statistically significant.

3 Results

3.1 Assessment of retinal degeneration in MASP-1- and/or MASP-3-deficient mice 7 d after NaIO₃ injection

To determine the roles of MASP-1 and/or MASP-3 in NaIO₃-induced retinal degeneration, morphological change of the RPE and retina was assessed by H&E staining using retinal cross sections of WT, MASP-1^{-/-}, MASP-3^{-/-}, and MASP-1/3^{-/-} mice 7 d after intravenous NaIO₃ injection. As shown in **Figure 1A**, the retina of WT mice showed depigmentation and disruption of the RPE and the RPE layer was poorly distinguishable. The inner and outer photoreceptor segments that constitute the PL were absent in the retina of WT mice, and their PL was atrophied with no measurable thickness. Furthermore, the ONL in the retina of WT mice was significantly thinned (**Figures 1B, C**). In contrast, those degenerations and the thinning of ONL were significantly attenuated in MASP-1^{-/-} ($P < 0.05$), MASP-3^{-/-} ($P < 0.001$), and MASP-1/3^{-/-} mice ($P < 0.01$) compared to WT mice (**Figure 1**). To determine whether the observed ONL thinning is dependent on NaIO₃ injection rather than genetic deficiency of MASP-1 and/or MASP-3, we analyzed the differences in ONL thickness of those deficient mice that were not injected with NaIO₃. There were no significant differences in ONL thickness between the groups (**Figures 1D, E**).

To examine the morphological damage of the RPE in the horizontal direction, the RPE/choroid flat mounted sections were stained with antibody against ZO-1, a component protein of the tight junctions between RPE cells. The results showed that RPE cells were severely damaged or lost their normal cell boundary patterns in the posterior central region in all groups 7 d after NaIO₃ injection (**Figure 2**). In the peripheral region, RPE cells exhibited elongated or intact RPE in MASP-1^{-/-}, MASP-3^{-/-}, and MASP-1/3^{-/-} mice, whereas were damaged in WT mice (**Figure 2C**). Necrotic area of RPE flat mounts, illustrated as areas within white lines in **Figure 2B**, immunostained with anti-ZO-1 antibody was quantified using Image J software and expressed as a percentage. As shown in **Figure 2D**, the percentage of necrotic RPE area to total RPE area

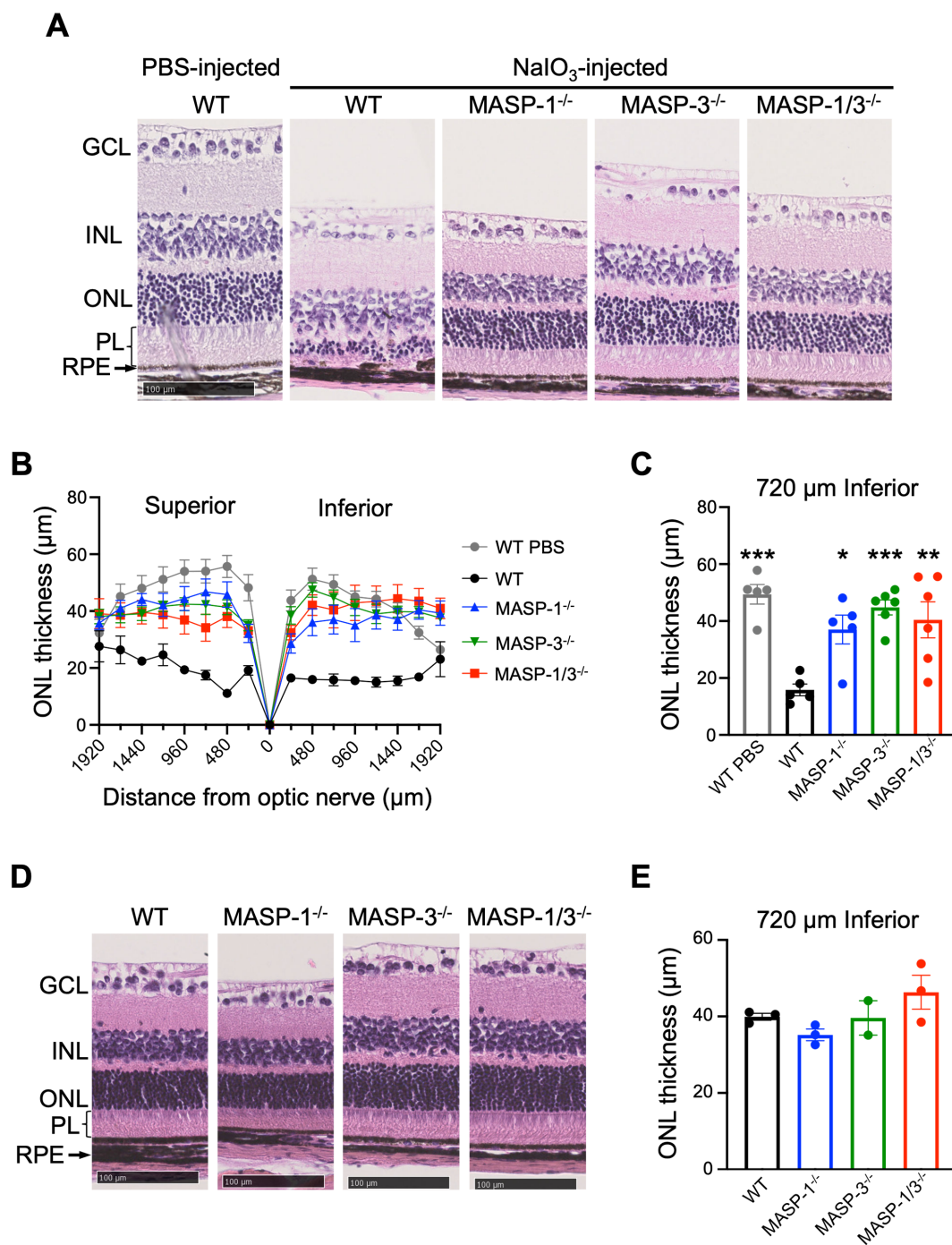


FIGURE 1

Assessment of retinal degeneration in MASP-1 and/or MASP-3 deficient mice 7 d after NaIO₃ injection. **(A)** Representative images of H&E-stained retinal sections from PBS-injected WT mice and WT, MASP-1^{-/-}, MASP-3^{-/-}, and MASP-1/3^{-/-} mice 7 d after NaIO₃ injection. Scale bars represent 100 μm. **(B)** ONL thickness measured in horizontal sections along superior-inferior axis of the retina. **(C)** ONL thickness at 720 μm inferior to optic nerve of the retina. Error bars indicate SEM of the mean (n = 5–6). Statistical comparisons were performed using Dunnett's multiple comparisons test (*P < 0.05, **P < 0.01, or ***P < 0.001 versus NaIO₃-injected WT group). **(D)** Representative images of H&E-stained retinal sections from WT, MASP-1^{-/-}, MASP-3^{-/-}, and MASP-1/3^{-/-} mice that were not injected with NaIO₃. Scale bars represent 100 μm. **(E)** ONL thickness at 720 μm inferior to optic nerve of the retina in WT, MASP-1^{-/-}, MASP-3^{-/-}, and MASP-1/3^{-/-} mice that were not injected with NaIO₃. Error bars indicate SEM of the mean (n = 2–3). Dunnett's multiple comparisons test showed no statistically significant differences between each deficient mice and WT mice. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; PL, photoreceptor layer.

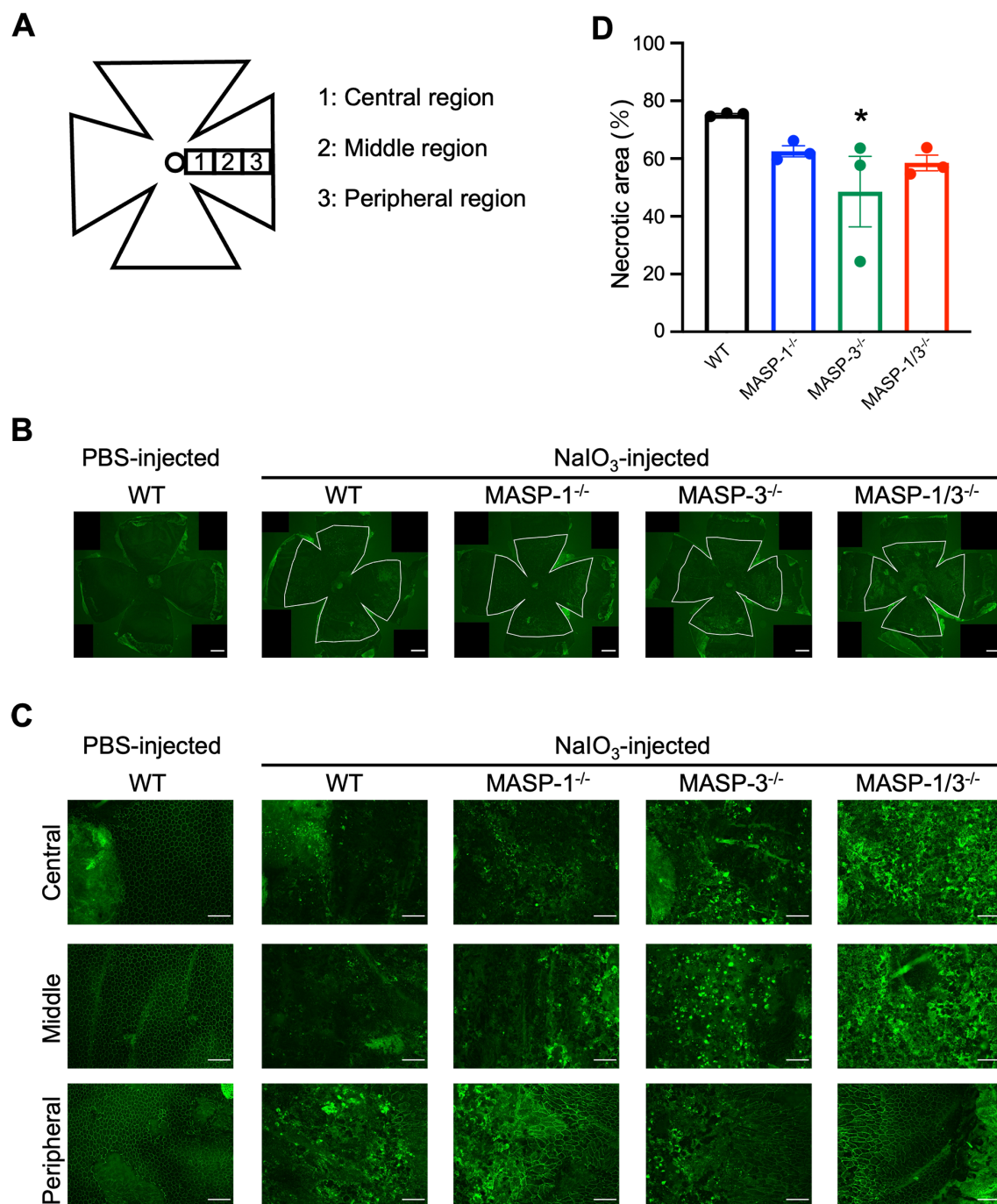


FIGURE 2

Assessment of morphological damage of RPE in MASP-1 and/or MASP-3 deficient mice 7 d after NaIO₃ injection. **(A)** RPE flat mounts were used to assess the central, middle, and peripheral regions of the retina. Central region is near the optic nerve. Middle and peripheral regions are 700 μ m and 1400 μ m from the central region, respectively. **(B)** Representative images of immunofluorescence of ZO-1 (in green) on RPE flat mounts at low magnification. The area of inside white lines shows the necrotic area of RPE flat mounts. Scale bars represent 500 μ m. **(C)** Representative images of immunofluorescence of ZO-1 (in green) on RPE flat mounts high magnification images of the central, middle, and peripheral region. Scale bars represent 100 μ m. **(D)** To quantify RPE damage, the percentage of necrotic RPE area to total RPE area in each mouse was calculated. Error bars indicate SEM of the mean ($n = 3$). Statistical comparisons were performed using Dunnett's multiple comparisons test (* $P < 0.05$ versus NaIO₃-injected WT group).

7 d after NaIO₃ injection was $75.30 \pm 0.36\%$ in WT mice, $62.53 \pm 1.92\%$ in MASP-1^{-/-} mice (not significantly decreased compared to WT mice), $48.57 \pm 12.20\%$ in MASP-3^{-/-} mice (significantly decreased at $P < 0.05$ compared to WT mice), and $58.50 \pm 2.73\%$

in MASP-1/3^{-/-} mice (not significantly decreased compared to WT mice). Only MASP-3^{-/-} mice showed statistically significant decrease in the percentage of necrotic RPE area compared to WT mice, however, both MASP-1^{-/-} and MASP-1/3^{-/-} mice also showed

a trend toward smaller necrotic RPE area than WT mice 7 d after NaIO₃ injection. These results suggest that MASP-1 and MASP-3 play a role in photoreceptor degeneration in the NaIO₃-induced murine dry AMD model.

3.2 Changes in the number of apoptotic photoreceptor cells in MASP-1 and/or MASP-3 deficient mice after NaIO₃ injection

To evaluate the effect of MASP-1 and/or MASP-3 deficiency on photoreceptor degeneration, TUNEL staining was performed on cryosections of enucleated mouse eyes. TUNEL-positive cells in the ONL of WT mice were almost absent on day 1 and then increased on day 2 and 3 after NaIO₃ injection (Figure 3A). Comparisons on day 2 after NaIO₃ injection showed a trend toward fewer TUNEL-positive cells in the ONL of MASP-3^{-/-} ($P = 0.19$) and MASP-1/3^{-/-} mice ($P = 0.12$) compared with WT mice (Figures 3A, B). Comparison on day 3 after NaIO₃ injection showed statistically significantly fewer TUNEL-positive cells in the ONL of MASP-3^{-/-} ($P = 0.02$) and MASP-1/3^{-/-} mice ($P = 0.01$) compared with WT mice (Figures 3A, C). On the other hand, there were no statistically significant differences in the number of TUNEL-positive cells in the ONL between WT and MASP-1^{-/-} mice on either day 2 or 3 after NaIO₃ injection (Figures 3B, C). These results suggest that the absence of MASP-3, but not MASP-1, prevented apoptotic photoreceptor cell death in the NaIO₃-induced murine dry AMD model.

3.3 Assessment of C3 deposition and activation in retina of MASP-1 and/or MASP-3 deficient mice 2 d after NaIO₃ injection

To assess the extent of complement activation in retinal degeneration, immunofluorescence staining of C3 was performed on the retinal tissue of mice 2 d after NaIO₃ injection. As shown in Figure 4A, C3 was detected in the PL of WT, MASP-1^{-/-}, MASP-3^{-/-}, and MASP-1/3^{-/-} mice 2 d after NaIO₃ injection. There was no statistically significant difference in the fluorescence intensity of C3 in the PL between the groups (Figure 4A). On the other hand, C3 was little-to-no detected in the ONL of all groups 2 d after NaIO₃ injection, and there was no significant difference in the fluorescence intensity of C3 in the PL between the groups (Figure 4A).

Since the anti-mouse C3 polyclonal antibody used in this study can detect both intact C3 and its activation products (e.g., C3b, iC3b, C3d, and C3dg), it is possible that the C3 detected in the PL of mice 2 d after NaIO₃ injection may not reflect *in situ* C3 activation. To address the issue, we performed Western blot analysis of C3 molecules in the protein lysates of neural retinas isolated from WT, MASP-1^{-/-}, MASP-3^{-/-}, or MASP-1/3^{-/-} mice 2 d after NaIO₃ injection. As shown in Figure 4B, intact C3 α -chain was detected in the lysates from all groups including the PBS-injected group. On the other hand, iC3b was not detected in the lysates from the PBS-

injected group, but was detected in those from all groups 2 d after NaIO₃ injection. Comparison of the data on the band intensities of iC3b and C3 normalized with that of GAPDH for each mouse revealed that there was no statistically significant difference in intact C3 level between the groups (Figure 4C). Notably, iC3b levels in the lysates from MASP-3^{-/-} ($P < 0.01$) and MASP-1/3^{-/-} mice ($P < 0.01$) were significantly lower than those from WT mice (Figure 4D). There was a trend toward lower iC3b levels in the lysates from MASP-1^{-/-} mice compared to those from WT mice, although the difference between MASP-1^{-/-} and WT mice did not reach statistical significance ($P = 0.129$, Figure 4D). To evaluate the degree of intraocular C3 activation in each mouse, the iC3b/C3 ratio was calculated using these band intensities. Consistent with the result of iC3b levels, the iC3b/C3 ratios in the lysates from MASP-3^{-/-} ($P < 0.001$) and MASP-1/3^{-/-} ($P < 0.001$) mice were significantly lower than those from WT mice (Figure 4E). The iC3b/C3 ratio in lysates from MASP-1^{-/-} mice also showed lower levels compared with those from WT mice, although the difference between MASP-1^{-/-} and WT mice did not reach statistical significance ($P = 0.394$). Thus, a significant reduction in C3 activation levels was observed in mouse retinas 2 d after NaIO₃ injection in the absence of MASP-3, but not in the absence of MASP-1.

3.4 Changes over time in C4 and MBL deposition in photoreceptor layer of mice after NaIO₃ injection

In the current study, we found that both MASP-1 and MASP-3 play critical roles in photoreceptor degeneration in the NaIO₃-induced murine dry AMD model. However, unlike MASP-3-deficient mice, no significant reduction in C3 activation levels was observed in the retina of MASP-1-deficient mice 2 d after NaIO₃ injection. To determine whether LP activation, in which MASP-1 plays a key role, is involved in the reduction of retinal degeneration, deposition of MBL-A and C4 in the retina of mice on days 1, 2, 3, and 5 after NaIO₃ injection was evaluated by immunofluorescence staining. As shown in Figure 5A, MBL-A was detected in the PL of WT mice on days 2, 3, and 5 after NaIO₃ injection. Whereas C4 was detected in the PL of WT mice on day 3 and 5 after NaIO₃ injection. Based on these observations, we assessed the levels of MBL and C4 deposition in the retinas of MASP-1^{-/-} and MASP-1/3^{-/-} mice on day 5 after NaIO₃ injection. As shown in Figures 5C, D, deposition of MBL-A and C4 was also observed in the PL of MASP-1^{-/-} and MASP-1/3^{-/-} mice, similar to that in WT mice. There were no significant differences in MBL-A and C4 deposition levels in the PL between the three groups. These results suggest that the absence of MASP-1 did not affect the levels of C4 deposition in the retinas of those mice 5 d after NaIO₃ injection.

4 Discussion

To clarify whether the LP is involved in the pathogenesis of dry AMD and to further investigate the involvement of the AP, we

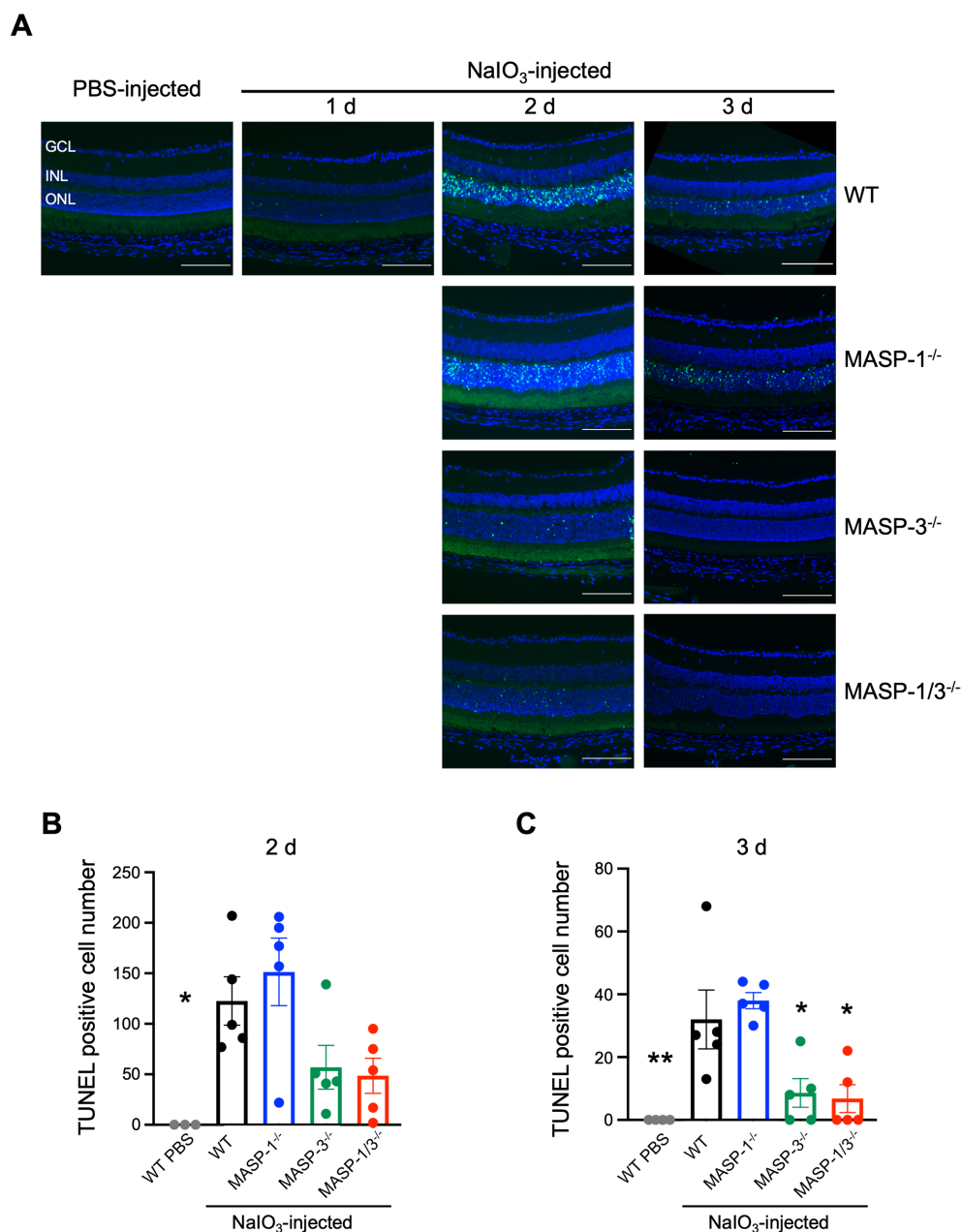


FIGURE 3

Changes in the number of apoptotic photoreceptor cells in MASP-1 and/or MASP-3 deficient mice after NaIO₃ injection. (A) Representative images of TUNEL (in green) and DAPI (in blue) stained retinal sections showing the entire compartment 400–700 μm from the optic disc. Scale bars represent 100 μm. Retinal sections of WT mice on days 1, 2, and 3 after NaIO₃ injection and those of MASP-1^{-/-}, MASP-3^{-/-}, and MASP-1/3^{-/-} mice on days 2 and 3 after NaIO₃ injection. (B, C) Quantitative analysis of the number of TUNEL positive cells in ONL on days 2 (B) and 3 (C) after NaIO₃ injection, counted within a compartment 400–700 μm from the optic disc. Error bars indicate SEM of the mean (n = 3–5). Statistical comparisons were performed using Dunnett's multiple comparisons test (*P < 0.05 and **P < 0.01 versus NaIO₃-injected WT group). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

performed histopathological analysis of the retina of a NaIO₃-induced murine dry AMD model using mice lacking MASP-1 and/or MASP-3. NaIO₃ is oxidative stress inducer and selectively damages RPE cell by increasing the ability of melanin to convert glycine to glyoxylate, which inhibits the enzymatic activity of intracellular energy generating processes (21). NaIO₃ also induces the production of reactive oxygen species (ROS), which injure mitochondria in RPE cells (22). The results presented in this

report indicate that the absence of MASP-1 or MASP-3 in a murine dry AMD model has a significantly beneficial effect on the development of the NaIO₃-induced retinal degeneration. Mice lacking MASP-1, MASP-3, or MASP-1/3 showed significantly reduced RPE depigmentation and destruction, PL atrophy, and ONL thinning after NaIO₃ injection compared with WT mice. Mice lacking MASP-3 or MASP-1/3 also showed significantly decreased numbers of TUNEL-positive apoptotic cells in the ONL and retinal

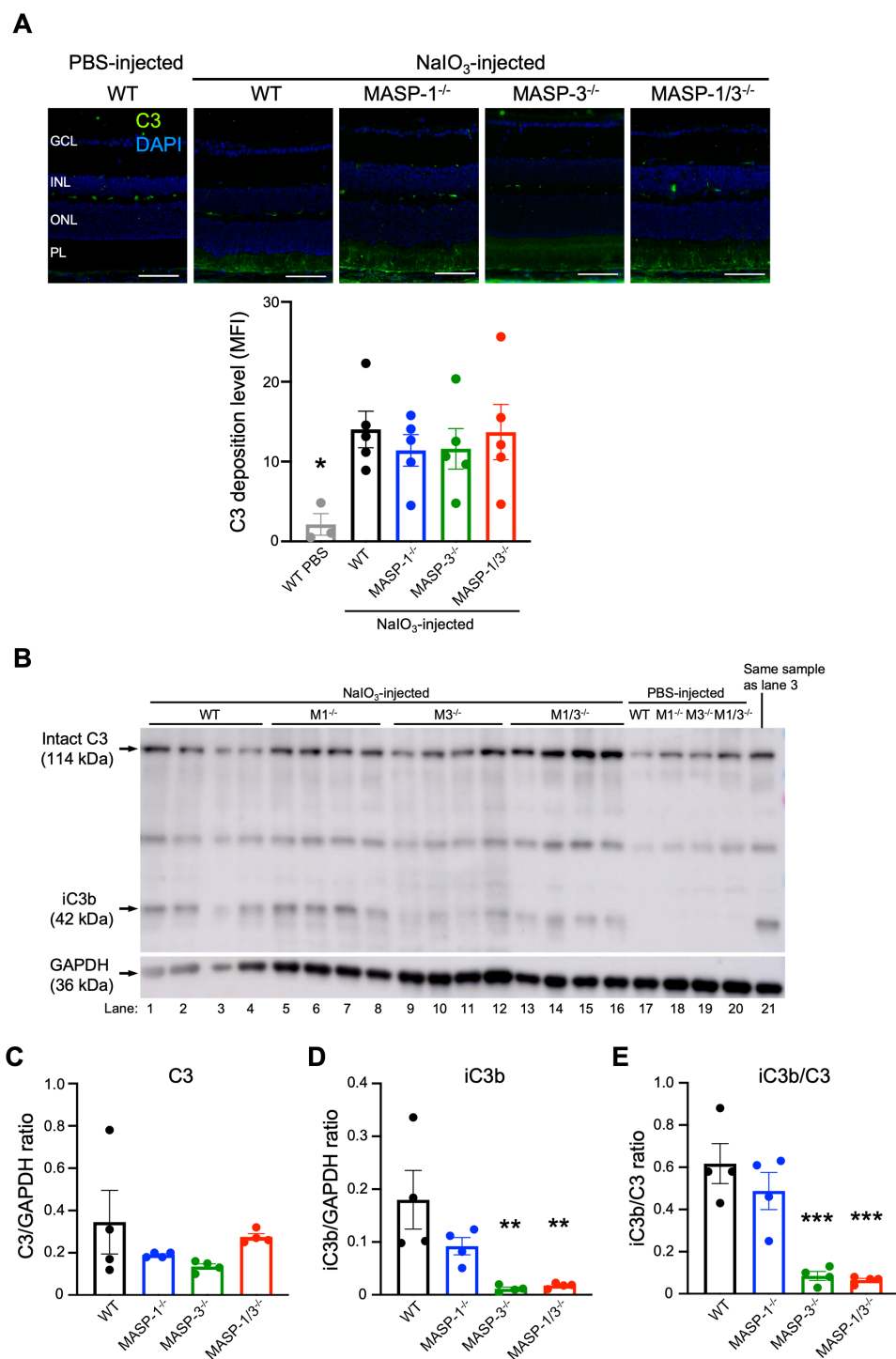


FIGURE 4

Assessment of C3 deposition and activation levels in retina of MASP-1 and/or MASP-3 deficient mice 2 d after NaIO₃ injection. **(A)** Representative images of C3 (in green) and DAPI (in blue) stained retinal sections from PBS-injected WT mice and WT, MASP-1^{-/-}, MASP-3^{-/-}, and MASP-1/3^{-/-} mice 2 d after NaIO₃ injection. Scale bars represent 50 μ m. The bar graph shows mean fluorescence intensity (MFI) of C3 staining in the photoreceptor layer (PL) of each mouse. Error bars indicate SEM of the mean ($n = 3-5$). Statistical comparisons were performed using Dunnett's multiple comparisons test (* $P < 0.05$ versus NaIO₃-injected WT mice group). **(B)** Western blot of C3 protein in the neural retina from WT, MASP-1^{-/-} (M1^{-/-}), MASP-3^{-/-} (M3^{-/-}), and MASP-1/3^{-/-} (M1/3^{-/-}) mice 2 d after NaIO₃ or PBS injection. Lane 3 was excluded from the quantitative analysis due to an error in sample addition, and lane 21, which is the same sample as lane 3, was used for the quantitative analysis. **(C–E)** Quantitative analysis of western blots for C3 **(C)** or iC3b **(D)** and the iC3b/C3 ratio **(E)** after normalization with GAPDH. Error bars indicate SEM of the mean ($n = 4$). Statistical comparisons were performed using Dunnett's multiple comparisons test (** $P < 0.01$ and *** $P < 0.001$ versus NaIO₃-injected WT group). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PL, photoreceptor layer.

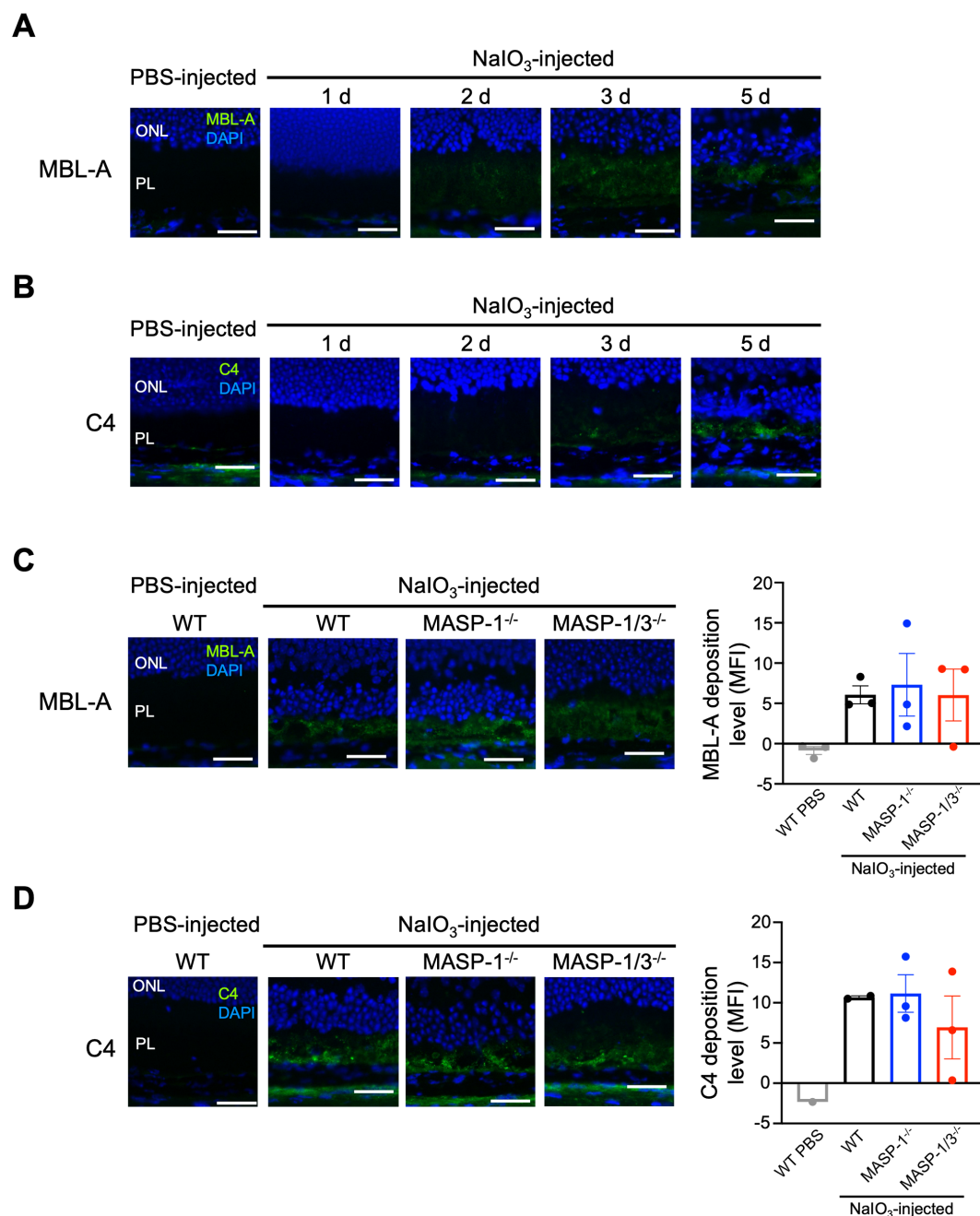


FIGURE 5

Changes in C4 and MBL-A deposition in photoreceptor layer of MASP-1^{-/-} and MASP-1/3^{-/-} mice after NaIO₃ injection. Representative images of MBL-A (in green) and DAPI (in blue) (A) or C4 (in green) and DAPI (in blue) (B) stained retinal sections from PBS-injected WT mice (7 d after injection) and WT mice on days 1, 2, 3, and 5 after NaIO₃ injection (n = 3 for A and n = 1 for B). Representative images of MBL-A (in green) and DAPI (in blue) (C) stained retinal sections from PBS-injected WT mice (7 d after injection, n = 3) and WT (n = 3), MASP-1^{-/-} (n = 3), and MASP-1/3^{-/-} (n = 3) mice on day 5 after NaIO₃ injection, or C4 (in green) and DAPI (in blue) (D) stained retinal sections from PBS-injected WT mice (2 d after injection, n = 1) and WT (n = 2), MASP-1^{-/-} (n = 3), and MASP-1/3^{-/-} (n = 3) mice on day 5 after NaIO₃ injection. Scale bars represent 20 μm. The bar graph shows MFI of MBL-A (C) or C4 (D) staining in the photoreceptor layer (PL) of each mouse. Error bars indicate SEM. Statistical comparisons were performed using Dunnett's multiple comparisons test (versus NaIO₃-injected WT mice group). ONL, outer nuclear layer; PL, photoreceptor layer.

C3 activation levels after NaIO₃ injection compared with WT mice. In contrast, these beneficial effects were not observed in mice lacking MASP-1 alone.

The first studies suggesting a link between AMD and complement can be traced back to reports showing the deposition

of various complement proteins along drusen and Bruch's membrane in patients with AMD (23). Subsequently, it was reported that genetic polymorphisms in FH, which selectively regulates the AP, are risk factors for both dry and neovascular AMD (8, 24–27). In addition, polymorphisms in the genes encoding

FB and C3 of the AP, as well as in the FH-like gene, have been reported to be associated with AMD (28–30). A strong association between AMD and the AP has also been reported in murine models of AMD. Rohrer and colleagues demonstrated that in a laser-induced murine neovascular AMD model, mice lacking either the FD or FB of the AP were protected from the development of CNV compared with WT mice (31, 32). In the present study, we used MASP-3-deficient mice to investigate whether the AP is also involved in the development of retinal degeneration in a NaIO₃-induced murine dry AMD model. As the result, we observed that mice lacking MASP-3 were protected from the development of retinal degeneration after NaIO₃ injection compared with WT mice.

MASP-3 is a splice variant transcribed from the *Masp1* gene, which also encodes the LP complement factor MASP-1 and its competitive protein MAP-1 (or MAP44) lacking the enzyme active site (3). We previously reported that in MASP-3-deficient mice, FD circulates in an inactive form, resulting in the loss of serum AP activity (4). This is because the active form of FD can efficiently cleave FB bound to C3(H₂O) or C3b to generate the AP C3 convertases C3(H₂O)Bb or C3bBb, respectively (3). C3(H₂O) is formed in the fluid-phase by spontaneous hydrolysis of the thioester of C3 in a process called “tick-over”. On the other hand, the initial C3b can be formed on the solid-phase by nearby C3(H₂O)Bb or by the CP/LP C3 convertase C4b2b. Once C3bBb is generated on the solid-phase, it cleaves C3 there, allowing efficient deposition of C3b on the target. The deposited C3b serves as a source of additional C3bBb and ultimately contributes to the amplification loop of the AP. In fact, approximately 80% of the C3 convertase deposited on the target surface originates from the AP amplification loop, rather than from the CP or LP (33, 34). Based on these reports, it is considered that MASP-3 plays an important role in complement activation by participating in the generation of the amplification loop C3 convertase C3bBb, which contributes significantly to C3 activation on the target surface. Thus, the significant decrease in retinal C3 activation levels observed in MASP-3-deficient mice 2 d after NaIO₃ injection is most likely due to a decrease in the generation of C3bBb in the retina.

Decreased generation of C3bBb in retinal tissue leads to decreased generation of the C3bBb-derived C5 convertase, C3bBb3b, and thus reduced production of the anaphylatoxin C5a and formation of the membrane attack complex C5b-9. The formation of C5b-9 on RPE cells triggers an increase in calcium influx into the cells, leading to mitochondrial damage and an increase in ROS production (35). Thus, complement activation mediates a feed-forward cycle of mitochondrial injury resulting in loss of RPE homeostasis, which is essential for maintaining photoreceptor cells through nutrient transport and outer segment phagocytosis (36). Indeed, MASP-3-deficient mice were protected from TUNEL-positive apoptotic photoreceptor cell death on day 3 after NaIO₃ injection and from RPE depigmentation and disruption, PL atrophy, and thinning of the ONL on day 7 after NaIO₃ injection. Furthermore, MASP-3-deficient mice showed a decrease in the percentage of necrotic RPE area 7 d after NaIO₃ injection, which is consistent with the results of anti-C5 antibody administration in a NaIO₃-induced murine dry AMD model (14).

Those mice administered with anti-C5 antibody showed the loss of central RPE integrity, but prevented the loss of peripheral RPE integrity, suggesting that C5 activation may affect the propagation of RPE cell death into the periphery (14, 37). Taken together, the present study suggested that MASP-3 acts as an exacerbating factor in NaIO₃-induced RPE cell injury and subsequent photoreceptor cell damage via activation of the AP.

MASP-1 activates MASP-2 and C2, contributing to the generation of the LP C3 convertase C4b2b. In the present study, mice lacking MASP-1 showed significantly attenuated ONL thinning on day 7 after NaIO₃ injection, similar to mice lacking MASP-3 or MASP-1/3. However, unlike mice lacking MASP-3 or MASP-1/3, mice lacking MASP-1 alone did not show a decrease in the number of TUNEL-positive apoptotic cells in the ONL or in the retinal C3 activation levels after NaIO₃ injection. These results suggest that the absence of MASP-1 alone has little-to-no effect on the retinal C3 activation levels in the NaIO₃-induced murine dry AMD model. To clarify whether there was an association between the LP and NaIO₃-induced retinal degeneration, we performed immunofluorescence staining for C4 and MBL-A in the retinal sections from mice after NaIO₃ injection. Deposition of MBL-A and C4 was clearly observed in the PL of WT mice 5 d after NaIO₃ injection, suggesting that the LP is activated in their retinal tissue. Notably, after NaIO₃ injection, TUNEL-positive cells and C3 deposition were observed before MBL-A and C4 deposition in their retinal tissue. These observations suggest that AP activation occurs first, followed by LP activation in the retina after NaIO₃ injection. MBL can bind to late apoptotic cells and enhance phagocytosis of apoptotic cells by macrophages (38). However, transcription levels of MBL in the human retina are minimal (15), suggesting that MBL deposits observed in the PL after NaIO₃ injection are unlikely to be derived from locally produced MBL. In the NaIO₃-induced murine dry AMD model, it is suggested that the blood-retinal barrier is disrupted, allowing circulating MBL-A to enter the retina and potentially play a role in the removal of apoptotic cells by opsonization. It is unclear whether the deposition of C4 observed in the PL after NaIO₃ injection is a result of LP activation. C4 can be activated through the CP as well as the LP. Indeed, it has been reported that the CP is involved in the development of NaIO₃-induced retinal degeneration (14). In the present study, no significant difference was observed in the levels of C4 deposition in the PL between MASP-1-deficient and WT mice, suggesting that the retinal C4 deposition observed 5 d after NaIO₃ injection was not caused by intraretinal activation of the LP. Further studies are needed to determine whether the LP is involved in the development of retinal degeneration in the NaIO₃-induced murine dry AMD model and in human dry AMD.

The question remains as to why retinal degeneration was attenuated in mice lacking MASP-1 alone, as in mice lacking MASP-3 or MASP-1/3. Recently, the rate of choriocapillaris flow deficit in participants with large drusen was measured at 6-month intervals, and impaired choriocapillaris blood flow was found prior to the development of nascent geographic atrophy (39). In a rabbit model of NaIO₃-induced dry AMD, fibrin deposition was observed in the choroidal interstitium and Bruch's membrane surrounding

the narrowed choriocapillaris 3 h after NaIO₃ injection (40). Furthermore, reduced retinal blood flow has been reported in a NaIO₃-induced murine dry AMD model (41). These results suggest that the blood coagulation system is involved in the development of retinal degeneration in human dry AMD and in the NaIO₃-induced models. Besides LP activation, MASP-1 cleaves coagulation factors such as fibrinogen and factor XIII to form fibrin clots, and also cleaves high-molecular-weight kininogen, a member of kallikrein-kinin system, to promote vasorelaxation and vasodilation (42). In fact, it has been reported that thrombin-like activity on mannan-coated plates, to which MBL/MASPs complexes can bind, was markedly reduced in the sera of MASP-1/3^{-/-} mice compared with that of MASP-2^{-/-} or WT mice (43). In the present study, the absence of MASP-1 may have reduced fibrin deposition in the choriocapillaris, which plays an essential role in RPE cell survival, thereby preserving blood flow and alleviating retinal degeneration after NaIO₃ injection. Interestingly, MASP-1 levels in plasma of patients with neovascular AMD were higher compared with healthy controls and patients with early AMD (44). Elevated levels of circulating MASP-1 may influence the reduction in choroidal blood flow prior to the development of retinal degeneration in AMD through activation of coagulation factors. However, further studies are required to clarify whether the blood coagulation system is involved in the development of retinal degeneration in the NaIO₃-induced models and in human dry AMD.

In summary, we demonstrated that MASP-1 and MASP-3 act as exacerbating factors in the NaIO₃-induced murine dry AMD model. MASP-3 is critically involved in apoptosis and C3 activation (i.e., AP activation) in the retina after NaIO₃ injection, suggesting that MASP-3 could be a therapeutic target for dry AMD. On the other hand, no clear evidence was observed that MASP-1 is involved in retinal degeneration in the NaIO₃-induced model via complement activation. Further studies are needed to define the role of MASP-1 in the development of retinal degeneration in the NaIO₃-induced murine dry AMD model.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by The Animal Experiments Committee of Fukushima Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

TO: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Writing – original draft, Writing – review & editing, Visualization. TM: Conceptualization, Formal Analysis, Investigation, Methodology, Validation, Writing – review & editing, Data curation, Project administration, Resources, Visualization, Writing – original draft. YI: Investigation, Methodology, Writing – review & editing. TS: Conceptualization, Validation, Writing – review & editing. HS: Conceptualization, Project administration, Supervision, Validation, Writing – review & editing, Data curation, Funding acquisition, Methodology, Resources, Visualization, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Expression of innate immunity genes in human hematopoietic stem/progenitor cells – single cell RNA-seq analysis

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Background: The complement system expressed intracellularly and known as complosome has been indicated as a trigger in the regulation of lymphocyte functioning. The expression of its genes was confirmed also in several types of human bone marrow-derived stem cells: mononuclear cells (MNCs), very small embryonic-like stem cells (VSELs), hematopoietic stem/progenitor cells (HSPCs), endothelial progenitors (EPCs) and mesenchymal stem cells (MSCs). In our previous studies, we demonstrated the expression of complosome proteins including C3, C5, C3aR, and cathepsin L in purified HSPCs. However, there is still a lack of results showing the expression of complosome system elements and other immunity-related proteins in human HSPCs at the level of single cell resolution.

Methods: We employed scRNA-seq to investigate comprehensively the expression of genes connected with immunity, in two populations of human HSPCs: CD34+Lin-CD45+ and CD133+Lin-CD45+, with the division to subpopulations. We focused on genes coding complosome elements, selected cytokines, and genes related to antigen presentation as well as related to immune regulation.

Results: We observed the differences in the expression of several genes e.g. C3AR1 and C5AR1 between two populations of HSPCs: CD34+LinCD45+ and CD133+Lin-CD45+ resulting from their heterogeneous nature. However, in both kinds of HSPCs, we observed similar cell subpopulations expressing genes (e.g. NLRP3 and IL-1 β) at the same level, which suggests the presence of cells performing similar functions connected with the activation of inflammatory processes contributing to the body's defense against infections.

Discussion: To our best knowledge, it is the first time that expression of complosome elements was studied in HSPCs at the single cell resolution with the use of single cell sequencing. Thus, our data sheds new light on complosome as a novel regulator of hematopoiesis that involves intracrine activation of the C5a-C5aR-Nlrp3 inflammasome axis.

KEYWORDS

innate immunity, complement cascade, toll-like receptors, NOD-like receptors, NLRP3 inflammasome, complosome, scRNA-seq

1 Introduction

Hematopoiesis and the immune system originate from a common hematopoietic/lymphopoietic stem cell. This close developmental relationship explains that cells belonging to both lineages often share the same receptors and respond to similar factors (1–7). Gene expression analysis performed on bulk RNA supported this developmental origin, explained by the common goal of both lineages being involved in tissue homeostasis, fighting invading pathogens, and promoting tissue repair (8–12). The complement, a crucial innate and adaptive immunity element, is a complex multiprotein system responsible for inflammatory processes and other biological effects (13–16). Initially, it was considered a serum-effective system in which proteins were synthesized in the liver and released into circulation (17, 18). Recent data demonstrated the presence of complement elements expressed intracellularly, which is crucial for the normal functioning of lymphocytes (19–21). Furthermore, the expression of its components was also detected in several types of human bone marrow-derived stem cells, including mononuclear cells (MNCs), very small embryonic-like stem cells (VSELs), hematopoietic stem/progenitor cells (HSPCs), endothelial progenitors (EPCs) and mesenchymal stem cells (MSCs) (22). Our previous studies demonstrated the expression of functional complosome proteins, including C3, C5, C3aR, and cathepsin L in purified human and murine HSPCs (18, 22). It has also been reported that HSPCs express several receptors that protect them from potential damage by circulating in peripheral blood (PB) activated complement proteins (23), some members of the toll-like receptor family (TLRs) (24, 25), and intracellular pattern recognition receptors (PRRs) (26, 27). Pattern recognition receptors (PRRs) are germline-encoded sensors that detect pathogen-associated (PAMPs) or damage-associated molecular patterns (DAMPs). They are highly expressed in innate immune cells, such as dendritic cells, macrophages, monocytes, and epithelial cells. Some elements of innate immunity, such as intracellular PRR, have been implicated, for example, in the specification of HSPCs in mammals during embryogenesis (28) and being involved in the early stages of hematopoiesis in the zebrafish (29, 30). Furthermore, bone marrow (BM) cells from TLR4^{−/−}, TLR9^{−/−}, and MyD88^{−/−} mice show a repopulating advantage over wild-type cells in lethally irradiated recipients (28), suggesting that TLR signaling supports HSPC maintenance.

To our best knowledge, there is still a lack of information about the expression and functioning of the complosome elements, obtained from single-cell sequencing experiments. This method (scRNA-seq) is becoming an increasingly widely used tool in biological and biomedical research and has advanced the understanding of a range of biological processes and molecular mechanisms occurring in cells. It potentially may have a clinical application and may be valuable in new diagnostic and therapeutic strategies (31). In the case of hematopoietic stem cells, scRNA-seq allowed to decode both human (32) and mice (33) hematopoiesis. Furthermore, the first hematopoietic stem cells in human embryo were traced by scRNA-seq (34). In our previous scRNA-seq

experiment (35), we identified several HSPCs subpopulations (14 clusters for both CD34+Lin-CD45+ and CD133+Lin-CD45+, with their basic division into 2 main cell groups. One, with confirmed expression of expression of two stem cell markers: CD34 and PROM1 (CD133). We call them primitive or quiescent clusters. And another one, with confirmed expression of lymphocyte (T and B), monocytes, macrophages, NK cells and granulocytes markers (e.g. CD2, CD3, CD19, CD14, FCGR3A, CD68). We call them ‘fate decision’ clusters (35). In the current study, we employed scRNA-seq and untargeted proteomic analysis using mass spectrometry to investigate the expression of genes immunity in two populations of purified human umbilical cord blood (UCB) HSPCs: CD34+Lin-CD45+ and CD133+Lin-CD45+, paying special attention to track the differences between primitive and ‘fate decision’ clusters. We analyzed our mRNA expression data sets employing the Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) technique, which allows us to visualize separate cell clusters (36, 37). We focused on genes encoding proteins involved in immune responses, encoding complosome elements, and selecting cytokines. To our knowledge, it is the first time that the expression of all these genes was studied in HSPCs at the single-cell resolution using single-cell sequencing. We observed some similarities and differences in gene expression between CD34+Lin-CD45+ and CD133+Lin-CD45+ cells. Moreover, scRNA-seq results revealed significant differences from previously published data using bulk mRNA analyses. Finally, the expression of some of these genes was subsequently evaluated by proteomic analysis.

2 Materials and methods

2.1 Isolation of human CD34+ and CD133+ HSPCs

Human umbilical cord blood (hUCB) was obtained from a healthy newborn delivered at the Department of Obstetrics and Gynecology, Medical University of Warsaw (Warsaw Bioethics Committee permission number KB/50/2022). Blood was diluted with phosphate-buffered saline (PBS) and carefully layered over Ficoll-Paque (GE Healthcare, Chicago, IL, USA). Next, it was centrifuged for 30 min at 400x g at 4°C. The mononuclear cells (MNCs) were collected, washed, and stained with the following antibodies: hematopoietic lineage markers (Lin) cocktail of antibodies, each FITC-conjugated: CD235a (clone GA-R2 [HIR2]), anti-CD2 (clone RPA-2.10), anti-CD3 (clone UCHT1), anti-CD14 (clone M5E2), anti-CD16 (clone 3G8), anti-CD19 (clone HIB19), anti-CD24 (clone ML5), anti-CD56 (clone NCAM16.2) and anti-CD66b (clone G10F5) (all BD Biosciences, San Jose, CA, USA); PE-Cy7-conjugated anti-CD45 (clone HI30, BioLegend, San Diego, CA, USA), PE-conjugated anti-CD34 (clone 581, BioLegend, San Diego, CA, USA) and APC-conjugated anti-CD133 (clone CD133, MiltenyiBiotec, Gladbach, Germany). Cells were then stained in the dark at 4°C for 30 min. After incubation, they were centrifuged and resuspended in RPMI-1640 medium containing 2% fetal bovine serum (FBS, Corning Inc, Corning, NY, USA). Finally, cells were

sorted according to the strategy described in numerous reports (38–40). Briefly, small events (2–15 μm in size) were included in the “lymphocyte-like” gate (P1) and then further analyzed for the expression of the Lin marker. Lin negative events were gated and subsequently analyzed for the expression of CD45 and CD34 or CD133 antigens. Populations of CD34+ HSPCs (CD34+Lin–CD45+) and CD133+ HSPCs (CD133+Lin–CD45+) were obtained on the MoFlo Astrios EQ cell sorter (Beckman Coulter, Brea, CA, USA).

2.2 Single-cell sequencing

After sorting, cells were directly proceeded using Chromium X Controller (10X Genomics, USA) and Chromium Next GEM Chip G Single Cell Kit (10X Genomics, USA). Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1, and Single Index Kit T Set A (10X Genomics, USA) were used for library preparation according to manufacturer's guidelines. Libraries were then pooled and run on Illumina NextSeq 1000/2000 (Illumina, San Diego, CA, USA) in P2 flow cell chemistry (200 cycles) with paired-end sequencing mode (read 1–28 bp, read 2–90 bp, index 1 – 10 cycles, index 2 – 10 cycles), assuming 25,000 reads per single cell.

2.3 Bioinformatic analysis

Downstream analysis was performed using Seurat (version 5.0.1), preceded by the 10X Genomics Cell Ranger pipelines (CellRanger version 7.2.0, 10x Genomics, USA) (41, 42). Raw sequencing files (BCL files) were demultiplexed and converted to fastq files using the bcl2fastq (version v2.20.0.422) within the 10X Genomics Cell Ranger mkfastq pipeline (43, 44). Then, the Cell Ranger count pipeline was used. Cells with less than 200 and more than 2500 transcripts and those with more than 5% of mitochondria-related transcripts were excluded from the analysis. Sequencing reads were mapped to a human genome GRCh38 (version 2020-A) acquired from the 10xGenomics website (<https://www.10xgenomics.com/support/software/cell-ranger/downloads#reference-downloads>). Gene expression measurements for each cell were normalized, and the normalized values were log-transformed (“LogNormalize” method) and then reduced to the first 2000 most highly variable genes. Non-linear dimensional reduction to visual clusters was performed using uniform manifold approximation and d projection (UMAP) implemented in Seurat (version 5.0.1.). Cell clusters were recognized based on differentially expressed genes, both positive (up-regulated genes) and negative (down-regulated genes) markers, using an adjusted p-value <0.05 and a log2FC >1. However, for this publication, we focused only on positive ones.

2.4 Data visualization

Data visualization was done using R (R Core Team) with the ggplot2 R package (version 3.4.4) (45). We selected hematopoietic

lineage differentiation cell markers (CD2, CD3, CD4, CD11b (ITGAM), CD14, CD16 (FCGR3A), CD19, CD34, CD41 (ITGA2B), CD45 (PTPRC), CD66b (CEACAM8), CD68, GYPA, PROM1 (CD133), CD117 (c-KIT) to determine their expression in CD34+lin-CD45+ and CD133+lin-CD45+ and confirm the hematopoietic character of the studied cells. Furthermore, we selected several genes related to the complosome system and immunity. They were as follows: TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, NOD1, NOD2, NOD3, NOD4, NOD5, NLR4, CIITA, NAIP, NLRP1, NLRP2, NLRP3, NLRP4, NLRP5, NLRP6, NLRP7, NLRP8, NLRP9, NLRP10, NLRP11, NLRP12, NLRP13, NLRP14, C1QA, C1QB, C1QC, C1R, C1S, C2, C3, C4, C5, C6, C7, C8, C9, CFB, CFP, C3AR1, C5AR1, C5AR2, CTSL, CFH, CFI, CD46, CD55, CD59, C1NH, CFHR1, CFHR2, CFHR3, CFHR4, CFHR5, CASP1, IL1B, IL18, PYCARD, MEFV, AIM2, MDAS, DDX58.

2.5 Data availability

The data sets supporting this article's results are available in the Sequence Read Archive (SRA) repository (<https://www.ncbi.nlm.nih.gov/sra>) and assigned a unique persistent identifier: PRJNA1128409 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1128409>).

2.6 Mass spectrometry

MoFlow-isolated CD133+lin-CD45+ and CD34+lin-CD45+ HSPCs were lysed in 50 μL of RIPA buffer. Samples were incubated for 60 min at 96°C and then sonicated for 90 min in a water bath. Samples were precipitated with the use of ice cold (–20°C) Acetonitrile (ACN, Merck, Darmstadt, Germany) in a volume 1:4 ratio and incubated for 120 min in (–20°C). After precipitation, samples were centrifuged (30 min, –9°C, 18000 $\times$ g), and the supernatant was removed. The protein pellet was dissolved in 40 mM ammonium bicarbonate (Merck, Darmstadt, Germany). Reduction and alkylation were performed using 500 mM DTT (in a final concentration of 20 mM) and 1 M iodoacetamide (IAA, in a final concentration of 40 mM). Proteins were in-solution digested for 16 h at 37°C using Trypsin Gold (Promega, Madison, WI, United States).

Liquid chromatography-mass spectrometry (LC-MS) analysis was carried out with the use of nano-UHPLC (nanoElute, Bruker, Billerica, MA, United States) coupled by Captive Spray (Bruker, Billerica, MA, United States) to ESI-Q-TOF mass spectrometer (Compact, Bruker, Billerica, MA, United States). A two-column separation method was used, that is, pre-column (300 $\mu\text{m} \times 5$ mm, C18 PepMap 100, 5 μm , 100 Å, Thermo Scientific, Waltham, MA, United States) and Bruker fifteen separation column (75 $\mu\text{m} \times 150$ mm, C18 1.6 μm) in gradient 2% B to 55% B in 45 min with the 300 nL/min flow rate.

2.7 Proteomic data analysis

The collected spectra were calibrated (lock mass calibrant) and analyzed using Data Analysis software (Bruker). They were then identified in ProteinScape (Bruker) using the MASCOT server. Proteins were identified using the online SwissProt database, and their annotation and biological significance were determined using UniProt.org and KEGG. All analyses were performed in the R version [4.3.3]. The following R packages were employed for data processing, statistical analysis, and visualization: tidyverse (46) for data manipulation and wrangling, readxl (47) for importing Excel files, dplyr (48) and purrr (49) for functional programming and data cleaning, and writexl (50) for exporting processed results to Excel.

3 Results

3.1 Cell subpopulations within CD34+Lin-CD45+ and CD133+Lin-CD45+

We identified several cell types and cell subpopulations in CD34+Lin-CD45+ (Figure 1A) and CD133+Lin-CD45+ (Figure 1B) cells, as it was previously published (35). Cluster 0 and 1 of CD34+Lin-CD45+ cells and clusters 1 and 4 of CD133+Lin-CD45+ cells were identified to express transcripts of CD34, CD133 (PROM1), and c-kit genes, corresponding to the most primitive cells evaluated in our study. Additionally, we also identified clusters 6 and 7 of CD34+Lin-CD45+ (Figure 1A) as cells expressing CD34, CD133^{dim}, and c-kit^{dim} and cluster 11 of CD133+Lin-CD45+ (Figure 1B) as primitive cells expressing CD34, CD133 and c-kit^{dim}. Furthermore, both HSCPs populations (Figures 1A, B) contain cell clusters with confirmed expression of myeloid (CD14, CD68) and lymphoid (CD2, CD4) progenitors. We call them “fate decision” clusters.

3.2 Expression of toll-like receptors

Our scRNA-seq data (Figure 2) revealed the expression of transcripts of TLR1 and TLR 2 receptors in primitive clusters (0 and 1 of CD34+lin-CD45+ and 1 of CD133+lin-CD45+), as well as fate decision clusters (2, 3 and 4 of CD34+lin-CD45+; and 0, 2 and 5 of CD133+lin-CD45+) Additionally, TLR4, TLR5, TLR6, and TLR8, were also expressed on cells in clusters enriched of CD2, CD4, CD14 and CD68 mRNA in both datasets (Figures 2A, B).

3.3 Expression of NOD-like receptors

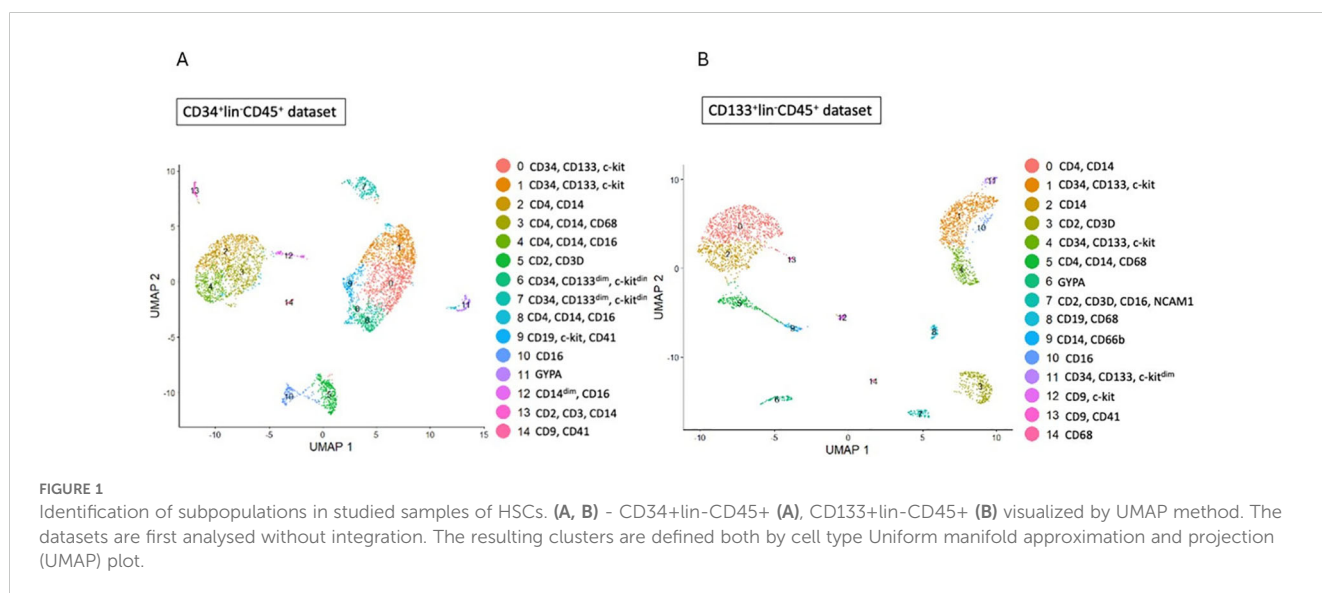
Next, we evaluated mRNA expression for NOD-like receptors in our scRNA-seq data sets:

-CARD domain – containing NODs/NLRCs

The nucleotide-binding oligomerization domain-like receptors, or NOD-like receptors (NLRs), are intracellular sensors known as pattern recognition receptors (51, 52). We detected transcripts for NOD1/NLRC1, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRZ1, CIITA/NLRA and NAIP in clusters 0 and 1 for CD34+Lin-CD45+ (Figure 3A) and clusters 1 and 4 for CD133+Lin-CD45+ (Figure 3B) enriched for mRNA for CD34, CD133, and c-kit (primitive clusters). The mRNA for these receptors was also expressed in other cell clusters with confirmed expression of mRNA for lymphocytic precursors.

-Pyrin domain – containing NODs/NLRPs

The expression of NLRP1, NLRP2, NLRP3, NLRP4/PAN2 was observed in clusters 0 and 1 of CD34+Lin-CD45+ (Figure 4A) and clusters 1 and 4 of CD133+Lin-CD45+ (Figure 4B), which are, as it was mentioned above the most primitive cell subpopulations of HPSCs (expressing CD34, CD133, and c-kit transcripts). Additionally, their expression was observed in “fate decision” subpopulations, with confirmed expression of CD4 and CD14 in



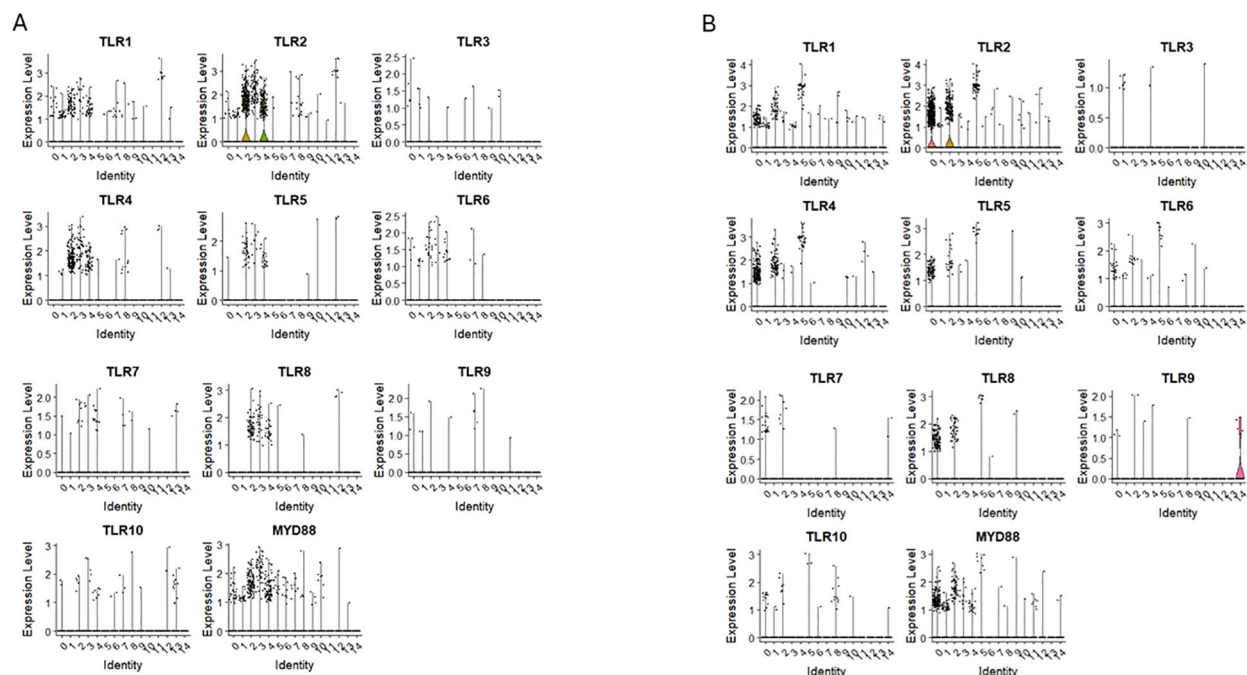


FIGURE 2

The expression of Toll-like receptor (TLRs) genes in CD34+lin-CD45+ (A) and CD133+lin-CD45+ (B) cells with the division to clusters. Violin plots shows differential expression of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10 and MYD88 in different subpopulations of both datasets.

both HSPCs populations. The same was found for NLRP12, which was also expressed in cell clusters expressing mRNA for lymphocytic precursors (CD4, CD14, CD68). Expression of NLRP5, NLRP10, NLRP11, and NLRP14 was not observed in both scRNA-seq datasets.

3.4 Expression of mRNA for RIG-1-like receptors and AIM2

We found the expression of RIG-1-like receptors MDAS (IFIHI) and DDX58 in clusters 0 and 1 of CD34+Lin-CD45+ (Figure 5A) and clusters 1 and 4 of CD133+Lin-CD45+ (Figure 5B).

AIM2 expression was found however, its level was very slight and observed only in single cells of both HSPCs populations.

3.5 Expression of mRNA for complement cascade/complosome

We found the expression of C3 and C5 in both HSPCs populations: CD34+lin-CD45+ (Figure 6A) and CD133+lin-CD45+ (Figure 6B) however it was on the slight level with just a few cells expressing mRNA of these genes. Interestingly, the expression of C3AR1, C5AR1, and C5AR2 (Figures 6A, B) was observed not in all cells of both HSPCs populations but in the subpopulations with

confirmed expression of CD4 and CD14 markers, corresponding to 'fate decision' cells, where the mechanisms of immunological actions start to become active. Because the expression of receptor genes is observed only in this group of cells, we can assume that their later immunological potential comes from there. We do not see the expression of C3, C5, C3AR1, C5AR1, and C5AR2 (Figures 6A, B) in the quiescent subpopulation of HSPCs with a confirmed expression of CD34, CD144, and c-KIT. Interestingly, in both HSPCs populations: CD34+lin-CD45+ and CD133+lin-CD45+.

3.6 Expression of mRNA for NLRP3 inflammasome components

Our scRNA-seq datasets revealed that mRNA for Nlrp3 inflammasome is expressed in clusters 0 and 1 for CD34+Lin-CD45+ and UMAP clusters 1 and 4 for CD133+Lin-CD45+ enriched for mRNA of HSPCs (Figure 4). In addition, Figures 7A, B show mRNA expression for crucial components of this pattern recognition receptor, including mRNA for IL1 β , IL18, PYCARD, and CASP1 in the same clusters. The NOD-like receptor protein 3 (NLRP3) inflammasome is a protein complex that regulates innate immune responses by activating caspase-1 and the inflammatory cytokines interleukin (IL)-1 β and IL-18 (53–55). We and others have demonstrated that Nlrp3 inflammasome is expressed in HSPCs and regulates the trafficking and metabolism of these cells (56–59).

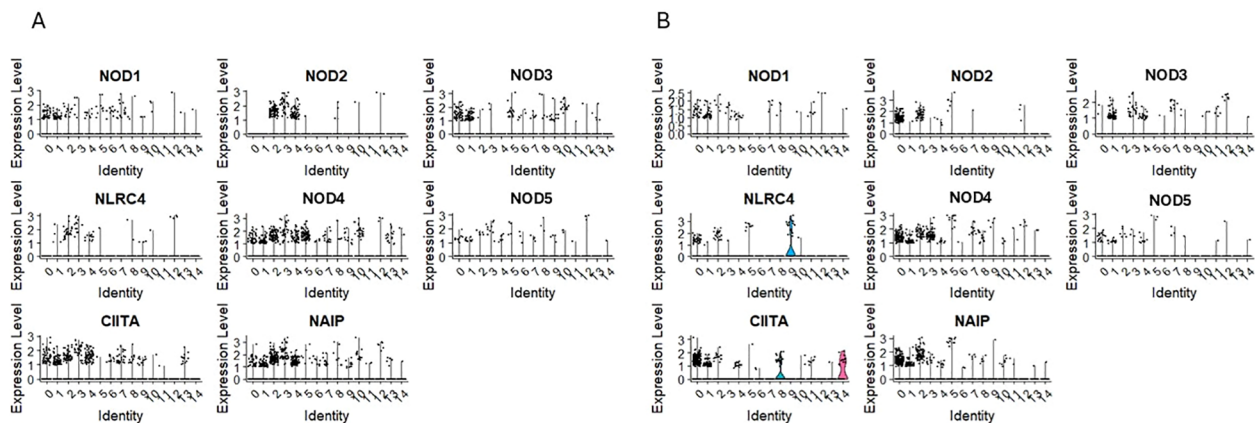


FIGURE 3

The expression of CARD domain-containing NOD-like receptor (NOD) genes in CD34+lin-CD45+ (A) and CD133+lin-CD45+ (B) cells with the division to clusters. Violin plots show differential expression of NOD1, NOD2, NOD3, NLRC4, NOD4, NOD5, CIITA, and NAIP in different subpopulations of both datasets.

3.7 Expression of mRNA for complement regulatory proteins

Opposite to complement system genes, which expression was mostly observed in ‘fate decision’ HSPCs subpopulations; the expression of complement regulatory elements was found in the primitive, quiescent subpopulations in both HSPCs datasets. Complement factor H (CFH) expression was found only in cells expressing CD34, CD133, and c-KIT in CD34+lin-CD45+ and CD133+lin-CD45+ cells (Figures 8A, B). Additionally, the expression of CD59 was found primarily on primitive subpopulations (Figures 8A, B); however, in the case of CD34+lin-CD45+, it was also found in cells expressing GYPA (Figure 8B).

3.8 Comparative analysis of proteins identified in HSPCs datasets

Using untargeted mass spectrometry for proteomic analysis, we identified 3161 and 4489 proteins for CD34+ and CD133+ HSPCs datasets (Figure 9A). Among those, 1257 proteins were common for both datasets. The highly enriched GO biological pathways and the number of proteins were involved in innate and adaptive immunity and inflammation (Figure 9B). Heatmap comparison of mean normalized intensities (Figure 9B) shows the comparative analysis of Toll-like receptors (TLR3, TLR4, TLR5) more abundant in CD133+lin-CD45+ dataset, proteins with pyrin (PYD) domain, that are most recognized for its role in signaling innate immunological responses, specifically in the formation of large

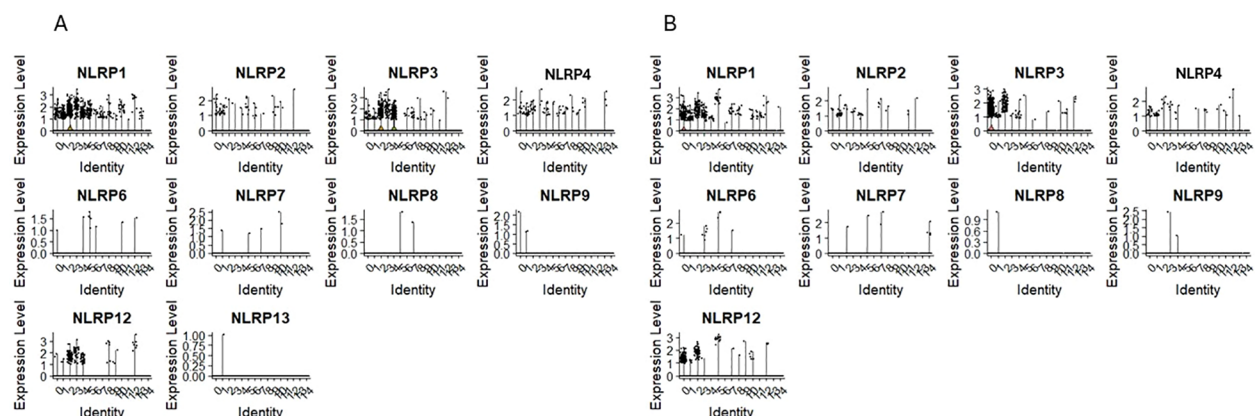


FIGURE 4

The expression of Pyrin domain-containing NOD-like receptor (NOD) genes in CD34+lin-CD45+ (A) and CD133+lin-CD45+ (B) cells with the division to clusters. Violin plots show differential expression of NLRP1, NLRP2, NLRP3, NLRP4, NLRP6, NLRP7, NLRP8, NLRP9, NLRP12 and NLRP13 in different subpopulations of both datasets.

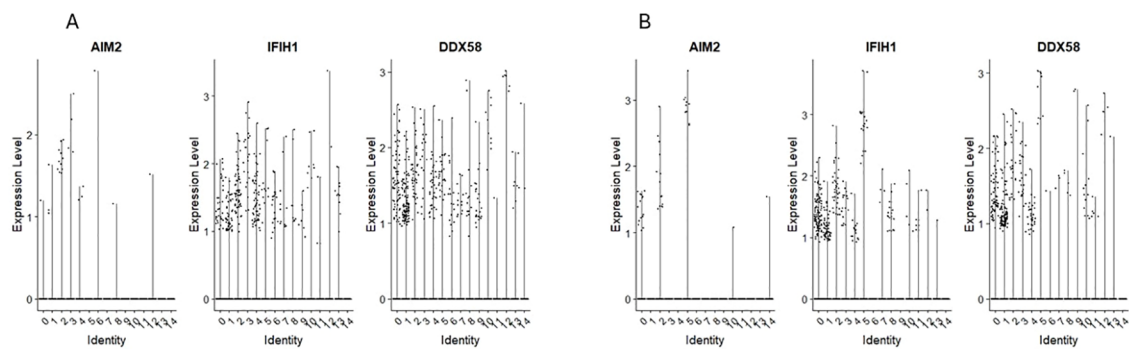


FIGURE 5

The expression of RIG-like receptors genes in CD34+lin-CD45+ (A) and CD133+lin-CD45+ (B) cells with the division to clusters. Violin plots show differential expression of AIM2, IFIH1, and RIG-1 in different subpopulations of both datasets.

protein complexes called inflammasomes, which enable the activation of caspase-1 by promoted proximity-mediated activation and the subsequent release of pro-inflammatory cytokines (60). We also detected the presence of AIM2 and ACS in our datasets, however a high abundance of AIM 2 was detected for CD34+lin-CD45+ HSCs, whereas higher mean normalized intensity was observed for ASC CD133+lin-CD45+ HSPCs.

Simultaneously, we detected increased caspase 2 and defensin for the CD133+ HSPCs dataset (Figure 9C). We also employed the Uniprot database and identified several immunomodulatory cytokines, including IL1, IFN, IL10, IL21 and IL-7 (Figure 9D).

4 Discussion

Using scRNA-seq, we confirmed that mRNA for some receptor and protein components of the immune system are expressed in purified human HSPCs, indicating cell subpopulations in which these genes are up or down regulated. There are published studies in which mRNA isolated as bulk from cells enriched for HSPCs were employed for RT-PCR analysis (18, 22, 61). To address this issue better, we used prospectively isolated by FASCs human umbilical

cord blood (UCB)-derived CD34+Lin-CD45+ and CD133+Lin-CD45+ cells and performed single-cell RNA-seq (scRNA-seq) analysis to detect mRNA of genes involved in immune responses. We analysed our mRNA expression data sets employing the Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) technique, which allows us to visualize separate cell clusters (36). Since both CD34+Lin-CD45+ and CD133+Lin-CD45+ cell populations are enriched for the cells at different levels of specification toward hematopoietic and lymphoid lineages, it was challenging to evaluate the expression of innate immunity markers in these cells. In particular, we were interested in UMAP clusters enriched for mRNA encoding antigens CD34, CD133, and c-kit receptors. We also employed untargeted mass spectrometry for proteomic analysis and identified several biological pathways and the number of proteins involved in innate and adaptive immunity and inflammation.

First, we evaluated the expression of membrane-bound and intracellular pattern recognition receptors (PPRs). PPRs are germline-encoded host sensors, which detect molecules typical for the pathogens (pathogen-associated molecular patterns – PAMPs) or release from damaged/apoptotic tissues (damage-associated molecular patterns DAMPs) (62–64). They are highly expressed

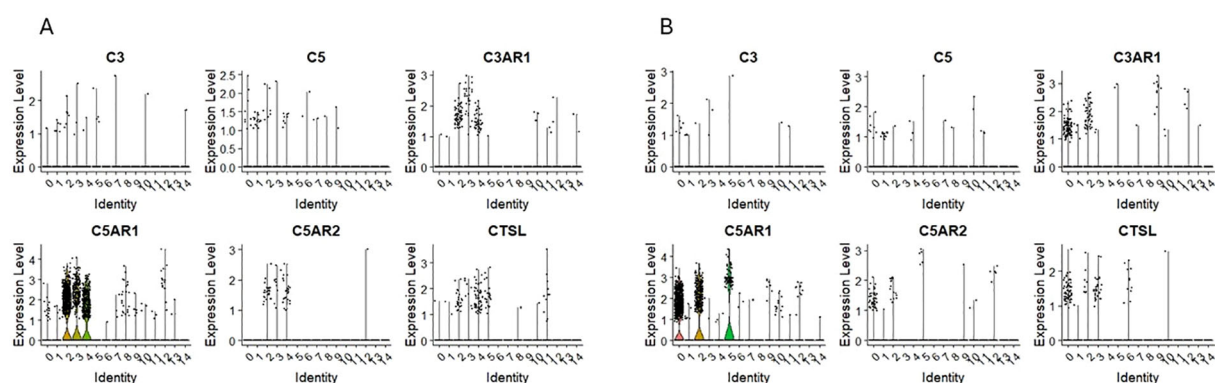


FIGURE 6

The expression of complement system genes in CD34+lin-CD45+ (A) and CD133+lin-CD45+ (B) cells with the division to clusters. Violin plots show differential expression of C3, C5, C3AR1, C5AR1, C5AR2, and CTSL in different subpopulations of both datasets.

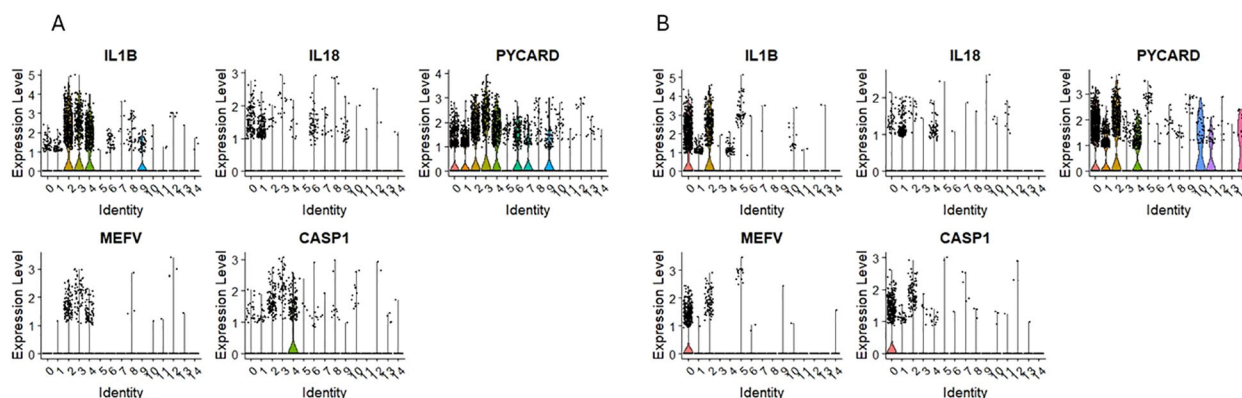


FIGURE 7

The expression of innate immunity genes in CD34+lin-CD45+ (A) and CD133+lin-CD45+ (B) cells with the division to clusters. Violin plots show differential expression of IL1B, IL18, PYCARD, MEFV, and CASP1 in different subpopulations of both datasets.

by innate immunity cells, including dendritic cells, macrophages, monocytes, and epithelial cells (64). Interestingly, as reported, BM cells from TLR4^{-/-}, TLR9^{-/-}, and MyD88^{-/-} mice have a repopulating advantage when transplanted competitively with wild-type marrow into lethally irradiated recipient animals (27, 65, 66). This suggests that TLR signaling may contribute to the maintenance of HSPCs under homeostatic conditions and that endogenous TLR ligands, such as oligosaccharide (LPS) produced by normal gut flora, may regulate HSPCs under steady-state conditions after interacting with selected TLRs (67). By employing scRNA-seq, we identified that cells enriched for mRNA encoding CD34, CD133, and c-kit express also TLR1, TLR2, and TLR6. We also detected mRNA for MYD88, which plays an important role in intracellular signalling from these receptors. Humans lack TLR11, TLR12, and TLR13 in contrast to rodents (62). The expression of Toll-like receptors is corroborated by mRNA expression for MYD88, an adaptor protein required for transducing signals from the activated Toll-like receptors (68). The

family of Toll-like receptors is a cell surface-expressed protein class that plays a key role in innate immune system responses. They are expressed on sentinel cells, including macrophages and dendritic cells, which recognize structurally conserved molecules derived from microbes (69). It has been postulated that some of them are also expressed in HSPCs (24). The receptors TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are located on the cell membrane, whereas TLR3, TLR7, TLR8, and TLR9 are located in cytosol (70, 71). In one of the reports, it has been postulated that human UCB CD34+ cells express TLR2 and TLR4 and are involved in the specification of CD34+ cells into megakaryopoiesis/thrombopoiesis (72). In another report, it was demonstrated that human HSPCs express TLR4 and can directly sense pathogens and, in response, produce cytokines that promote the emergency of granulopoiesis (73, 74). It has also been shown that signalling through TLRs 7/8 induces the differentiation of human BM CD34+ cells along the myeloid lineage and, in another report, that PAM3CSK4, a synthetic triacylated lipopeptide known as TLRs 1/2 agonist, instructs human HSPCs to

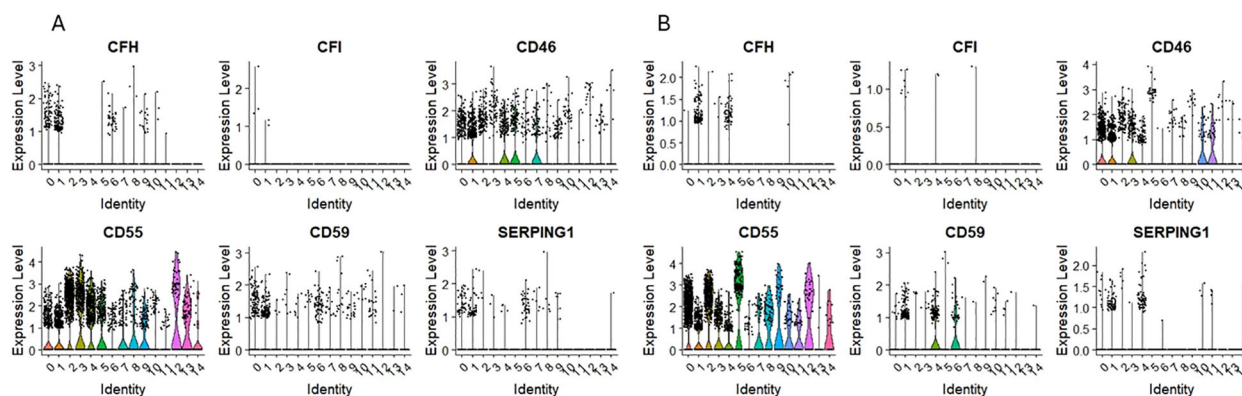


FIGURE 8

The expression of complement system regulatory genes in CD34+lin-CD45+ (A) and CD133+lin-CD45+ (B) cells with the division to clusters. Violin plots show differential expression of CFH, CFI, CD46, CD55, CD59, and SERPING1 in different subpopulations of both datasets.

Interferon-inducible protein AIM2, also known as absent in melanoma 2, is a protein that in humans is a cytoplasmic sensor

Next, based on exciting data that some types of cells, including lymphocytes, may express complement proteins (complosome) that may be functional inside cells (17, 20, 21), we evaluated the expression of mRNA for major components of the complement cascade in our purified from UCB HSPCs and focused on cell clusters enriched for mRNA for CD34, CD133, and c-kit. We noticed expressions of C5, C3, and C5aR1. Increased expression was observed mainly in the case of C3AR1 and C5AR1 but also C5AR2 in both HSPCs populations. which are receptors of C3a and C5a subunits. C3AR1 receptor, after binding to C3a, can initiate a signaling cascade leading to inflammatory reactions such as mast

Analysis of annotated proteins to CD34+lin-CD45+ and CD133+lin-CD45+ HSPCs dataset. **(A)** Vienna plot of annotated proteins for both datasets. **(B)** Comparative analysis of the mean normalized heat map for CD34+ and CD133+HSPCs dataset presents annotated common proteins corresponding to GO biological processes regulating immune response, inflammation, and cell death. **(C, D)** The mean normalized intensity displays the abundance of annotated proteins playing a pivotal role in the inflammation.

cell degranulation, cytokine secretion, and leukocyte chemotaxis. Respectively, the C5AR1 receptor, after binding to C5a, one of the most potent inflammatory mediators, can lead to various cellular responses, including increases in blood vessel permeability, neutrophil chemotaxis, and the release of proinflammatory cytokines (87). A low level of expression of C3 and C5 genes with the increased expression level of C3AR1, C5AR1, and C5AR2 genes may indicate a later stage of complosome activation in these cells. Novel data indicates that complement proteins are not synthesized only in the liver. Still, normal lymphocytes also express them and as reported, activate intracellular complement receptors intracrine-dependently (20). This novel regulatory loop operating in lymphocytes has been named complosome (17, 21), and in addition to lymphocytes (20), we have demonstrated it to be expressed in normal HSPCs (22). Some complement protein components expression corroborated our previous studies with bulk mRNA extractions (18, 22). We confirmed Dr. Kemper's data that complosome is expressed at higher levels in cells committed to lymphocytic lineage (18). Furthermore, we found that it is also expressed in HSPCs, playing an important role in the regulation of their trafficking, metabolism and proliferation (23). Our data with human HSPCs reveal that the more critical component of the complosome is C5. However, since these cells do not express all the components of proximal ComC proteins and C3 activating cathepsin L, other proteases must be involved in the activation/cleavage of C5.

As it was mentioned above, the expression of complosome regulatory elements was found in the primitive, quiescent subpopulations in both HSPCs datasets. Complement factor H (CFH) expression was found only in cells expressing CD34, CD133, and c-KIT in CD34+lin-CD45+ and CD133+lin-CD45+ cells. This regulatory protein controls the activity of the complement system to prevent damage to host tissues. It inhibits the alternative pathway by binding to C3b, accelerating the decay of C3 convertase (C3bBb), and acting as a cofactor for Factor I-mediated cleavage of C3b. Due to this, by differentiating self from non-self, the complement system does not attack its cells. The expression of CFH in quiescent cells confirms their role in preserving inactive cells for further needs. SERPING1 (C1NH) is a gene that codes for a C1 inhibitor. It is crucial in regulating the complement system's functions, controlling inflammation, and preventing excessive complement activation. We observed its expression mostly in primitive, quiescent subpopulations of HSPCs. Some expression traces were observed in other clusters of both datasets, but their level was very low. CD59, also known as protectin, is a membrane-bound glycoprotein that plays a critical role in regulating the complement system functioning, mainly by inhibiting the formation of the membrane attack complex (MAC). In our study, its expression was found primarily on primitive subpopulations; however, in the case of CD34+lin-CD45+, it was also found in cells expressing GYPA. The complement cascade is tightly regulated to protect host cells, including HSPCs, from indiscriminate attacks by its activated components (13). Complement inhibitors include the plasma serine proteinase inhibitor serpin (C1 inactivator) (88). The classical pathway is

also inhibited by surface-bound proteins CD55 (also known as decay accelerating factor or DAF) (89) and CD46 (also known as membrane co-factor protein or MCP) (90). Furthermore, CD59 is an essential regulatory protein of the complement terminal pathway, the membrane attack complex (MAC) (91). In contrast, complement factor H (CFH) is a key regulator of the alternative complement pathway, ensuring that the complement activation is directed against pathogens or harmful materials while preventing damage to host tissue (92). At the same time, complement factor I (CFI), also known as C3b/C4b inactivator, controls complement activation by cleaving C3b and C4b in both cell-bound or fluid phases (93).

Thus, our data on purified human HSPCs analysed based on transcriptome signature and categorized into subpopulations revealed that cells expressing mRNA of CD34, CD133, and c-kit also express several genes characteristic of innate and acquired immunity cells. It is important to realize that even though we sorted cells based on their very early hematopoietic development phenotype characteristic for HSPCs, only a few such cells are clonogenic in *in vitro* functional progenitor assays. These clonogenic cells, however, correspond to UMAP clusters enriched in mRNA for CD34, CD133, and c-kit.

We also employed untargeted mass spectrometry for proteomic analysis and identified several biological pathways and the number of proteins involved in innate and adaptive immunity and inflammation. We observed some similarities and differences between both populations of HSPCs.

For example, Toll-like receptors (TLR3, TLR4, TLR5) and proteins with pyrin (PYD) domain were more abundant in CD133+lin-CD45+ datasets. On the other hand, AIM2 and ACS proteins were detected in both datasets.

5 Conclusion

In this study, we provided a comprehensive analysis of human hematopoietic stem and progenitor cells (HSPCs) purified from umbilical cord blood (UCB) using single-cell RNA sequencing (scRNA-seq) and proteomic approaches. Our findings confirm that mRNA for receptors and proteins associated with innate and adaptive immunity are expressed in HSPCs, particularly in subpopulations enriched for CD34, CD133, and c-kit markers. Using scRNA-seq and UMAP clustering, we identified specific patterns of gene expression that shed light on the roles of these immune components in early hematopoietic development.

Notably, we detected the expression of Toll-like receptors (e.g., TLR1, TLR2, and TLR6) and the adaptor protein MYD88, suggesting potential involvement of these receptors in HSPC maintenance and lineage specification. Additionally, cytoplasmic pattern recognition receptors, including NOD-like and RIG-I-like receptors, were expressed in primitive HSPC clusters, implicating these receptors in hematopoietic development and immune response regulation. Furthermore, our proteomic analysis corroborated these findings and revealed differences in the

abundance of immune-related proteins between CD34+Lin-CD45+ and CD133+Lin-CD45+ cell populations.

Importantly, we observed the expression of complement components, such as C3AR1, C5AR1, and C5AR2, and regulatory proteins like CFH and SERPING1 in quiescent HSPCs. These findings highlight the potential involvement of intracellular complement signaling (complosome) in preserving HSPC integrity and modulating their response to environmental stimuli.

Collectively, our results provide new insights into the immune properties of human HSPCs, underscoring their potential role in innate and adaptive immune responses. These findings enhance our understanding of HSPC biology and may inform future therapeutic strategies aimed at harnessing the immune capabilities of these cells for regenerative medicine and immune modulation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>, PRJNA1128409.

Ethics statement

The studies involving humans were approved by Warsaw Bioethics Committee permission number KB/50/2022. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JJ: Formal analysis, Investigation, Methodology, Software, Writing – original draft. KT: Data curation, Resources, Software,

Writing – original draft. DW: Data curation, Resources, Writing – original draft. KB: Data curation, Methodology, Resources, Writing – original draft. MZR: Supervision, Writing – original draft, Writing – review & editing. MK: Conceptualization, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Non-canonical extracellular complement pathways and the complosome paradigm in cancer: a scoping review

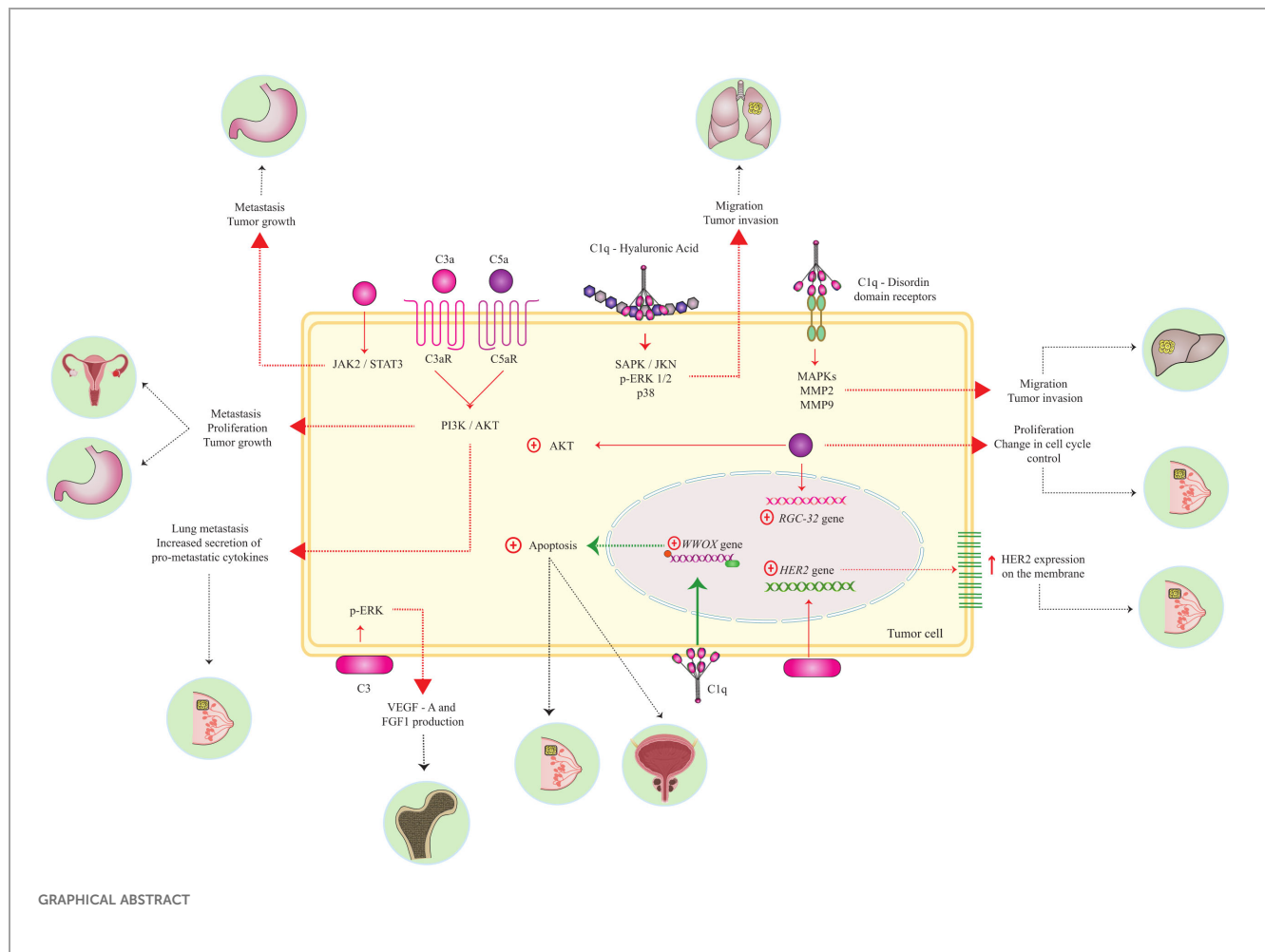
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The Complement System (CS) comprises three catalytic pathways that can be activated by specific immune triggers. However, within the tumor microenvironment (TME), CS intracellular components, recently named as complosome, play roles that extend beyond the activation and regulation of its pathways. The interaction between TME elements and tumor cells alters the local immune response, leading to inflammation, cell proliferation, and tumor invasion. Our focus is on understanding the significance of complosome and non-canonical pathways in cancer. In this scoping review, we analyzed 45 articles that discussed the various roles of CS components in carcinogenesis. Many CS components, including C1q, C3a-C3aR, C5a-C5aR, factor H, and properdin, some of them at the intracellular level, may play a dual role in tumor progression, demonstrating either anti-tumor or pro-tumor activity independent of complement pathway activation. The specific function of each component can influence both the type and stage of tumor cells. There is a notable lack of studies on the role of the lectin pathway in tumor development, and this knowledge gap must be addressed to fully understand the role of complosome in cancer. Nevertheless, the activation of CS and the roles of its components in complosome pathways are crucial steps in tumor development.

KEYWORDS

C3, C1q, C5, cancer, complement system, non-canonical activation pathway



Highlights

- C1q has an anti-tumor role through the *WWOX* oncogene in breast and colon cancers.
- The C3a-C3aR and C5a-C5aR axis activate the PI3K/AKT and ERK/MEK1-2 pathways.
- Activation of C3 plays a negative regulatory role for the oncogene Her2.
- C5a promotes overexpression of the *RGCC* gene, which controls cell cycle progression.
- Properdin showed an antitumor function by regulating pro-apoptotic factors.

1 Introduction

The complement system (CS) comprises a set of soluble, membrane-bound, and regulatory proteins that are involved in homeostasis processes. It serves as a central component of innate immunity while also providing a critical link to adaptive immunity. CS is known as a key mediator of inflammation, coagulation cascade, in the elimination of immune complexes and apoptotic cells (1–3).

Canonical complement activation induces proteolytic cleavage of key molecules through three pathways: the classical (CP), lectin (LP), and alternative (AP) pathways. CP activation occurs through antibody binding to the C1q-C1r/C1s complex (4, 5). LP is activated through the recognition of pathogen-associated, damage-associated, and altered cell-associated molecular patterns (PAMPs, DAMPs, and ACAMPs) by pattern recognition receptors (PRRs, as mannose-binding lectin -MBL, ficolins, and collectins), thereby activating the MBL-associated serine proteases MASP-1 or MASP-2 (mannan-binding lectin-associated serine proteases -1 or -2). Activation of the CP and LP pathways generate a crucial C3 convertase - C2aC4b. Yet the AP is continuously activated at low levels by the spontaneous hydrolysis of native C3, also circulating as C3(H₂O), generating the C3bBb convertase (Figure 1). C3 convertases (C2aC4b or C3bBb) cleave C3 molecules into C3a and C3b fragments (6). The C3a fragment is an anaphylatoxin that may bind to its cell receptor, C3aR, influencing several immunological pathways (as discussed in this review), whereas C3b continues the cascade, leading to the formation of C5 convertase, which cleaves C5 into C5a and C5b. C5a is an effective chemotactic factor and proinflammatory mediator and like C3a, acts as an anaphylatoxin with several biological roles. C5b binds to C6,

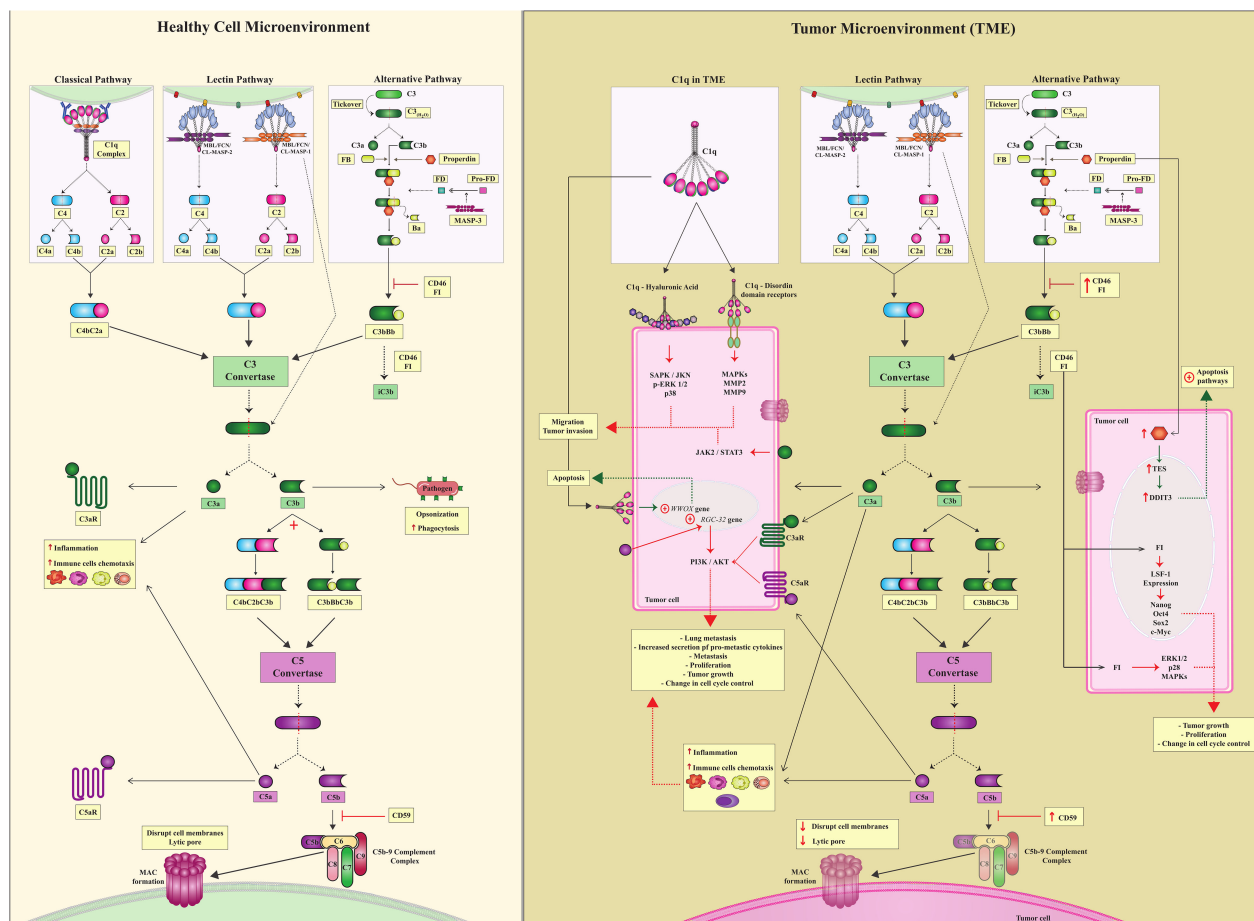


FIGURE 1

Canonical complement system and complosome pathways. (Healthy Cell Microenvironment) There are three canonical CS pathways in the microenvironment of healthy cells: classical, lectin, and alternative pathways. Different triggers activate all of them; however, these three pathways converge to form C3 convertase, which is a central complement enzyme. C3 convertase cleaves C3 into C3a and C3b. C3a is a potent anaphylatoxin capable of attracting immune cells and contributing to inflammation. C3b continues in the complement cascade and forms another essential enzyme, C5 convertase. C5 convertase cleaves C5 into C5a and C5b. C5a, just as C3a, is an anaphylatoxin, and C3b follows the cascade and leads to the formation of the C5b-9 complement complex, which culminates in the formation of MAC. To ensure controlled complement activation, complement regulators must act efficiently. (TME) In the tumor microenvironment, in addition to the canonical action of the complement system, there is also a complosome. Complosome proteins play a role in different intracellular pathways, leading to pro- or anti-tumor profiles in these cells. Tumor cells are pink. Green dotted arrows indicate the anti-tumor profile, and red dotted arrows indicate a pro-tumor profile.

C7, C8, and C9 and forms the membrane attack complex (MAC), which may disrupt cell membranes by causing lytic pores (Figure 1) (5).

Recent evidence suggests that the role of the CS extends well beyond the three traditional catalytic pathways activated in the extracellular environment (7–10). To recognize this, two new terms have been introduced by complement researchers in the past decade: *complosome* and *non-canonical pathways* (NCP). They encompass the roles of complement proteins, regulators, and receptors that do not depend on activation of the canonical pathways CP, LP and AP. Although these concepts remain under discussion (9, 11), they will be used in this scoping review to emphasize the rather unexpected involvement of complement components in cancer development. Specifically, the term NCP will refer to complement activation within the extracellular environment, without the direct involvement of any of the three

canonical pathways. In contrast, the term *complosome* will be used to describe the activity of intracellular complement components (Figure 1).

NCPs are less well defined but have gained increasing recognition for their roles in immune regulation and the modulation of inflammation. CS components that do not directly involve the activation of the CP, LP or AP in the extracellular space include serine proteases as MASPs, which interact with other proteins and receptors, such as protease-activated receptor 4 (PAR4) in endothelial cells (12–14), leading to pro-inflammatory and/or pro-thrombotic responses (15). They involve complex mechanisms, including activation via PRRs, tissue-specific factors, or indirect modulation through cytokine signaling, and contribute to the complement system's broad regulatory functions in immunity, particularly in inflammation, tissue injury, and pathogen defense.

The complosome comprises all CS proteins able to act in intracellular processes independently of the activation of the canonical pathways. It represents a recent and evolving perspective, still requiring new study designs to achieve a more comprehensive definition (9, 11, 16, 17). Despite ongoing discussion regarding which pathways and functions of CS proteins can be specifically attributed to the complosome, it is evident that these proteins have the potential to regulate intracellular pathways through endosomes (18, 19), mitochondrial membranes (20, 21) and direct gene regulation (22, 23). The core complosome components are C3 and C5, as well as their respective membrane receptors. Furthermore, the complosome can be found in immune and non-immune cells, as well as in different tissues (11). Although still poorly investigated in the TME, the complosome may hold an additional key for a pro- or anti-tumor outcome (16).

Cancer is one of the most common health problems worldwide. In 2020, the International Agency for Research on Cancer reported 19.3 million new cases and almost 10 million deaths from cancer worldwide (24–26). The mechanisms underlying cancer development and progression are not completely understood. The TME is considered to be a key promoter of carcinogenesis, along with genetic and epigenetic factors (27–31). Crosstalk between TME elements and tumor cells modifies the local immune response, inducing inflammation and suppressing the anti-tumor response, thereby facilitating tumor proliferation and invasion (32–35). The hypothesis that chronic inflammation is closely related to malignant tumor transformation and progression has been substantiated by human epidemiological data and genetic experiments (36). However, the interplay between non-canonical complement pathways, the complosome and the TME has been largely out of the spotlight in cancer research. In this scoping review, we discuss the available data published till 2020 at the light of recent evidence, focusing on understanding the implications of activating these poorly known arms of the CS, within tumoral cells and the TME.

2 Materials and methods

2.1 Data sources and literature search strategy

For this scoping review, we followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for Scoping Reviews Checklist (37). We performed a systematic search of the role of the complement system in TME on two main databases: PubMed and Web of Science. For the descriptors, we selected MeSH Terms in PubMed (“neoplasms”[MeSH Terms]) AND (“complement system proteins”[MeSH Terms]) and topics (Ts) in Web of Science (cancer AND (Complement system)) AND (OPEN ACCESS) AND (ARTICLE). We retrieved a total of 550 articles from PubMed and 69 from the Web of Science, and excluded duplicate articles using Excel (n=32).

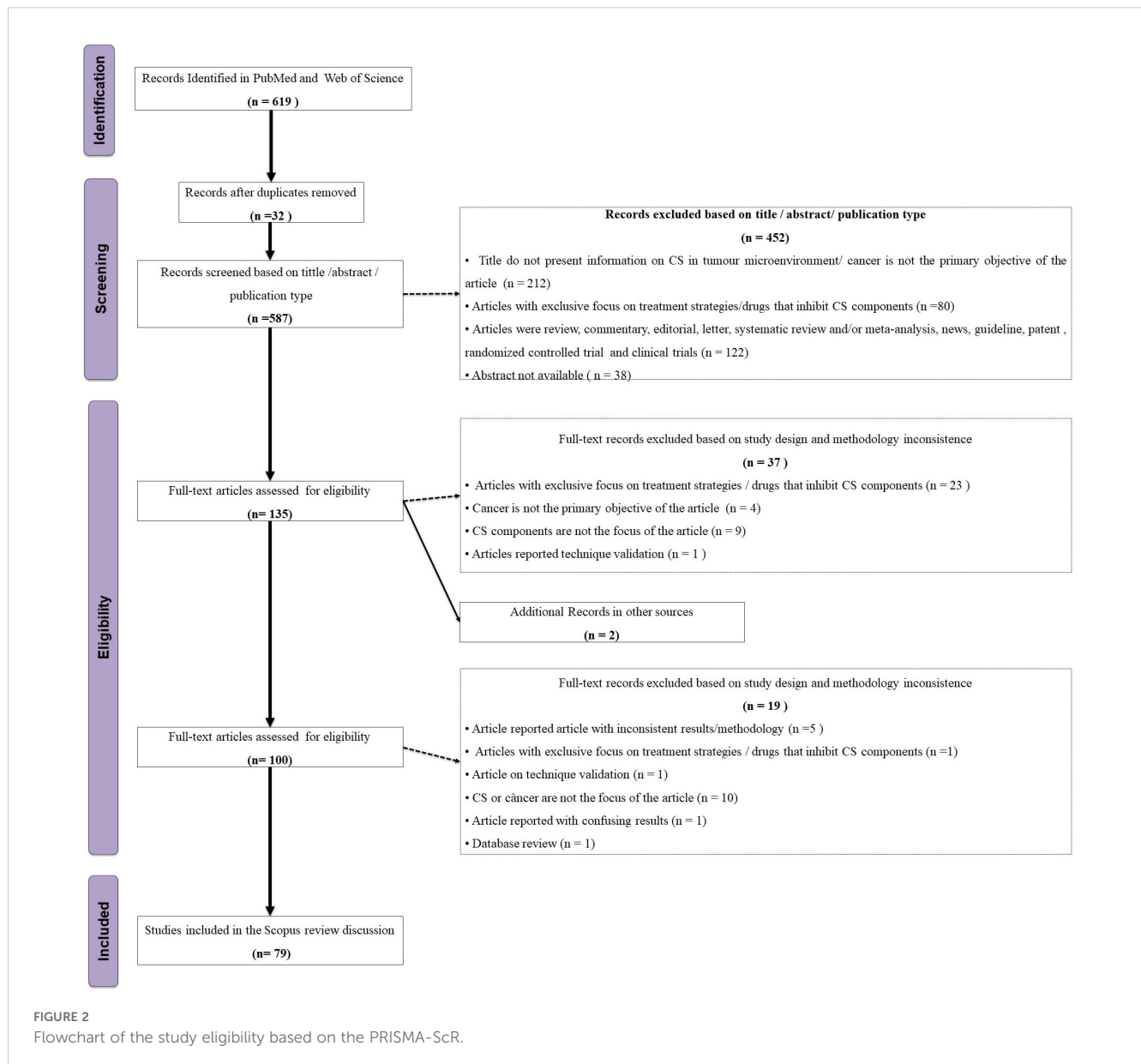
2.2 Study eligibility – inclusion and exclusion criteria, and limitations

Independently of each other, two of us (CFO-T and AGM) screened the titles and abstracts of 619 articles for the following inclusion criteria: articles in English, with available abstract, open access, in humans or animal models, *in vivo*, *in vitro*, or *in silico*, and published from November 26th, 2010 to November 26th, 2020 (Figure 2). Exclusion criteria were: articles whose subjects were not components of the CS and cancer; studies classified either as review, commentary, editorial, letter, systematic review and/or meta-analysis, news, guidelines, clinical trials/controlled trials, and patents. We further excluded articles without an abstract and those with an exclusive focus on treatment strategies/drugs that inhibit CS components, retrieving a total of 135 articles, grouped according to the investigated topic. Next, five of us (CFO-T, HMBSP, NMDLS, PDF, and TPB) distributed the 135 articles among themselves according to their area of expertise and assessed their full content. We discussed and resolved any doubts by consensus, retrieving 45 studies for the final analysis (Figure 2, Supplementary Table 1). We extracted the following information from the selected studies: first author’s name, year of publication, title, experimental model, cancer type, complement components, anti/pro-tumoral role of complement components in the cancer context, and experimental methods. This review is limited by the inclusion of articles published up to 2020 and accessible through open access. As a result, relevant studies published after this period or behind paywalls may not have been considered. Additionally, as with any scoping review that relies on keyword searches in databases, some articles may have been unintentionally excluded due to variations in search terms or database indexing. Nevertheless, we made every effort to incorporate all relevant studies within the defined scope of this review.

3 Results

3.1 C1q complex

The C1 complex is composed of three proteins, C1q, C1r, and C1s. C1q is the recognition unit which is bound to two molecules each of the serine proteases C1r and C1s (C1q-C1r2-C1s2) (38, 39). C1q has a structural protein configuration consisting of a globular region and collagen-like domain. The globular region comprises three different subunits, A, B, and C, which form a trimer that is capable of recognizing a wide range of ligands. Apart from its role in CP activation, C1q interacts with complement receptor 1 (CR1), promoting phagocytosis of opsonized elements by mononuclear phagocytes and regulating their cytokine profile. C1q-stimulated macrophages upregulate pro-phagocytic genes, enhancing their phagocytic activity and altering cytokine expression, thereby suppressing the expression of IL-1 (interleukins 1 alpha and beta) and increasing secretion of interleukin-10 (IL-10), IL-1 receptor



antagonist, monocyte chemoattractant protein-1, and interleukin-6 (IL-6) (40–42). It also plays an important anti-inflammatory role in maintaining homeostasis by aiding immune cells such as monocytes/macrophages and red blood cells in clearing immune complexes and apoptotic cells. Below we present our principal results, regarding C1q's CP-independent role in cancer.

3.1.1 C1q and C1r/C1s gene expression and protein levels in cancer

C1q-deficient mice lacking the *C1qa* gene exhibited reduced B16 melanoma tumor mass and vascular density, as well as prolonged survival. C1q further induces proliferation, migration and murine melanoma cell adhesion, corroborating the hypothesis that C1q promotes melanoma progression *in vivo* independent of the CP activation (43). In line with this, tumor-associated macrophages (TAMs), resembling M2-like macrophages, as well

as monocytoïd B cells, contribute to elevated C1q levels in the TME. C1q-producing TAMs were associated with an immunosuppressed TME in clear cell renal cell carcinoma (ccRCC), characterized by high expression of immune-checkpoint molecules (PD1, LAG3, PDL1 and PDL2) (44). High C1q concentrations also occur in the TME of both human low-grade gliomas (LGGs) and glioblastoma multiforme (GBMs). In GBM, C1q was specifically associated with vascular endothelial cells, and its source could again be attributed to TAMs (45). C1q protein was diffusely present in the TME and expressed by TAMs in basal-like breast cancer and lung adenocarcinoma, whereas in clear cell renal cell carcinoma it was found in the small vessels and on the cell membrane of tumor cells - besides the TME. Expression of all three genes encoding C1q chains was associated with tumor growth and a poor prognosis in these types of lung and kidney cancer, despite representing a favorable prognosis in Her-2 positive and basal-type breast cancer (Figure 3)

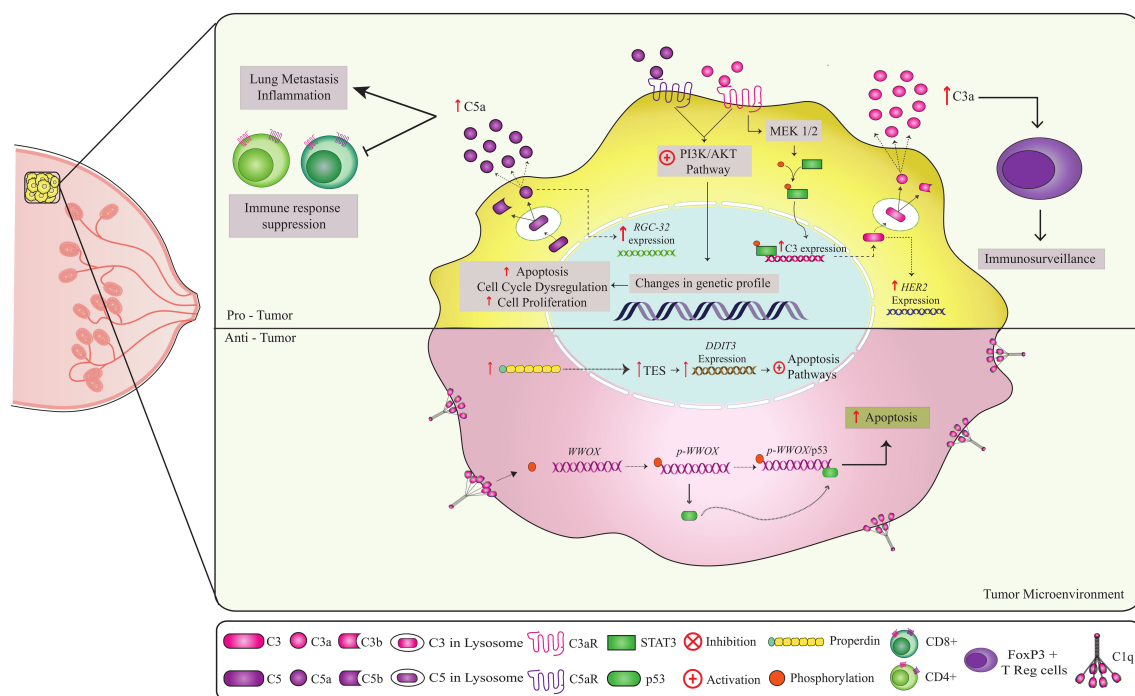


FIGURE 3

Non-canonical pathways involving complement components in breast cancer. Complement components play a role in both anti-tumor and pro-tumor responses. C1q and properdin have anti-tumor effects in breast cancer (51, 53, 91, 92). Binding of C1q to the cell at the breast tumor membrane leads to Tyr33 site phosphorylation of the WWOX protein, enabling the binding of p53 to WWOX gene. The p-WWOX-p53 complex is carried to the mitochondria and nucleus, where it activates apoptotic pathways (51). The high expression of intracellular properdin in tumor cells induces stress on the endoplasmic reticulum, leading to the expression of molecules that act in the increase of TES transcription, causing an increase in the expression of the DDIT3 gene that activates apoptotic pathways. Thus, both C1q and properdin contribute to the elimination of tumor cells. In contrast, on the C3a-C3aR and C5a-C5aR axes, CD46 and CD59 may have a pro-tumor role. Activation of the C3a-C3aR axis leads to PI3K/AKT and MEK1/2 pathway activation (63, 64). The PI3K/AKT pathway is a key regulator of the cellular processes involved in cell growth, metabolism, motility, survival, and apoptosis. Uncontrolled activation of this pathway promotes tumor cell survival and proliferation, leading to tumor progression and resistance to cancer therapy. Intracellular production of C5a leads to the C5a-C5aR axis, which can activate the PI3K/AKT pathway. C5a also promotes overexpression of the RGC-32 gene (complement response gene 32), which controls cell cycle progression. High expression of the regulators CD46 and CD59 can potentially worsen prognosis and inhibit apoptosis, respectively. In addition, C3 has been shown to be involved in tumor immunosurveillance, probably through interactions with FOXP3+ T (Tregs), which are essential for the maintenance of immune tolerance and immune homeostasis. Thus, the combined action of these components can have a crucial effect on clinical outcomes.

(46). Notably, there was no evidence of C3d nor C4d immunoreactivity and thus for complement activation in gliomas (45). In fact, deposition of other CS components seems to be infrequent in most tumors, suggesting that C1q operates in the TME independently of CP activation (43).

3.1.2 C1q or C1r/C1s NCP signaling in cancer

In vitro studies have shown that C1q binds to high molecular weight hyaluronan (HMW-HA) in the extracellular matrix, triggering adhesion and proliferation of mesothelioma cells. This occurs through an increased phosphorylation of ERK1/ERK2 (extracellular signal-regulated kinases), SAPK/JNK, and p38, regardless of CP activation in a NCP manner (47). In a similar way, C1q has been shown to bind *in vitro* to discoidin domain tyrosine kinase receptors (DDR) at the membrane of hepatocellular carcinoma cell lines. The C1q-DDR complex interacts with MMP2 and MMP9, two zinc-dependent matrix metalloproteinases that degrade extracellular matrix, promoting tumoral cell migration and

tissue invasion (48). These data support the idea that C1q may increase the aggressiveness of liver cancer, as well as of mesothelioma, though through different mechanisms. Yet in breast and prostate cancer cell lines, extracellular C1q was involved in initiating a signaling pathway that activates the expression of the WWOX (WW domain-containing oxidoreductase) tumor-suppressor gene. In these particular tumor types, diminished C1q deposition was associated with an enhanced cell proliferation and less apoptosis (Figure 3) (22, 23).

In contrast to previous examples, cutaneous squamous cell (cSCC) lines exhibit high expression levels of *C1r* and *C1s* mRNA and protein, while lacking expression of genes producing C1q proteins. These cell lines secrete C1s and C1r, which leads to their activation in a C1q-independent manner. Consequently, activation of latent MMP9 occurs, accompanied by inhibition of extracellular signal-related kinase 1/2 and Akt activation (49). These findings suggest a potential role for these serine proteases in promoting cSCC tumor growth, angiogenesis, and metastasis.

3.2 C3 component

The canonical AP can contribute to up to 80% of the overall complement response and remains active even after activation of the CP and LP. These pathways converge to form and activate C3 convertase, which is a central step of the cascade that leads to the cleavage of C3, producing C3a and C3b. Moreover, AP C3 convertase amplifies C3b deposition on cell membranes, facilitating immune surveillance. C3a acts as a potent anaphylatoxin and induces chemotaxis, cell activation, and inflammation by binding to the C3aR receptor. The conformational change from C3 to C3b exposes previously hidden binding sites, enabling distinct interactions and efficient immune surveillance. Interactions between C3b and complement receptors/regulators, such as CR1/CD35 and CD46, was shown to lead to its degradation into iC3b and/or C3dg fragments, allowing immune adhesion, phagocytosis, and adaptive stimulation (Figure 1) (6).

3.2.1 C3 gene expression and protein levels in cancer

In the neuT mouse model of breast cancer, C3 accumulates specifically in the vessels and stroma undergoing tumorigenesis in the mammary gland, while remaining absent in the surrounding tissue (Figure 3). These cancer cells express high levels of *Her2* and of the complement regulator CD55, preventing C3 cleavage by the C3 convertase. In the neuT C3^{-/-} knockout mice, autochthonous

mammary carcinomas started earlier and grew faster, reaching larger volumes in less time and presenting greater multiplicity, sooner seeding lung metastases. Tumors in these C3^{-/-} mice showed 6- to 8-fold higher levels of Her2/neu expression. Thus, C3 may limit Her2 expression and, consequently, cell proliferation. The número of regulatory T cells (Tregs) did also increase in tumors from neuT-C3^{-/-} mice, contributing to a more immunosuppressive environment and favoring tumor growth. Although the number of tumor vessels was similar between neuT and neuT-C3^{-/-} mice, the vessels in C3-deficient tumors were wider and more permeable, resulting in better nutrient and oxygen supply to the tumor (Figure 3) (50).

3.2.2 C3 and C3aR NCP signaling in cancer

In lung cancer, CD4⁺ T cells present intracellular C3 cleavage by cathepsin and C3a signaling inhibits the production of multiple cytokines by CD4⁺ T cells, independently of FOXP3⁺ Tregs (51) (Figure 4). *In vitro* studies revealed that iC3b is inactivated by prostate-specific antigen (PSA) in prostatic fluid, generating a novel 37 kDa fragment independently of factors H and I, smaller than that produced by the canonical pathways. Notably, the new iC3b fragments appear to promote an immunosuppressive prostatic tumor profile, although their biological function is still unclear (52).

C3a has the potential to activate ERK/MEK1-2 kinases promoting cell proliferation and apoptosis resistance, regulating synaptic plasticity and p53 phosphorylation, e.g. human bone osteosarcoma epithelial cells (U2-OS) exposed to normal serum

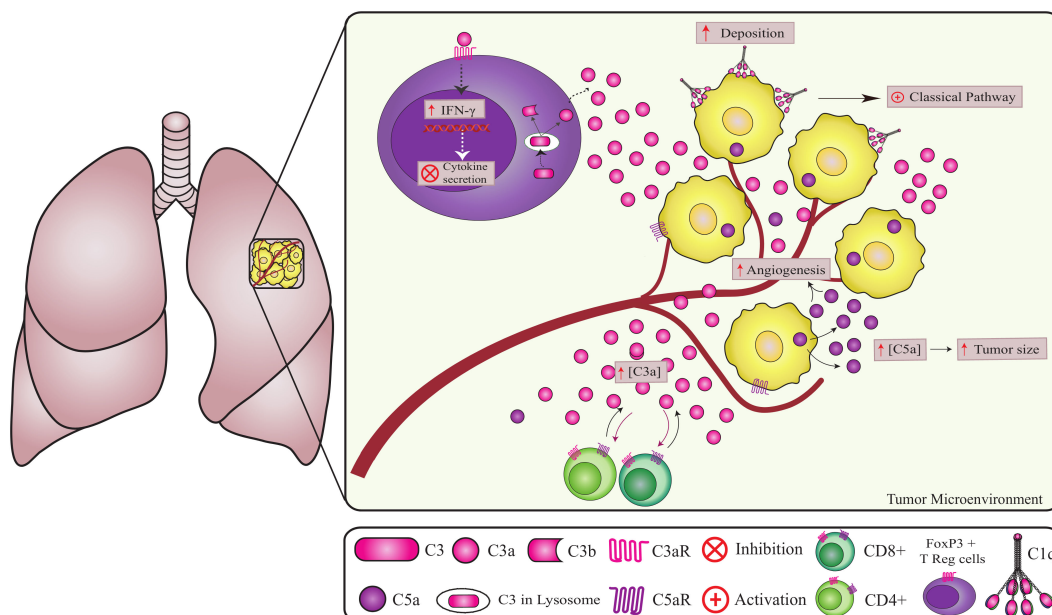


FIGURE 4

Non-canonical pathways involving complement components in lung cancer. Complement components play a crucial role in lung cancer progression. C1q promotes angiogenesis independent of CS activation (2). However, the origin of intracellular C5a in cancer cells remains unknown. Increased extracellular C5a levels lead to tumor growth and angiogenesis (72, 74). The high deposition of factor H on the tumor cell membrane contributes to tumor progression and can be considered a marker for lung adenocarcinomas. A high concentration of C3 in the TME reduces CD4⁺ T cells, promoting metastasis and tumor growth. In addition, CD4⁺ T cells can produce and cleave C3 intracellularly via cathepsin leading to the regulation of IFN γ (interferon-gamma) secretion (60, 61).

exhibited increased ERK phosphorylation compared to U2-OS treated with heat-inactivated serum (53). In gastric cancer, deposition of C3 fragments on tumor cells activates the JAK2/STAT3 pathway, increasing levels of phosphorylated STAT3 (promoting cell proliferation and migration) and IL-6 (54). Ovarian cancer cells secrete high amounts of C3, whose non-canonical cleavage (independent of CP, AP and LP) leads to C3a production in the TME. C3a binding to C3aRs on the tumoral cell membrane activate the PI3K/AKT/mTOR pathway, boosting protein synthesis, cell proliferation, survival, and motility by increasing AKT mRNA levels and phosphorylating p85, a PI3K regulatory subunit (55). Similarly, in ovarian cancer mice models (C57BL/6), the C3a-C3aR interaction triggers PI3K/AKT pathway activation, enhancing C3 secretion, tumor growth, and proliferation via a C3-dependent autocrine loop (55). Finally, C3a-C3aR signaling through PI3K-AKT promote lung metastases of breast cancer in a murine model, modulating carcinoma-associated fibroblasts to increase secretion of pro-metastatic cytokines and expression of extracellular matrix components (Figure 3) (56).

3.3 C5 component in cancer

The final phase of the CS cascade starts with the formation of C5 convertase, which catalyzes the cleavage of the C5 component into C5a and C5b (Figure 1). C5a plays a role as a potent anaphylatoxin and chemoattractant for neutrophils, monocytes, and macrophages (57). Additionally, intracellular C5a modulates cytokine expression in various cell types, playing a role in activating coagulation pathways and inducing the formation of neutrophil extracellular traps (NETs). The C5a receptor (C5aR), a member of the rhodopsin family of G protein-coupled receptors, is predominantly expressed by myeloid cells in the lung and liver. Activation of C5aR triggers diverse local responses depending on the cell type, with implications in cancer, particularly in the generation and modulation of anti-apoptotic responses, among other functions (Figure 1) (58).

3.3.1 C5 and C5aR gene expression and protein levels in cancer

In lung (59), colon (60), ovarian (36), and bile duct cancers (60), C5a production does not appear to be associated with the activation of the CP or AP, a matter that still needs to be clarified (59, 60). *In vitro*, lung cancer cells produced C5a independently of the expression of factor B, properdin, C1q, C1r, C1s, C1, and C4 (Figure 4) (59). However, C5a production was reduced by inhibitors of trypsin-like serine proteases. MASP-1, a serine protease from the LP with trypsin-like activity, might play a role in producing tumoral C5a. However, further experiments using inhibitors of LP serine proteases are required to confirm this possibility (59).

3.3.2 C5a and C5aR NCP signaling in cancer

Similarly to C3, C5 activation plays a pro-tumoral role in different types of cancer. Certain types of cancer cells, such as those found in lung, colon, ovarian, and bile duct cancers, secrete C5a into the TME, independently of CP and AP, initiating an autocrine loop that enhances cell proliferation and fosters metastasis. Mice with lung cancer, if treated with C5aR antagonist, slow the growth of the tumor and decrease angiogenesis (59, 60).

In lung cancer tissue and cell lines, C5a contributes to an immunosuppressive microenvironment by recruiting myeloid-derived suppressor cells and inducing the expression of several immunomodulators, including arginase 1 (ARG1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), IL-6, IL-10, lymphocyte-activation gene 3 (LAG3), and programmed cell death 1 ligand 1 (PD-L1 or CD274) (59). Similarly, in ovarian cancer, elevated levels of C5a impact the levels of immunoregulators in the TME, such as arginase, inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), and tumor necrosis factor alpha (TNF- α), thereby reducing the infiltration of CD4+ and CD8+ T cells and promoting tumor growth (36). High C5a levels stimulate lymphoma with decreased infiltration of CD4+ and CD8+ T cells, while low levels of C5a in the TME decrease tumor progression, thus, the local concentration of C5a, independent of complement activation, seem to be critical to determine its role in tumor progression (61).

In human breast cancer cell lines, C5a induces the overexpression of the *RGCC* gene (response gene to complement 32 protein, involved in cell cycle progression), by activating the Akt pathway (Figure 3) (62). Additionally, C5a-C5aR signaling facilitates lung metastasis, a common occurrence in breast cancer, by suppressing the responses of CD8+ and CD4+ T cells and promoting the generation of regulatory T cells (Tregs) *in situ* (Figure 3) (63). The mechanism of this C5aR-mediated T cell suppression in the metastatic target involves the recruitment of immature myeloid cells and increased production of transforming growth factor β (TGF- β) and IL-10, favoring the generation of Treg and Th2-oriented responses that render CD8+ T cells, dysfunctional (Figure 3) (63).

In gastric cancer, C5a-C5aR recognition regulates p21 expression through the PI3K/AKT axis. Moreover, increased C5aR expression by the MKN1 and MKN7 gastric cancer cells enhances their invasiveness and promotes liver metastasis, which is associated with a poor prognosis (64). In these cells, C5a-C5aR signaling promotes the conversion of RhoA-GDP (RhoA-guanosine diphosphate) to RhoA-GTP (RhoA-guanosine triphosphate) in the cytosol, leading to cytoskeletal rearrangement and increased invasive capacity (65). In fact, in renal cell carcinoma the activation of the C5a-C5aR axis was indicated as a prognostic marker, with C5a stimulating ERK and PI3K-dependent invasion in renal cell carcinoma cells expressing C5aR (66, 67).

In metastatic pancreatic invasive ductal adenocarcinoma, podocalyxin-like protein 1 (PODXL1) activates C5aR on the cell membrane, increasing cellular motility. This activation confers invasive and metastatic properties to PDAC cells (68). Furthermore, C5aR1 signaling induces the secretion of chemokine C-C motif ligand 2 (CCL2) by melanoma cells. This attracts immunosuppressive populations of myeloid cells, supporting tumoral growth and facilitating the infiltration of leukocytes with an immunosuppressive profile in the TME. Conversely, C5aR2 plays a more limited yet beneficial role in restraining tumor growth (69).

In summary, C5a/C5aR signaling plays a significant pro-tumoral activity in several tumor types. Binding of C5a to C5aR in tumor cells correlates with reduced overall survival and recurrence-free survival, while also elevating the incidence of microvascular invasion and metastasis of gastric cancer and renal cell carcinoma (Figures 1, 3, 4) (66, 67).

3.4 The final complement lytic pathway in cancer

The final complement lytic pathway serves as the common intersection for the three canonical complement pathways, which culminate with the disruption of plasma membranes of target cells or microorganisms (Figure 1) (70). Complement regulators restrict CS activation on most tumors. However, sublytic MAC deposition on cancer cells raises intracellular Ca^{2+} levels, altering cellular functionality and causing cellular damage (70). MAC activity is crucial for maintaining cellular and tissue homeostasis and promoting protection against infections. It may also maintain tumor-associated inflammatory signaling, assuming a dual role in cancer (71, 72).

3.4.1 The role of MAC and C7 in cancer

Exposure of cancer cells to sublytic levels of MAC alters the expression of G protein and Ca^{2+} signal transduction (ITPRIP, RGS16), transcription factors (EGR1, EGR2), inflammatory response genes (Interferon Regulatory Factor 1 - IRF1), as well as four other extracellular protein genes (Amphiregulin - AREG, C-X-C Motif Chemokine Ligand 1 - CXCL1, Matrix Metalloproteinase 3 - MMP3, and MMP13), impacting cell proliferation and survival (73). In this way, sublytic MAC deposition on cancer cells emerges as a potent stimulator of tumor progression.

C7 is an important protein in the MAC formation. Increased amounts of C7 protein in the nuclei of hepatocellular carcinoma cell lines promotes *in vivo* cell growth, by upregulating late SV40 factor (LSF-1) protein levels and the expression of stemness factors such as POU Class 5 Homeobox 1, SRY-Box Transcription Factor 2, and MYC Proto-Oncogene Transcription Factor (encoded by OCT4, SOX2, and MYC, respectively) (74). These alterations in gene expression sustain the replication capacity of liver tumor-initiating cells, while suppression of C7 inhibited the establishment of human tumors in NOD/SCID (non obese diabetic/severe combined immunodeficiency) mice (74).

On the other hand, in non-small cell lung cancer (NSCLC), C7 overexpression suppresses colony formation *in vitro* and lower C7 expression was associated with worse outcome, advanced clinical stage and grade, increased likelihood of relapse and death (75). Therefore, the anti- or pro-tumorigenic activity of C7 is influenced by the different cancer types. While these components act as independent drivers of tumor immune response and surveillance, further investigation is needed to clarify their *in vivo* effects on carcinogenesis and tumor progression.

3.5 Complement regulators in cancer

Complement activation leads to various biological processes extending beyond its protective function and can, in some cases, pose harm. Therefore, safeguarding self-tissues from complement-mediated damage is crucial, achieved through the actions of several soluble regulators or membrane-bound counterparts. Different cell types rely on a combination of complement regulatory proteins (CRPs) to modulate the cascade at various points and in diverse manners. Noteworthy membrane-bound CRPs in tumors include CD46, CD55, and CD59, while soluble ones encompass factor I, factor H, and properdin (76, 77). Although CD46, CD55, and CD59 are pivotal complement regulators expressed across most cell types and tumor cells, they exhibit a double-edged sword role. While they avert complement-mediated autologous lysis in normal cells, their aberrant overexpression impedes complement-mediated lysis, thereby fostering tumor cell survival and progression. Consequently, membrane-bound CRPs may serve as biomarkers of malignant transformation (78–81).

CD59, a prevalent regulator expressed in most tumor cells, is a glycosylphosphatidylinositol (GPI)-anchored membrane protein that impedes C9 polymerization and the attachment of C9 units to the C5b-8 complex and MAC through its physical incorporation into the complex (79), thereby preventing membrane disruption and cell lysis. CD55, referred to as a complement decay-accelerating factor (DAF), expedites the decay of C3 and C5 convertases by swiftly dissociating the catalytic subunit C2a or Bb from the cell surface, thus preventing the generation of anaphylatoxins, opsonins, and MAC. Additionally, CD55 can recognize C4b and C3b fragments produced during C4 or C3 activation (78). These CRPs exert significant modulation over complement activity and may profoundly influence tumor progression.

The factor H (FH) is a plasma glycoprotein that acts as a soluble inhibitor of complement, where its binding to self markers such as glycan structures prevents complement activation and amplification on cell surfaces (82, 83). FH ensures the complement system spares host tissues from damage, by destabilizing the AP C3 convertase complex (C3bBb). Its dysfunction in age-related macular degeneration and atypical hemolytic uremic syndrome highlights its importance in maintaining the balance between effective immunity and self-damage. In contrast, properdin stabilizes C3bBb, amplifying pathogen opsonization, inflammation, and MAC formation. Properdin is unique as one of the few complement components that act as a positive regulator in the

system (84). As a plasma glycoprotein, the complement factor properdin (CFP) is the only known positive regulator of the CS. It plays a crucial role in stabilizing surface-bound alternative pathway (AP) C3 and C5 convertases (64, 85).

3.5.1 CD59, CD55 and CD46 gene expression and protein levels in cancer

Head and neck cancer cells exhibit high expression of CD46, CD55, and CD59 (86). CD59 is highly expressed on tumoral cells, protecting them against complement lysis, and aiding in immune evasion. CD59 expression correlated with TAM infiltration in pancreatic cancer tissues. Pancreatic cancer-educated macrophages upregulate CD59 expression on pancreatic cancer cell lines through STAT3 phosphorylation via the IL-6R/STAT3 signaling pathway and protect them from complement-dependent cytotoxicity (81). Similarly, in human ovarian A2780 cancer cells, IL-6 stimulates CD59 expression at low concentrations, but at high concentrations or with IL-8, it presents a post-transcriptional inhibitory effect (87). Indeed, CD59 immunoreactivity is detected in up to 50% of ovarian tumors and at the border areas between normal and malignant tissue (87). Similarly, increased CD55 and CD59 expression was observed on cell membranes and in the TME of ovarian and uterine cancers (80, 88). Additionally, CD55 overexpression predicts poorer clinical outcomes in colorectal cancer, with stromal CD55 overexpression correlating with unfavorable prognostic markers (78). Beyond its role in inhibiting canonical complement action within the TME, CD59 was shown to impede apoptosis of breast cancer cells, thereby contributing to tumor development (79).

High CD46 expression is an unfavorable prognostic factor associated with lower relapse-free survival in breast cancer (89). Notably, the *CD46* gene promoter has two binding sites for STAT3. If induced by STAT3 and IL-6, CD46 expression protects cancer cells against complement lysis and fosters a pro-tumoral profile in breast and prostate cancers (89). On the other hand, low CD46 expression rates in cervical cancer correlate with increased patient's survival (90). CD46 shifts its expression profile in cervical cancer cells, compared to normal female reproductive tissue, suggesting its involvement in tumorigenesis.

3.5.2 Complosomes with FH and properdin in cancer

As is the case with complosome involving C7, intranuclear FH promotes via LSF-1, the expression of *NANOG*, *OCT4*, *SOX2*, and *MYC* genes. On their turn, these stemness factors promote liver tumor formation and growth *in vivo* (Figure 1). Notably, increased FH expression, particularly within the nucleus, suggests enrichment in tumor cells (74). Similarly, FH and its isoform FHL-1 expression is specifically induced during cutaneous carcinogenesis (cSCC) and exert effects on intracellular pathways, being negatively regulated by the inhibition of ERK1/2, p38, and MAPK pathways, while being upregulated by IFN γ , IL-1b, TGF- α , TGF- β , and TNF- α . FH/FHL-1 silencing inhibited tumoral cell proliferation and migration, pointing to a critical protumorigenic role (91).

In the complosome network, properdin presented an anti-tumoral role through a novel tumor suppressor pathway

identified in breast cancer (Figures 1, 3). High expression of intracellular properdin induces the transcription of the Testin LIM domain protein (TES), subsequently leading to increased expression of DDIT3 (DNA-Damage-Inducible Transcript 3), a pro-apoptotic transcription factor associated with the endoplasmic reticulum stress response. Consequently, increased properdin expression modulates cell apoptosis, playing an anti-tumoral role in breast cancer (Figure 3) (92). Furthermore, *in vitro* studies have demonstrated that bone marrow-derived macrophages (BMDM) from properdin-deficient mice can stimulate B16F10 melanoma cell lines. This properdin deficiency results in low levels of IL-1b mRNA and higher levels of arginase-1, monocyte chemoattractant protein-1 (MCP-1), and IL-10 mRNAs, suggesting that a properdin-deficient tumor microenvironment may induce a profile of M2 macrophages with pro-tumoral activity (92).

Although there is currently no significant association between properdin expression and cancer prognosis, this regulator plays an important anti-tumoral role in complosome pathways in breast cancer³ (93). In summary, FH acts as a pro-tumoral factor in hepatocellular carcinoma, while properdin serves as an anti-tumoral factor suppressing breast cancer/melanoma. These two CRPs likely play a role in complosome pathways by modulating tumor formation and growth.

4 Discussion

Research over the past decade has firmly established that CS components play a pivotal role in driving hallmark features of cancer. These elements contribute to critical tumor processes such as angiogenesis, invasion, metastasis, immunomodulation, metabolic reprogramming, microenvironmental remodeling, unchecked proliferation, and therapy resistance. While canonical complement activation pathways are implicated in many of these mechanisms, emerging evidence highlights the substantial involvement of CS components through activation-independent pathways. Its role in cancer is context-dependent, shaped by its cellular source, interaction partners, and downstream signaling pathways.

We found little evidence for complosome activity in cancer, but rather for NCP-mediated activation of intracellular signaling pathways, suppressing tumors in certain settings (e.g., for C1q, by activating *WWOX* in breast and prostate cancer cells (22, 23), and more commonly driving malignancy by stromal interactions, receptor signaling, and matrix remodeling, (e.g. C1q by engaging receptors as DDR1 and HA to activate MAPK signaling or modulate MMPs, fostering tumor aggression) (47, 48). A recent study published after the timeframe of our scoping review described a pro-tumorigenic C1q-driven mechanism in pleural mesothelioma (PM). The researchers identified a functional interplay between hyaluronidase-2 (*HYAL2*)—a prognostic marker associated with poor PM outcomes—and the hyaluronic acid-binding protein gC1qR (globular C1q receptor/HABP1/p32). C1q binding to hyaluronic acid (HA) via gC1qR activates intracellular signaling pathways that up-regulate the expression of both *HYAL2* and hyaluronan synthase *HAS3* mRNA and protein expression.

Through this NCP, C1q both increases HA synthesis and stimulates its degradation into low-molecular-weight fragments, creating a feedback loop that supports tumor growth and maintenance (94, 95).

Beside complement regulators and receptors, components normally produced in the liver, as C1q, C1r, C1s, C3 and C5 are locally synthesized and deposited in different tumor types and in the stroma regardless of complement activation, being predominantly tumorigenic in the majority of cancer types. C3a-C3aR and C5a-C5aR binding activate the PI3K/AKT/mTOR pathways, which promote cell proliferation, protein synthesis, survival and motility. Concurrently, C3 cleavage fragments stimulate the JAK2/STAT3 and ERK pathways, driving cellular reprogramming that promotes tumor progression. These pathways collectively orchestrate pro-tumorigenic changes such as enhanced survival, proliferation, and immune evasion (56).

Thus, tumor fate depends on all the dynamic interactions between complement components, immune cells, and signaling pathways in the TME. A tumor-friendly TME would probably present one or more of the following features: PI3K/AKT and MEK1/2 signaling via C3a-C3aR and/or C5a-C5aR axes, JAK2/STAT3 activation driven by tumor-deposited C3 fragments, promoting growth/metastasis, anti-apoptotic effects via CD59-mediated inhibition of terminal complement complexes, C5a-induced Akt-dependent oncogenic RGC-32 expression, overexpression of CD55, CD46, and CD59 on tumor cells, Factor H overexpression, enhancing stemness (via *LSF-1*) and angiogenesis. On the contrary, TME-driven tumor suppression would include C1q-mediated phosphorylation of the WWOX tumor suppressor, Properdin-dependent activation of the TES-DDIT3 tumor suppressor pathway, CD4+/CD8+ T-cell infiltration due to high intratumoral C3 expression and C3-mediated inhibition of *HER2* oncogene expression.

While no studies meeting our inclusion criteria (Section 2.2) directly demonstrated LP components influencing carcinogenesis via complosomes or NCPs, two references are worth mentioning. Ficolin-2 was found to inhibit epithelial-mesenchymal transition in HCC, both *in vivo* and *in vitro*, by decreasing TGF beta levels and phosphorylation of Smad2 and Smad3 (96). FCN-2 was also shown to interact with Toll-like receptor 4 (TLR4) and drive macrophage polarization toward the M1 phenotype. This activation enhanced antigen presentation to CD8+ T lymphocytes, ultimately reducing tumor growth (CT26 colon carcinoma, Lewis lung carcinoma, and Hca-f hepatocarcinoma) across three distinct murine models (BALB/c, C57BL/6, and C3H/He). Notably, FCN-A knockout mice exhibited accelerated CT26 colon and Lewis lung carcinoma progression – a phenomenon reversed by FCN-2 or FCN-A supplementation (97). In fact, LP components seem very relevant in oncogenic processes (98, 99). Their absence from currently characterized carcinogenic pathways reflects the paucity of mechanistic studies, underscoring the need for systematic

functional characterization of these molecules in oncogenic processes.

A central unresolved question persists: do CS components drive tumorigenesis, or are they merely bystanders in the TME? While some studies suggest individual CS elements may foster tumor-friendly niches, this perspective conflicts with cancer's multifactorial etiology. Instead, we propose that CS components act as dynamic regulators of the TME, through direct involvement in complement activation cascades, in NCP and complosomes leading to receptor signaling, and crosstalk with stromal cells. Critically, the TME itself dictates whether CS components promote or suppress tumor progression. For example, tumor cells and TME-associated immune cells (e.g., macrophages, monocytes) can locally produce CS proteins. These elements may then paradoxically support or antagonize tumor growth depending on contextual factors like tumor type, stage, and immune landscape (44, 45). This complexity underscores the need for cancer-specific investigations to unravel the precise role and therapeutic potential of each complement component.

Author contributions

CdFO-T: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. AdM: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Supervision, Visualization, Writing – review & editing. HP: Data curation, Investigation, Methodology, Validation, Writing – review & editing. NS: Conceptualization, Investigation, Methodology, Validation, Writing – review & editing. PF: Conceptualization, Investigation, Methodology, Validation, Writing – review & editing. TdPBG: Conceptualization, Investigation, Methodology, Validation, Writing – review & editing. AB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. IdM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1519465/full#supplementary-material>

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Reduced hemolytic complement activity in the classical pathway (CH50) is a risk factor for poor clinical outcomes of patients with infections: a retrospective analysis of health insurance claims in Japan

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Purpose: To evaluate whether low CH50 (a comprehensive measure of hemolytic activity of the classical complement pathway) is associated with infection-related coagulopathy, organ dysfunction, and poor clinical outcomes.

Methods: This was a retrospective study using Japanese health insurance claim data (2014–2023). Adult patients whose CH50 values were measured within one week of admission were included. We divided the patients into three groups based on the normal CH50 range: Low CH50 (< 25 U/mL; n=168), Normal CH50 (25 ≤, < 48 U/mL; n=1273), and High CH50 (48 ≤ U/mL; n=1285).

Results: Of 2,726 patients who met the inclusion criteria, logistic regression models demonstrated that decreased CH50 is a significant predictor of 180-day mortality (OR: 0.98–0.99). Cumulative survival rates in the Low CH50 group at 28 days and 180 days were both unfavorable (both p < 0.0001, Log-rank test). CH50 was significantly inversely correlated with SOFA, SIC, ISTH-overt DIC, and JAAM-2 DIC scores, and was also correlated with C3 and C4 levels. Diminished CH50 may be particularly useful in diagnosing SIC (specificity; 79.2%) and excluding ISTH-overt DIC (sensitivity; 90.5%). Moreover, patients with low levels of both CH50 and C3 had an extremely high mortality rate (25.0%).

Conclusion: Low CH50 after infection is not only significantly associated with multiple organ failure and coagulopathy but is also an independent risk factor for poor prognosis. Complement activation after infection may help to avert organ damage and to improve clinical outcomes.

KEYWORDS

CH50, infection, health insurance claims database, outcome, coagulopathy

Introduction

Innate immunity serves as the first responder of the immune system. When foreign substances or microorganisms invade the body, the innate immune system recognizes them and triggers a rapid immune response that promotes phagocytosis and elimination of pathogens (1). The complement system, which is the central element in the innate immune system, is important in monitoring pathogens. When the complement cascade is activated, it triggers a sequential series of enzymatic reactions that help eliminate pathogens through bacterial opsonization, leukocyte recruitment and triggering, and pathogen destruction (2). More than 50 components comprise three major, trigger-dependent cascades that are modified by multiple regulators and receptors to maintain homeostasis against invaders (3). Three main types of complement cascades have been identified: classical (antibody-dependent), alternative (the mannose-binding lectin dependent), and lectin pathway (recognizing carbohydrate patterns on microbial surfaces). These pathways converge at C3 convertase, leading to the terminal pathway that forms membrane pores in target cells (2).

Recently, the concept “immunothrombosis” has been introduced, and infection induced complement activation not only amplifies the coagulation cascade through mechanisms such as platelet aggregation and thrombin generation, but also triggers immune defense (4). As an immunothrombosis, coagulation activation due to bacterial infections is acceptable in controlling the infection locally during the compensatory phase, but when this balance is disrupted, the disease can evolve into disseminated intravascular coagulation (DIC), causing thrombotic events in multiple organs (5). Previous studies have demonstrated that the prevalence of DIC is increasing in septic patients with complement activation, whereas marked complement activation has already been observed in severe sepsis and septic shock (6, 7). These observations suggest that complement activation is critical in controlling severity of infection and progression of coagulopathy.

CH50, an indicator of comprehensive hemolytic activity of the classical pathway (CP), is useful in diagnosing severity and predicting prognosis in lung cancer (8), heart failure (9), venous thromboembolism (10) and burn injuries (11). As a comprehensive measure of CP functionality, the CH50 test can identify when any component is reduced, missing, or inactive throughout the entire cascade, including those required for the cytolytic membrane attack complex (12, 13). On the other hand, several studies on complement

activation in infectious diseases have reported that activation of anaphylatoxins, C3a and C5a, and the membrane attack complex (C5b-9) are useful predictors of disease severity, organ damage, and clinical outcomes; however, it is difficult to measure these markers in general clinical practice in Japan (6, 14, 15). Furthermore, studies on infection-induced changes in CH50 are few, making it difficult to provide feedback to clinical practice (7, 16–19).

In this study, we evaluated whether low CH50 values are associated with infection-related coagulopathy, organ dysfunctions, and poor clinical outcomes, utilizing big data from Japanese health insurance claims and various clinical data sources. We believe that this study is significant in that it provides a more comprehensive analysis by utilizing Japanese medical data, whereas previous clinical studies have faced limitations regarding the number of cases and measurement markers.

Methods

Data source

This study used commercially available, anonymized, insurance claim data from the Japan Medical Data Center (JMDC Inc.; Tokyo, Japan). The JMDC database includes a payer database with 17 million members registered since 2005, a personal health record database of 5.7 million members, a hospital database of 860 hospitals, a pharmacy database of 5700 pharmacies, and an electronic medical record database with 190 contracted medical institutions that represents approximately 14% of the total Japanese population, as of 2024 (JMDC Database. Available from: <https://www.jmdc.co.jp/en/>). When medical institutions claim medical treatment costs, diagnostic names of diseases are registered based in the International Classification of Diseases, 10th revision (ICD-10) codes. We received an anonymized dataset from JMDC in September 2023. Then, we extracted and analyzed these data with necessary parameters using specific IDs.

Study design and setting

This was a retrospective study that used the JMDC database. Patients who were admitted to hospitals due to infection and whose

CH50 values were measured within one week of admission were included in the study (Supplementary Table S1). Patients younger than 18 years, patients with undiagnosed infections, patients with missing C3 or C4 data, patients with pregnancy, and patients with cardiac arrest on admission were excluded from the study. In addition, for patients with a history of multiple hospitalizations, only data from the earliest hospitalization were included. The present study was conducted and reported in accordance with the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) statement.

Selection of participants

Enrolled patients were divided into three groups based on the initial CH50 value: Low CH50 group (< 25 U/mL), Normal CH50 ($25 \leq, < 48$ U/mL), and High CH50 ($48 \leq$ U/mL). This classification was based on the normal range of CH50 (25–48 U/mL).

Data collection and definitions

In this study, the following factors were collected and analyzed: patient demographic factors (age, male, Charlson morbidity index, intensive care unit (ICU) admission, sepsis diagnosis), clinical indices related to organ damage (sequential organ failure assessment (SOFA) total score, every component of SOFA, such as respiratory, coagulation, liver, circulatory, central nervous system (CNS), and kidney, No of organ failure), coagulation/fibrinolysis abnormalities (sepsis-induced coagulopathy (SIC) score (20), SIC diagnosis, International society on thrombosis and hemostasis (ISTH) overt DIC score (21), ISTH overt DIC diagnosis, New Japanese association for acute medicine DIC score (JAAM-2 DIC score) (22), JAAM-2 DIC diagnosis, blood test findings (white blood cell (WBC), Neutrophils, Lymphocytes, Monocytes, hemoglobin (Hb), platelet count (Plt), prothrombin time-international normalized ratio (PT-INR), activated partial thromboplastin time (APTT), Fibrinogen, fibrin/fibrinogen degradation products (FDP), D-dimer, antithrombin (AT), albumin (Alb), C-reactive protein (CRP), lactate (Lac)), complement values (CH50, C3, C4), treatment modalities (ventilation, renal replacement therapy (RRT), use of vasopressor, heparin treatment, intravenous immunoglobulin (IVIG) therapy, AT therapy, recombinant thrombomodulin (rTM) therapy, serine protease inhibitors (SPI) treatment, history of blood transfusion during the first 7 days, each blood products transfusion), presence of bleeding/thrombotic complications and DIC during hospitalization, hospital length of stay and clinical outcomes. The recently proposed JAAM-2 score excludes the systemic inflammatory response syndrome (SIRS) component from the original JAAM DIC score, and the cutoff value for DIC diagnosis is a total of at least three points.

We based definitions of infection and sepsis on the Global Burden of Diseases, Injuries, and Risk Factors Study 2017 (23). All blood markers except serum complement values and clinical indices

were evaluated on the day of admission. Normal values for complement C3 and C4, as well as CH50, were referenced to the normal ranges (C3; 86–160 mg/dL, C4; 17–45 mg/dL, CH50; 25–48 U/mL). The number of organ failures was counted for each organ component with a SOFA score ≥ 3 . Diagnoses of SIC and ISTH-overt DIC were defined as scores of ≥ 4 and ≥ 5 , respectively.

Analysis

The median with interquartile range was used to represent continuous variables and the number with percent (%) was used to represent nominal variables. The Kruskal-Wallis test was employed to analyze continuous variables, and the Chi-square test was used for categorical/nominal data. The Steel-Dwass test was also used to analyze multiple comparisons and to set the significance level (alpha) to 0.05. We defined statistical significance of all analyses as $p < 0.05$. Logistic regression analysis was conducted to analyze whether CH50 is an independent predictor of mortality within 180 days. In addition, several models were created by increasing the number of factors extracted from the results of univariate analyses, and the odds ratios for CH50 were analyzed to assess the robustness of these models. Kaplan-Meier curves were constructed, and log-rank tests were performed to analyze 28-day, and 180-day survival based on the CH50 value. Correlation analysis was performed to evaluate factors correlated with CH50. Spearman's rho was employed for correlation coefficients. As above, correlation coefficients between CH50 and C3, and CH50 and C4 were determined. Finally, receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic performance of CH50 for SIC, ISTH-overt DIC, and JAAM-2 DIC. Area under curve (AUC), cut-off values, sensitivity, and specificity were calculated. The cut-off value of CH50 was calculated using the Youden index. All statistical analyses were performed utilizing JMP[®] Pro 16.1.0 software (SAS Institute, Cary, NC, USA).

Results

From the original JMDC database, 20,528 patients fulfilled the inclusion criteria. After excluding 17,802 cases that met the exclusion criteria, 2,726 patients remained in the analysis of this study (Figure 1). Patients were categorized into the following three groups, according to their CH50 values: Low CH50 (< 25 U/mL; $n = 168$), Normal CH50 ($25 \leq, < 48$ U/mL; $n = 1273$), and High CH50 ($48 \leq$ U/mL; $n = 1285$).

Patient characteristics are shown in Table 1. The Low CH50 group was the oldest, with the highest Charlson index, and the highest rate of ICU admission. The percentage of sepsis was highest in the Low CH50 group.

The median total SOFA score was the highest in the Low CH50 group (Table 2). A similar tendency was identified in terms of each component of the SOFA score such as coagulation, circulatory, and CNS. In addition, the median number of organ failures in the Low CH50 group was also the highest. On the other hand, the median

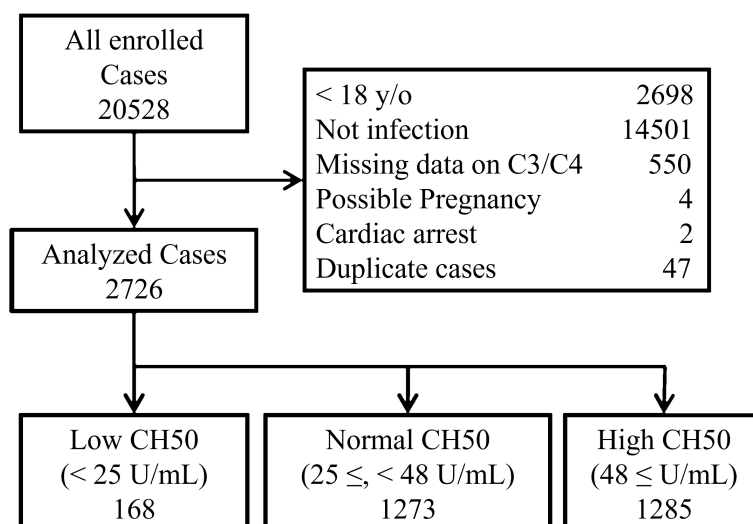


FIGURE 1
Study flow diagram.

SIC score, ISTH-overt DIC score and JAAM-2 DIC score were highest in the Low CH50 group. The percentage of patients meeting the SIC criteria was highest in the Low CH50 group. Statistically similar trends were also observed for the ISTH-overt DIC and the JAAM-2 DIC scores, although numbers of these cases were limited. Comparisons between CH50 groups showed statistically significant differences in all scores (Supplementary Figure S1). The median CH50 value in each group was 18 U/mL, 40 U/mL, and 55 U/mL, respectively (Table 2). Consistent with the change in CH50, C3 and C4 values showed a similar trend.

According to treatment modalities, the Low CH50 group had the highest rates of ventilator management, renal replacement therapy, vasopressor use, heparin treatment, administration of antithrombin or thrombomodulin (which are used for DIC in Japan), and SPI use (Table 2). Furthermore, the Low CH50 group had the highest percentage of blood transfusions during the first week of hospitalization (approximately 15%), and statistically significant differences were confirmed for each transfusion product.

Other laboratory data are shown in Supplementary Table S2. White blood cell counts were similar in all groups, but the Low CH50 group had the largest neutrophil fraction and the smallest lymphocyte and monocyte fractions. Other blood count items, Hb and Plt, were lowest in the Low CH50 group. As for coagulation

markers, the Low CH50 group showed the most prolonged PT-INR and APTT, and the highest D-dimer. A significant insufficiency of fibrinogen and a decrease in AT levels were also confirmed in the Low CH50 group. The Low CH50 group had the lowest Alb level and the least elevated CRP.

Table 3 shows complications after hospitalization and clinical outcomes. DIC was significantly more common in the Low CH50 group, while bleeding and thrombotic complications were not significantly different in any group. The longest hospital stays, as well as the highest mortality rate during the hospital stay, 28 days (15.5% vs 6.8% vs 3.2%, $p < 0.0001$), 90 days, and 180 days (19.6% vs 9.9% vs 5.1%, $p < 0.0001$) were also confirmed in the Low CH50 group. Overall cumulative survival rates for 28 days (Figure 2A) and 180 days (Figure 2B) were determined. At both time points, a decrease in CH50 was prognostically unfavorable by a statistically significant difference (both $p < 0.0001$, Log-rank test). Then, several logistic regression models were constructed using factors extracted from univariate analysis to determine whether a decrease in CH50 is a reliable predictor of 180-day mortality in patients with infections (Table 4). Lower CH50 was consistently a significant predictor of 180-day mortality in both models (OR: 0.978-0.987).

Clinical signs and most markers were significantly correlated with CH50 (Supplementary Table S3). Therefore, to evaluate the

TABLE 1 Patients characteristics in the analysis (n=2726).

Characteristic	Low CH50 (n=168)	Normal CH50 (n=1273)	High CH50 (n=1285)	P value
Age, y/o	77 [66, 87]	75 [60, 83]	71 [54, 81]	< 0.0001
Male, % (n)	53.0 (89)	53.3 (678)	53.8 (691)	0.96
Charlson morbidity index	6 [5, 9]	6 [4, 8]	5 [3, 7]	< 0.0001
ICU admission, % (n)	14.3 (24)	8.7 (111)	5.1 (65)	< 0.0001
Sepsis, % (n)	51.8 (87)	44.9 (571)	41.9 (538)	0.03

Continuous values are expressed as median [Q1, Q3]; $p < 0.05$ was considered significant.
ICU, intensive care unit.

TABLE 2 Values of complement factors and treatments by CH50 levels (n=2726).

Characteristic	Low CH50 (n=168)	Normal CH50 (n=1273)	High CH50 (n=1285)	P value
SOFA	11 [5, 12]	6 [3, 8]	4 [2, 6]	< 0.0001
respiratory	2 [0, 3]	1 [0, 3]	0 [0, 2]	0.22
coagulation	1 [1, 2]	0 [0, 2]	0 [0, 1]	0.005
liver	0 [0, 1]	0 [0, 1]	0 [0, 1]	0.17
circulatory	3 [1, 4]	0 [0, 2]	0 [0, 0]	< 0.0001
CNS	2 [0, 3]	1 [0, 3]	0 [0, 2]	0.007
kidney	2 [0, 3]	1 [0, 2]	0 [0, 2]	0.13
No. of Organ Failure	2 [1, 3]	1 [0, 2]	0 [0, 1]	< 0.0001
SIC score	1 [0, 2]	0 [0, 1]	0 [0, 0]	< 0.0001
≥ 4, % (n)	9.1 (15/165)	2.6 (32/1223)	0.8 (10/1227)	< 0.0001
ISTH-overt DIC score	2 [0, 3]	1 [0, 2]	0 [0, 2]	0.001
≥ 5, % (n)	18.4 (7/38)	4.1 (12/293)	0.7 (2/283)	< 0.0001
JAAM-2 DIC score,	2 [1, 4]	1 [0, 2]	1 [0, 1]	< 0.0001
≥ 3, % (n)	37.7 (29/77)	22.2 (106/478)	16.8 (86/512)	< 0.0001
CH50, U/mL	18 [13, 22]	40 [34, 44]	55 [51, 63]	< 0.0001
C3, mg/dL	70 [52, 88]	102 [85, 120]	131 [113, 150]	< 0.0001
C4, mg/dL	17 [10, 23]	27 [22, 33]	34 [29, 41]	< 0.0001
Ventilation, % (n)	17.3 (29)	9.8 (125)	4.7 (60)	< 0.0001
RRT, % (n)	8.3 (14)	4.2 (53)	2.4 (31)	0.0002
Vasopressor, % (n)	22.0 (37)	11.6 (148)	7.7 (99)	< 0.0001
Heparin, % (n)	25.6 (43)	21.3 (271)	16.1 (207)	0.0003
IVIG, % (n)	1.2 (2)	2.7 (34)	2.3 (30)	0.48
AT, % (n)	3.0 (5)	1.5 (19)	0.2 (2)	< 0.0001
rTM, % (n)	3.6 (6)	1.0 (13)	0.9 (11)	0.006
SPI, % (n)	7.7 (13)	4.1 (52)	2.2 (28)	0.0002
Transfusion_7days, % (n)	14.9 (25)	9.4 (119)	4.0 (51)	< 0.0001
RBC_7days, U	0 [0, 0]	0 [0, 0]	0 [0, 0]	< 0.0001
FFP_7days, U	0 [0, 0]	0 [0, 0]	0 [0, 0]	< 0.0001
PC_7days, U	0 [0, 0]	0 [0, 0]	0 [0, 0]	< 0.0001

Continuous values are expressed as medians [Q1, Q3]; $p < 0.05$ was considered significant.
SOFA, sequential organ failure assessment; CNS, central nervous system; SIC, sepsis induced coagulopathy; ISTH, international society on thrombosis and hemostasis; DIC, disseminated intravascular coagulation; JAAM, Japanese association for acute medicine; RRT, renal replacement therapy; IVIG, intravenous immunoglobulin; AT, antithrombin; rTM, recombinant thrombomodulin; SPI, synthetic protease inhibitor; RBC, red blood cell; FFP, fresh frozen plasma; PC, platelet concentrates.

predictive accuracy of CH50 for infection-induced coagulopathy or DIC, ROC curve analyses were performed (Supplementary Figure S2). The AUC was highest for ISTH-overt DIC (AUC: 0.764), with a sensitivity of 90.5% and specificity of 59.4% at the CH50 cutoff value of 44.0 U/mL. Based on the normal range of C3, C4, and CH50, we categorized all patients into three-by-three groups and evaluated the mortality rate of each population (Supplementary Table S4). Clinical outcomes were poorer in the low C3 (17.4%) and low C4 group (12.9%). Mortality rate was particularly high in patients with

low CH50 and low C3 (25.0%), as well as in patients with low CH50 and normal C4 (22.6%).

Discussion

In this retrospective study using Japanese big data from health insurance claims and clinical data sources, a low CH50 value was an independent risk factor for poor prognosis in patients with

TABLE 3 Complications and clinical outcomes by levels of CH50 (n=2726).

Characteristic	Low CH50 (n=168)	Normal CH50 (n=1273)	High CH50 (n=1285)	P value
Complications after admission				
DIC, % (n)	4.2 (7)	1.9 (24)	1.3 (17)	0.03
Bleeding, % (n)	9.5 (16)	7.1 (90)	6.8 (87)	0.43
DVT, % (n)	5.4 (9)	4.5 (57)	5.5 (71)	0.47
PE, % (n)	2.4 (4)	5.3 (68)	6.2 (80)	0.11
Hp stay, days	19 [12, 31]	16 [10, 30]	15 [9, 27]	0.005
Mortality rates				
Hospital, % (n)	19.6 (33)	10.0 (127)	5.1 (66)	< 0.0001
28 days, % (n)	15.5 (26)	6.8 (86)	3.2 (41)	< 0.0001
90 days, % (n)	19.1 (32)	9.3 (118)	4.6 (59)	< 0.0001
180 days, % (n)	19.6 (33)	9.9 (126)	5.1 (65)	< 0.0001

Continuous values are expressed as median [Q1, Q3]; p <0.05 considered as significant.
DIC, disseminated intravascular coagulation; DVT, deep vein thrombosis; PE, pulmonary embolism; Hp, hospital.

infection. CH50 was also useful in diagnosing organ damage and coagulopathy. Moreover, C3 and C4, which showed the same trend as CH50, suggested that in combination with CH50, it could further identify patient populations with poor clinical outcomes.

Limited evidence reported recently demonstrated that the relationship between CH50 and mortality in septic patients is inconclusive (16, 17). These studies had much smaller sample sizes, different definitions of sepsis, and mortality rates more than twice as high as the present study. In addition, a report analyzing 19 patients with bacterial meningitis demonstrated that complement values such as CH50, C3, and C4 of all patients were within the normal range (24). Considering the above, the findings of this study, which analyzed 2,726 patients with infectious diseases, are highly significant in that they reveal changes in complement markers that can be used clinically.

Decreased CH50 was also significantly associated with organ damage and coagulation abnormalities induced by infection. Not only the total SOFA score and the number of damaged organs, but also the organ dysfunction of each component of the SOFA score, including coagulation, circulation, and CNS were significantly associated with low CH50 value. This suggests that different organs have different susceptibilities to effects of infection-induced complement activation, like the CP. More interestingly, the mortality rate is lower in the high CH50 group than in the normal CH50 group. This means that high CH50 values associated with infection may be a pathophysiologically more congruent response in the acute phase of inflammatory response (25). Indeed, hypocomplementemia due to COVID-19 infection has been reported to be a risk for secondary bacterial infections

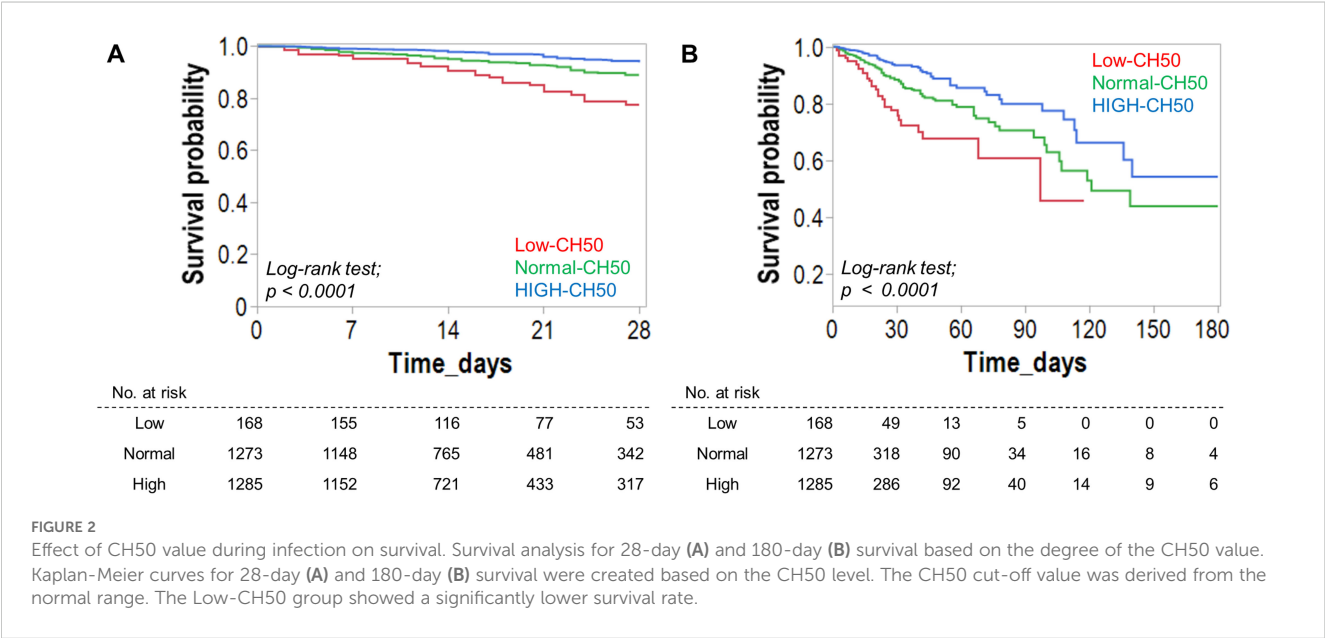


TABLE 4 Multivariate analysis for mortality within 180 days (n=2726).

Characteristic	Univariate analysis			Multivariate analysis1			Multivariate analysis2			Multivariate analysis3		
	OR	95%CI	P value	OR	95%CI	P value	OR	95% CI	P value	OR	95%CI	P value
Age	1.05	1.04-1.07	< 0.0001	1.04	1.03-1.06	< 0.0001	1.06	1.04-1.07	< 0.0001	1.06	1.05-1.08	< 0.0001
Charlson morbidity index	1.18	1.13-1.22	< 0.0001	1.08	1.03-1.14	0.002	1.09	1.03-1.15	0.003	1.10	1.04-1.16	0.001
CH50	0.96	0.96-0.97	< 0.0001	0.98	0.97-0.99	< 0.0001	0.99	0.97-0.997	0.01	0.99	0.98-0.998	0.03
SIC score	1.67	1.49-1.86	< 0.0001	1.47	1.31-1.66	< 0.0001	1.32	1.16-1.51	< 0.0001	1.14	0.99-1.32	0.07
Ventilation	15.57	11.27-21.50	< 0.0001				14.42	9.94-20.92	< 0.0001	9.13	6.13-13.58	< 0.0001
Vasopressor	9.01	6.65-12.21	< 0.0001							4.09	2.72-6.14	< 0.0001

p < 0.05 considered as significant.

SIC, sepsis induced coagulopathy; OR, odds ratio; CI, confidence interval.

through reduced opsonization and decreased hemolytic ability of MAC (12). In other words, complement activation is inadequate during this acute phase of infection, it can be assumed that the infection is poorly controlled, leading to organ failure and poor clinical outcomes.

In this study, strong positive correlations were demonstrated between both C3 and C4 with CH50. This confirms that CH50 primarily reflects comprehensive complement activation of the CP (26). In clinical studies of severe sepsis, C3 and C4 were reduced because C3 and C4 are degraded and activated to C3a and C4a, respectively (27). Furthermore, in a recently reported prospective study of severe sepsis and septic shock, the complement system was markedly activated, with significantly decreased C3 and C5 while significantly increased C3a and C5a, although CH50 was not measured (6). However, no correlation was found between complement activation and inflammatory markers, organ damage, or clinical outcomes. This suggests that in severe sepsis, remarkable complement activation has already developed and is not a useful predictor of clinical severity or outcome. Regarding the prognosis for each of the CH50 and C3 categories in this study, the mortality rate was higher in cases with low C3, and the worst mortality rate increased to 25.0% in the group with low CH50 and low C3. With respect to the combination of CH50 and C4, the mortality rate was higher than 10% in the normal or low CH50 and normal or low C4 groups. These differences are important in analyzing the impact of each complement pathway on complement activation in infectious diseases on clinical outcomes.

There are some limitations to the present study. First, although complement values can be routinely measured as a clinical test in Japan, it is unclear what the purpose of the measurement was in this study. In addition, complement values were measured within one week of admission, not necessarily on the day of admission; thus, it is difficult to define a causal relationship between actual complement values and pathophysiology of infection. Second, there is limited information on infectious diseases. For example, data on the source of infection, the need for drainage, the use of antimicrobial agents, and other treatment-related data were not analyzed in this study. In addition, it should be noted that the definition of sepsis in this study varies by age group. Fourth, information on indications and treatment details provided is

limited in this study. Finally, the choice of specific laboratory tests to be performed is at the discretion of the physician in charge, leading to potential selection bias.

Conclusions

Low CH50 in patients with infection was not only significantly associated with multiple organ failure and coagulopathy but was also an independent risk factor of poor prognosis. CH50 also showed a strong positive correlation with C3 and C4, and patients with low levels of both CH50 and C3 had an extremely high mortality rate. These findings suggest that infection-induced activation of the complement pathway is a normal acute-phase immune response, and that early recognition of these reactive disturbances may help to avoid organ damage and to improve clinical outcomes.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Datasets generated and/or analyzed during the present study are not publicly available due to ethical restrictions associated with personal information, but anonymized data are available from the corresponding author upon request. Requests to access these datasets should be directed to Hiroyuki Koami, hkoami@cc.saga-u.ac.jp.

Ethics statement

The studies involving humans were approved by Osaka Medical and Pharmaceutical University. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because Due to the retrospective nature of this study and the use of anonymized data.

Author contributions

HK: Project administration, Formal analysis, Methodology, Data curation, Visualization, Investigation, Writing – original draft, Conceptualization. YF: Writing – review & editing, Validation. YH: Writing – review & editing. AS: Writing – review & editing. HO: Writing – review & editing. AM: Writing – review & editing. KS: Writing – review & editing. KN: Writing – review & editing. RS: Writing – review & editing. SI: Writing – review & editing. TO: Writing – review & editing. SN: Writing – review & editing. MK: Writing – review & editing. HM: Writing – review & editing. YU: Validation, Project administration, Writing – review & editing. KY: Validation, Project administration, Writing – review & editing. KO: Writing – review & editing. YS: Writing – review & editing, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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HK investigated and established the research methodology. HK, KY and YU managed the project. YS supervised the entire study. YF, KY and YU conducted the validation of the study. Data curation, formal analysis, visualization of the study and writing of the original draft were performed by HK. Review and editing the manuscript were performed by YF, YH, AS, HO, AM, KS, KN, RS, SI, TO, SN, MK, HM, YU, KY, KO, and YS. All authors revised the manuscript for important intellectual content. All authors read and approved the final version of the manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1601690/full#supplementary-material>

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Impact of smoking on the complement system: a narrative review

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Smoking is a major cause of morbidity and mortality, resulting in an increased risk of cardiovascular, respiratory, inflammatory, and degenerative diseases. In this review, we highlight the complex interactions between smoking and activation of different components of the complement system, in order to underscore the notion that its dysregulation underlies—mechanistically—as well as exacerbates the progression of a host of disease processes. Moreover, we also briefly delve into components of tobacco smoke—including chemical constituents like tobacco glycoprotein (TGP) and particulate matter (PM), toxic metals, and other mainstream cigarette smoke chemicals—that have been identified as possible culprits in complement activation. In doing so, this review makes important and meaningful contributions to the ongoing efforts of combating the global health crisis posed by tobacco use, all while emphasizing the need for multifaceted strategies that include not only public health measures and educational efforts, but also innovative research that focuses on understanding and mitigating the biological mechanisms underlying smoking-related health conditions.

KEYWORDS

smoking, complement system, inflammation, cardiovascular disease, lung disease, e-cigarette, tobacco glycoprotein, particulate matter

Introduction

While data show a declining trend, tobacco use remains a leading cause of preventable diseases, disabilities, and deaths in the U.S., as per the Centers for Disease Control and Prevention (CDC). Indeed, around 28.3 million adults and 2.8 million middle and high school students in the U.S. use tobacco products (1). Smoking and secondhand smoke exposure lead to nearly 500,000 premature deaths annually, while 16 million Americans suffer from serious smoking-related illnesses. In terms of the economic impact, the U.S. incurs over \$225 billion in annual medical expenses to address diseases caused by smoking (1). While these numbers reflect a persistent public health challenge, they also underscore the critical need for effective strategies to further reduce tobacco use as well as mitigate its

profound and detrimental impact on health. Consequently, exploring the multifaceted mechanisms of how smoking can participate in disease pathogenesis is essential for highlighting evidence to educate the public, and for developing targeted approaches to reduce its impact on health.

There is ample evidence that links smoking to several health issues that span virtually every system within the human body, which underscores its role as a primary contributor to morbidity and mortality not only in the U.S. but worldwide. To this end, cardiovascular diseases, including heart attacks and stroke, are significantly more common among smokers (2), as are a variety of cancers (3). Respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and emphysema, are markedly exacerbated by smoking, which also compromises the body's ability to fight infections, leading to increased susceptibility to pneumonia and tuberculosis (4). Smoking was also found to impact reproductive health, thereby contributing to fertility problems (5), as well as pregnancy complications (6), and/or even adverse outcomes for the offspring due to maternal exposures (7). This broad spectrum of smoking-related disorders not only reflects the significant and extensive impact tobacco use exerts on human health, but also highlights the critical need for a better/further understanding of the underlying pathological processes.

The detrimental health effects of smoking can be attributed to a complex interplay of 'harmful' mechanisms that are triggered by more than 7,000 chemicals known to be present in tobacco smoke, of which hundreds are toxic and at least 69 are known carcinogens (8, 9). To this end, these chemicals have been found to produce a host of effects such as initiate oxidative stress, promote inflammation, induce DNA damage, and impair cellular and systemic immune responses, collectively contributing to the development and progression of various diseases (9). Among the less explored yet pivotal pathways affected by smoking is the complement system—which is a key component of the immune system that enhances (aka complements) the ability of antibodies and phagocytic cells to clear pathogenic invaders (10). While the complement system plays a crucial role in defending against infection and disease, its aberrant activation by smoking could in fact lead to excessive inflammation, tissue damage, and a cascade of immune responses that contribute to the pathogenesis of smoking-related diseases (11, 12). In fact, complement system activation has been linked to a number of major chronic disease conditions including cancer, cardiovascular, autoimmune, and renal diseases (13–15). Furthermore, therapeutic targeting of the complement system components yielded very promising results in the treatment of different disease conditions, which is exhaustively reviewed in (16). Therefore, summarizing what is known about the interplay between smoking and the complement system should provide potential/crucial insight that is currently lacking from a clinical and research stand points, thereby paving the way for researchers to focus on this important system; and also for guiding the development of targeted therapeutic strategies and interventions designed to ameliorate the related adverse health effects associated with tobacco use.

In summary, by examining the intricate ways in which smoking influences the complement system, which, as indicated before, is a fundamental aspect of the immune response, we aim to provide a fresh perspective on the pathophysiological processes underlying smoking-related health conditions. And in doing so, this review contributes to a more comprehensive approach to combating the global health crisis posed by tobacco use, while emphasizing the need for multifaceted strategies that include not only public health measures and educational efforts but also innovative research focused on understanding and mitigating the biological mechanisms through which smoking inflicts its harm.

Overview of the complement system

The complement system is a critical component of the immune system, serving as a bridge between innate and adaptive immunity. It comprises of over 50 glycoproteins, both soluble and membrane-bound, that work together in a complex network of protein-protein interactions. This network functions to recognize and eliminate pathogens through a series of proteolytic reactions that result in key immune processes, including opsonization of pathogens, recruitment of inflammatory cells via anaphylatoxins (e.g., C3a, C5a), and direct lysis of target cells through the formation of the membrane attack complex (MAC) (17, 18).

There are three canonical pathways that are known to initiate the activation of the complement system, those are: 1. classical, 2. alternative, and 3. lectin pathways (18), each of which is triggered/activated by different signals. To this end, the classical pathway begins with the attachment of the C1q molecule either to antibodies that are bound to pathogens, or directly to pathogens. C1q is part of the C1 complex, which comprises a single C1q molecule linked to two molecules each, namely C1r and C1s. Once activated (C1s), it acts on the next two components of the classical pathway, cleaving C4 and then C2 to generate two large fragments, C4b and C2b, which together form the C3 convertase of the classical pathway (Figure 1). As for the lectin pathway, it is initiated by the binding of pattern-recognition molecules like mannose-binding lectin (MBL) to specific sugars on the surface of microorganisms or damaged host cells. When the MBL complex—whose components are MASP-1 and MASP-2—binds to a pathogen surface, this leads to cleavage of C4 and C2. Finally, the alternative pathway is continuously active at low levels due to the spontaneous hydrolysis of C3, a condition known as “C3 tick over” (19). This pathway can quickly amplify the immune response once a pathogen or other non-pathogenic invaders are detected, thereby leading to the generation of a distinct C3 convertase, namely C3bBb (18, 20).

At the heart of the action of the complement system is the cleavage of the C3 protein into C3b and C3a by the C3 convertases formed by the trigger of the canonical pathways (classical, alternative, and lectin). C3b then tags pathogens for destruction through a process called opsonization, making them easier targets for immune cell phagocytosis. C3a, along with another fragment called C5a that is generated from the cleavage of C5 by C5

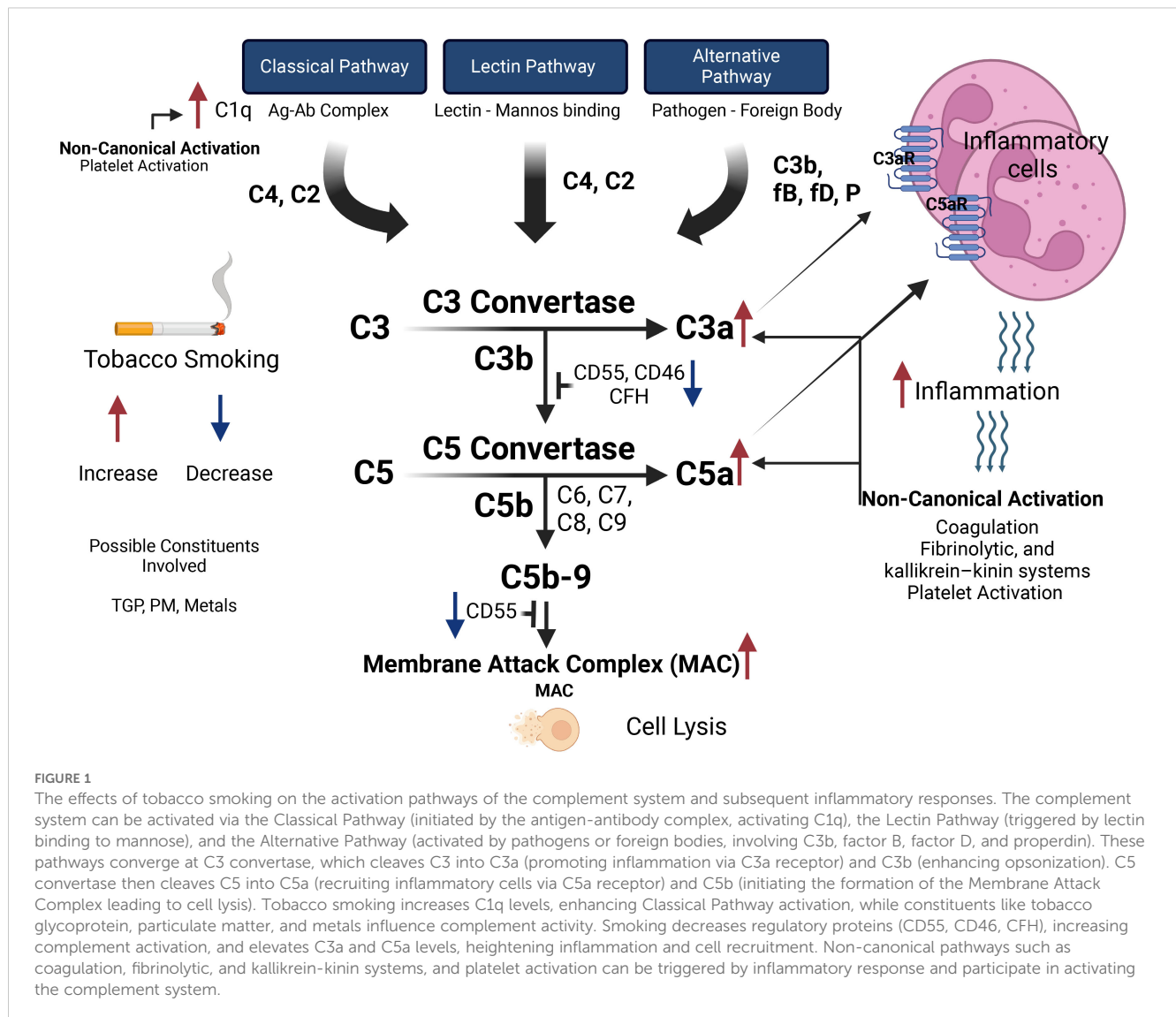


FIGURE 1

The effects of tobacco smoking on the activation pathways of the complement system and subsequent inflammatory responses. The complement system can be activated via the Classical Pathway (initiated by the antigen-antibody complex, activating C1q), the Lectin Pathway (triggered by lectin binding to mannose), and the Alternative Pathway (activated by pathogens or foreign bodies, involving C3b, factor B, factor D, and properdin). These pathways converge at C3 convertase, which cleaves C3 into C3a (promoting inflammation via C3a receptor) and C3b (enhancing opsonization). C5 convertase then cleaves C5 into C5a (recruiting inflammatory cells via C5a receptor) and C5b (initiating the formation of the Membrane Attack Complex leading to cell lysis). Tobacco smoking increases C1q levels, enhancing Classical Pathway activation, while constituents like tobacco glycoprotein, particulate matter, and metals influence complement activity. Smoking decreases regulatory proteins (CD55, CD46, CFH), increasing complement activation, and elevates C3a and C5a levels, heightening inflammation and cell recruitment. Non-canonical pathways such as coagulation, fibrinolytic, and kallikrein-kinin systems, and platelet activation can be triggered by inflammatory response and participate in activating the complement system.

convertases, plays a significant role in inflammation and immune modulation by robustly attracting inflammatory cells (C3a and C5a are also called anaphylotoxins) (18). It is important to note that the complement system is finely tuned to prevent excessive activation and damage to host tissues. Indeed, there are various regulatory proteins (Table 1) that ensure that complement activity is precisely controlled, such that there is balancing of the need for a rapid response to invaders all while preserving/protecting the host's cells (30).

In addition to the canonical pathways of activation, the complement system can also be activated through non-canonical routes. To this end, enzymatic activity from systems such as coagulation has been shown to activate the complement system, which was illustrated to be mainly due to the ability of FXa, FXIa and plasmin to cleave both C5 and C3, and robustly generate C5a and C3a, with the latter being proinflammatory molecules (31). It was also shown that thrombin can cleave C5, especially in the absence of C3 substituting for the C3-dependent C5 convertase (32) a process that confirms a close and intertwined relationship

between coagulation and the complement system. Other non-canonical pathways such as fibrinolysis, and kallikrein-kinin systems can directly cleave complement proteins, bypassing the need for convertases (33, 34). It was also shown that platelets can contribute to activating the complement's classic pathway through the paracrine complement activating mediator chondroitin sulfate A, which is expressed on the platelet surface upon activation and binds C1q along with many other complement components (35). It was also reported that microparticles released from activated platelets can participate in activating the complement system (36). In addition, thrombin-activated platelets were shown to initiate the alternative complement cascade (37) whereas the platelet p-selectin was found to independently activate, at least, parts of the complement components (38). These non-canonical pathways may play roles in various conditions, including inflammatory diseases and thrombo-inflammation, both of which are important contributors to the pathogenesis of a host of different diseases states. It is also worth mentioning that the complement system in and of itself is increasingly recognized for its non-

TABLE 1 Complement System Regulators.

Complement system regulators	Type	Mechanism of regulation
Factor H and related proteins	Fluid-phase	Facilitates the decay of C3 convertases, acts as a cofactor for inactivation of C3b, and recognizes host surfaces to prevent self-attack (18).
Factor I (CFI)	Fluid-phase	Serine protease that cleaves and inactivates C3b and C4b in the presence of cofactors such as Factor H, MCP (CD46), or C4BP (21).
C4b-binding protein (C4BP)	Fluid-phase	Acts as a cofactor for inactivation of C4b, playing a role in limiting classical and lectin pathway activations (22). Decay-accelerating activity for the classical C3 convertase and as a cofactor for the cleavage of C3b (23, 24).
CD46 (MCP)	Membrane-bound	Regulate complement activation on cell surfaces by acting as cofactors (CFI) for the inactivation of C3b and C4b (25, 26)
Decay-Accelerating Factor (DAF)	Membrane-bound	Accelerates the decay of C3 and C5 convertases, preventing the amplification of complement responses on host cells (26).
CD59	Membrane-bound	Prevents the formation of the MAC by inhibiting the assembly of C5b-9 complexes, protecting host cells from lysis (27).
Carboxypeptidases N, R, B2	Fluid-phase	Regulate magnitude of complement activation of C3a and C5a, reducing their inflammatory potential (28).
C1-esterase inhibitor	Fluid-phase	Inhibits the classical and lectin pathway proteases (C1r, C1s, MASPs), controlling the activation of these pathways (29).

canonical roles, which go beyond the innate immunity domain. These include regulating neuronal development, tissue repair, and metabolic homeostasis (39, 40). Indeed, there is evidence showing that complement system activity also operates intracellularly, where it interacts with innate sensors such as the pyrin domains-containing protein 3 (NLRP3) inflammasome and mitochondrial anti-viral signaling protein (MAVS), to coordinate responses to cellular stress and danger signals (41). However, this area is still under active investigation and more work is needed to solidify this non canonical role of the complement system in the development of disease conditions.

Understanding the fundamental “operations” of the complement system has provided crucial insight into how its delicate equilibrium is maintained, hence ensuring a robust state of homeostasis. However, external factors, particularly lifestyle choices, such as smoking, can significantly disrupt this balance, leading to aberrant complement activation and contributing to the pathogenesis of various conditions. In the next section we will delve into the specific impact of smoking on the complement system and its different components in the context of diseases states. We will also highlight the pathophysiological consequences and the underlying mechanisms through which smoking exacerbates disease progression via complement system dysregulation.

Smoking-induced dysregulation of the complement system: pathways to pathology

The impact of smoking on complement activation was explored very early in a study that examined its effects on serum complement using Alternative Complement pathway hemolytic activity (ACH50) assay. This work revealed that the alternative complement pathway is

activated, particularly in chronic elderly smokers (42) but this work did not explore this relationship in the context of disease conditions. Nonetheless, separate studies explored the complex interplay between smoking and the complement system across various health contexts, underscoring the significant impact of cigarette smoke on cardiovascular, respiratory, and immune system functions, as well as its role in the parthenogenesis of diseases that impact these systems, including development of cancer. Collectively, these studies showcase the critical role of complement activation in the pathophysiology of smoke-induced damage and disease.

Complement activation and smoking induced cardiovascular diseases

The activation of complement components has been shown to predict adverse cardiovascular events, such as myocardial infarction (43). Furthermore, complement factors such as C3 and C4 were found to be linked to the presence of atherosclerosis (44, 45). In fact, the deposition of the complement components C1q, C3, and C4 and generation of the terminal complement complex C5b-9 in atherosclerotic lesions was unequivocally illustrated (by immunohistochemistry) (46). There is also evidence that in patients with type 2 diabetes who have experienced myocardial infarction (MI), elevated levels of soluble sC5b-9 can be/are indicative of an increased risk for future cardiovascular events (47). This suggests that the complement system is significantly involved/plays a major role in the development of cardiovascular disorders. Therefore, exploring complement activation triggers such as smoking offers opportunities to enhance our understanding of the pathophysiology of the aforementioned diseases.

The association between plasma complement C3 levels and coronary heart disease (CHD) within the context of smoking behavior was examined in the CODAM (Cohort on Diabetes and

Atherosclerosis Maastricht; sample size $n = 562$) study (48). This study revealed a significant interaction between high plasma C3 levels and heavy smoking in relation to the prevalence of CHD. Specifically, while no substantial association between C3 levels and CHD prevalence was observed among never and light smokers, a strong association emerged in those who are heavy smokers. This association persisted even after adjustments for traditional cardiovascular risk factors, including the metabolic syndrome, C-reactive protein levels, and insulin resistance; which clearly indicates that the relationship between high C3 levels and CHD in heavy smokers is independent of these factors (48). Furthermore, separate reports suggested that complement activation, specifically, elevated levels of C3a and C5a are linked to cardiovascular complications and adverse cardiovascular events, respectively (49, 50).

In a different but related context, another study investigated the impact of tobacco (smoke and extract) and shear stress on complement activation in human endothelial cells (51). The results showed that tobacco smoke extract and shear stress both enhanced the deposition of the complement component C4d on endothelial cells, but did not significantly affect the deposition of C3b or C5b-9, suggesting limited activation of the complement system. This work also revealed and in a similar manner to tobacco extract, that the combination of tobacco smoke and shear stress significantly increases C4d deposition, confirming a synergistic effect on complement activation. Importantly, despite this activation, increases in C3b or C5b-9 were not observed similar to smoke extract. Accordingly, this regulation could be attributed to the concurrent increase in the expression of complement regulatory proteins CD35 (CR1) and CD55 on endothelial cells that are exposed to both stressors (52). These findings together suggest a mechanism to prevent excessive complement activation and potential vascular injury. The same research team conducted a similar set of experiments using endothelial cell lines (HUVEC) and human bone marrow microvascular endothelial cells (BMEC) and showed increased C4d deposition after treatment with tobacco smoke or exposure to shear stress, whereas their combined effects resulted in a two-fold increase in C4d deposition (53). This was accompanied by a 50% increase in surface gC1qR/p33 receptors and a 33% increase in ICAM-1 expression (53). These studies indicate that the interplay between smoking, shear stress, and complement system in inducing endothelial activation is complex. This complex relationship might also hint that the impact of smoking and shear stress on the complement system in the context of cardiovascular disease could be moderated/mediated by other unknown shear stress variables such as blood viscosity, internal diameter, physical dimension of the blood vessels, and blood velocity as well as unknown smoking variables such as the specific chemical composition and concentration; which are areas that require more examination.

The decline in traditional smoking seems to have been associated with increased prevalence of novel tobacco products such as electronic/e-cigarettes, in part because of misconceptions regarding their safety (54). The activation of complement components was also illustrated in the context of endothelial cells

in response to e-cigarettes (vapor) exposure. To this end, one study showed a significant increase in complement deposition and expression of the receptors for C1q. Interestingly, these results were independent of nicotine and the exposure to e-vapor was just as harmful as tobacco smoke extracts (55). E-cigarette role in activating complement components was also assessed *in vitro* in immortalized Kupffer cells, to determine their role as promoters of cardiovascular diseases through propagating the state of inflammation (56). The study did show- after exposure to e-cigarette extracts- an increase in the deposition of complement products C1q, C3b, C4d and C5b-9 on Kupffer cells, that also coincided with increased surface expression of globular head of C1q (gC1qR) and receptor for the collagen region of C1q (cC1qR) (56).

More recently, a study that examined the impact of e-cigarette extracts on platelets- which are major players in development of cardiovascular disease (57)) (58)- and as expected, showed enhanced function, including platelet aggregation, adhesion and secretion. Interestingly, it was observed that exposure of platelets to e-cigarette extracts induced significant up-regulation in the expression of the platelet receptor for the globular head of C1q (gC1qR) and receptor for the collagen region of C1q (cC1qR). Furthermore, a marked increase in the deposition of C3b and C4d was also observed; which was inhibited by pure nicotine, suggesting an antagonistic role (58). These data highlight the complexity of the interaction between complement components, their target cells and the state of the surrounding environment. However, whether such impact of e-cigarettes on the complement can actually lead to the development of pathology clearly remains an area that requires further investigation.

It is noteworthy that a study evaluated the combined effects of aging and cigarette smoking on stroke outcomes and the potential benefits of anti-complement mitigation strategies, in mice. The study used B4Crry, a single-chain variable fragment (scFv) antibody derived from the B4 IgM monoclonal antibody (a self-reactive pathogenic natural IgM antibody). B4Crry is linked to murine complement receptor 1 (CR1)-related gene/protein γ (CRRY), an ortholog of the human complement receptor 1 (CR1), and serves as a targeted complement inhibitor that blocks all complement pathways at the central C3 activation step (59). This study did highlight the exacerbated inflammatory effects of aging and smoking on stroke outcomes, which were significantly mitigated by complement inhibition, thereby underscoring the critical role the complement system plays in the pathophysiology of stroke, including in the context of smoking (60).

Finally, another study investigated the interaction between smoking and a complement gene polymorphism (C4B*Q0, a silent allele of the C4B gene) in promoting cardiovascular disease morbidity and mortality (61). It was found that among smokers, carriers of the C4B*Q0 allele had a significantly higher frequency of cardiovascular disease compared to non-carriers and non-smoking subjects. The authors hypothesized that low levels of C4B can lead to an impaired ability to eliminate immune complexes (that is, the removal of immune aggregates through erythrocyte complement receptor CR1) (61, 62). Indeed, a less efficient and reduced ability to tag and clear immune complexes (aggregates of antigens and

antibodies) can lead to accumulation of the immune complexes in tissues and the bloodstream, leading to chronic inflammation and potential tissue damage (26). Thus, this work underscores the impact of the interaction between genetic predisposition and smoking in the development of cardiovascular diseases, and emphasizes the importance of identifying genetic risk factors that may enhance the detrimental effects of smoking on cardiovascular health (61).

Collectively, the aforementioned studies/findings illustrate the complex role of complement activation and its interplay with environmental factors such as smoking, and highlight the delicate balance between activation and regulation and how that influences the progression of disease.

Complement activation and smoking induced respiratory diseases

Studies have suggested that the complement system plays a multifaceted role in respiratory system defense mechanisms, and hence could potentially contribute to the development of pathological processes. For instance, activation of complement pathways can lead to severe respiratory inflammation and tissue damage (63, 64). Furthermore, complement activation is also thought to participate in progression of various chronic lung diseases, including cancer (65–68). In light of this notion, exploring the impact of smoking on complement activation as a mechanism of lung disease development is warranted. To this end, the role of C1q, a complement protein 1 complex component, in the development of emphysema through cigarette smoke exposure was examined (69). This work revealed that chronic cigarette smoke exposure downregulated C1q in lung antigen-presenting cells in both humans and mice. This downregulation was associated with enhanced lung inflammation, increased Th17, which is a subset of CD4+ T helper cells, and the promotion of emphysema, suggesting that cigarette smoke-induced reduction of C1q contributes significantly to the loss of peripheral tolerance. This occurs when the mechanisms that normally keep self-reactive immune cells “in check” fail, thereby allowing them to get activated and attack the body’s own tissues (70), which leads to emphysema (69).

Another study conducted in the context of investigating the mechanism of complement-induced lung emphysema demonstrated that cigarette smoke extract has the capacity to modify the complement (C3) and activate the alternative pathway, *in vitro* (71). This work detailed that cigarette smoke activates the alternative pathway of complement by specifically modifying C3 and that these modifications include cleavage of its thiolester bond and formation of C3 multimers. Together, these findings elucidate the molecular mechanism by which cigarette smoke activates the complement system and provide insight into how that potentially contributes to the inflammatory processes observed in the lungs of smokers (71). Along the same lines, it was demonstrated that exposure to cigarette smoke-dependent activation of the complement system results in increased neutrophil and monocyte chemotactic activity. This was

evidenced by the significant attraction of neutrophils and monocytes to serum exposed to cigarette smoke. The increase in chemotactic activity was partially attenuated by EDTA, suggesting activation of the alternate complement pathway, which does not require the presence of antibodies. Further analysis revealed cleavage of properdin factor B and C3 in smoke-exposed serum, along with detection of C5a, which indicates complement activation (72). These findings suggest that complement activation by cigarette smoke may play a role in directing the influx of neutrophils and monocytes into the lungs of smokers, and contribute to the pathogenesis of smoking-induced lung diseases. In this connection, a separate study investigated the effects of smoking on peripheral leukocyte counts and serum components related to leukocytes, such as complement in smokers without lung diseases, and showed contrasting findings. Thus, it was found that peripheral leukocyte numbers increased with the duration and quantity of smoking, independent of age or gender, however though, serum levels of complement (CH50 and C3a) did not significantly change with smoking. Additionally, there was no correlation between neutrophil counts in the peripheral blood and bronchoalveolar lavage fluid, indicating that the increased leukocyte numbers in smokers might not be due to complement activation (73). While these contrasting results can be attributed to differences in methodology and experimental settings, they do nonetheless highlight the need for more careful examination of the link between smoking and complement activation in the context of lung diseases, under different real-life experimental settings.

Complement components were also investigated as biomarkers of lung malignancy. To this end, a study highlighted that lung tumor cells, compared to non-malignant bronchial epithelial cells, activate the classical complement pathway more robustly via direct binding of C1q, leading to the generation of elevated levels of C4d, a stable degradation product of complement activation. This elevation in C4d is consistently observed in lung tumor tissues, bronchoalveolar lavage fluid, and plasma of lung cancer patients, and correlates with poor prognosis (74). Importantly, these increases are not observed in smoking-related inflammatory lung conditions such as emphysema or COPD, emphasizing the specificity of C4d as a biomarker for malignancy rather than for smoking-induced inflammation. Interestingly, despite heightened complement activation, lung tumor cells evade complement-mediated cytotoxicity by expressing and binding complement factor H (CFH), a key regulatory protein that inhibits the alternative pathway of complement activation (75, 76). This allows tumor cells to harness the immunomodulatory effects of complement activation—such as inflammation and microenvironment remodeling—while avoiding its destructive consequences. Together, these findings reveal a unique tumor-associated complement activation signature in the lung, with potential utility in distinguishing malignant from non-malignant smoking-related lung pathologies as well as for informing prognosis.

Evidence indicates that smoking is the most important factor for developing lung cancer (77). To this end, and consequently, the interaction between cigarette smoking and the complement components such as factor H (CFH) variant Y402H (rs1061170) in relation to lung cancer risk was examined. In this study that

included 1000 lung cancer cases and 1000 controls (Chinese population), it was found that the CFH Y402H genotypes were significantly overrepresented among lung cancer patients compared to controls, with the 402His/His or 402His/Trp genotypes associated with a higher risk of lung cancer (OR = 1.50, 95% CI = 1.12–2.00). Importantly, the increased cancer risk associated with CFH variants was evident among smokers but not non-smokers, suggesting a smoking-related genetic risk factor for lung cancer. Further analysis showed that the risk increased with higher cumulative smoking doses, which suggests that the CFH Y402H polymorphism may interact with cigarette smoking and amplify lung cancer development risk in the Chinese population (78).

Collectively, these studies highlight the intricate interplay between the different complement system components and smoking in modulating the risk of lung diseases, including lung cancer. These findings also further underscore the importance of considering both genetic susceptibility and lifestyle factors in understanding and potentially mitigating disease risk.

Complement activation, smoking, inflammatory mucosal and macular degeneration diseases)

As indicated before, the activation of the complement system is a significant factor in the development and exacerbation of inflammation (10, 79), and it plays a multifaceted role in various inflammatory disorders (80, 81), contributing to tissue damage (82) and disease progression (83). Similarly, smoking is intricately linked to inflammation through various mechanisms, such as oxidative stress (84), alteration of immune responses (85, 86), which supports its capacity in inducing chronic inflammation (87), all of which can contribute to the development of a range of diseases. Given this intertwined relationship between smoking, inflammation, and complement activation, it is imperative to highlight this notion, in an attempt to offer a better understanding of the potential mechanisms that can lead to the genesis of disease. To this end, *in vitro* experiments demonstrated that exposure of human respiratory epithelial cells to cigarette smoke extract resulted in complement activation, as evidenced by increased levels of complement activation fragments (C4a, C3a, and C5a) and immunofluorescent staining for C3d (88). Moreover, an *in vivo* study using complement-deficient ($C3^{-/-}$) mice exposed to cigarette smoke showed a significant reduction in nasal damage. This suggests that cigarette smoke activates the complement system, and contributes to mucosal damage, and that complement deficiency provides a protective effect against smoke-induced nasal injury (88). Similarly, it was demonstrated that extracts from various smokeless tobacco products, including loose leaf chewing tobacco, dry snuff, and moist snuff, depleted the complement hemolytic activity in normal human serum, and that they did so in a dose-dependent manner (89). This complement depletion was largely due to the consumption of C3, a key component in the complement cascade. The presence of significantly elevated levels of complement cleavage fragments

iC3b, Bb, and- particularly with moist snuff- C4d, indicates activation of both the alternative and classical pathways. This complement activation by smokeless tobacco extracts suggests a potential mechanism for initiating inflammation of the oral mucosa in users of these products (89).

With regard to macular degeneration, it has been shown that chronic smoke exposure leads to ocular damage due to involvement of the alternative complement pathway. This was demonstrated by the finding that deficiency of alternative complement pathway (using $CFB^{-/-}$ mice) can ameliorate age-related macular degeneration (AMD) associated with smoking (90). In a similar context, retinal pigment epithelium (RPE) cells exposed to smoke revealed AMD through the process of oxidative stress and alternative complement pathway activation. This activation is linked to endoplasmic reticulum stress and subsequent lipid accumulation within the RPE, highlighting a synergistic role of oxidative stress and complement activation in AMD pathogenesis. Furthermore, using antioxidants and inhibitors to block the alternative complement pathway signaling led to mitigation of smoke-induced damage in the RPE and prevented or slowed AMD progression (12). Additionally, the relationship between smoking and complement activation in inducing AMD was also shown to be dependent on the translocation of antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) in RPE cells, which led to an increase in C3a and C3b as well as a decrease in complement regulators such as CD46, CD55, and CD59. Additionally, Nrf2 knockdown amplified cigarette smoking-induced increases in C3a and C3b, indicating that Nrf2 might- at least in part- play a protective role against cigarette smoking-induced pro-inflammatory responses in RPE cells (11). Another important aspect in the pathogenesis of AMD was related to a genetic risk linked to the region spanning from the CFH gene to the F13B gene on chromosome one. The data supported the notion that smoking was associated with increased terminal complement component and C-reactive protein levels in various parts of the macula, indicating elevated levels of complement activation and inflammation associated with both genetic risk at the CFH-to-F13B locus and cigarette smoking (91).

Complement–coagulation interplay under smoke and vape exposure

Evidence has demonstrated that the complement and coagulation systems/cascades do not operate in isolation; rather they exhibit significant cross-talk (31). For example, thrombin can generate the complement fragment C5a even in the absence of the usual complement convertases (32). Likewise, coagulation factor XIIa, kallikrein, and plasmin can cleave complement proteins to produce active fragments (92). Reciprocally, complement activation produces inflammatory mediators (C3a, C5a) that can enhance the pro-coagulant activity on cell surfaces, thereby promoting clot formation. This bidirectional “liaison” means that an insult activating one system often propagates a cascade in the other, creating a “thromboinflammatory” response (93). In healthy

conditions, regulatory mechanisms keep both systems in check, but environmental stressors like tobacco smoke or other forms of environmental exposure can destabilize this balance toward pathological activation. To this end, we highlighted evidence of smoking/vaping-induced complement activation, which participates in disease development. Similarly, smokers exhibit higher circulating levels of coagulation/clotting biomarkers, such as fibrinogen, D-dimer (fibrin degradation product), and homocysteine, reflecting a tendency toward hypercoagulation (94). Additionally, when the complement and coagulation systems are co-activated by smoking or vaping, they can amplify each other's effects. For example, complement C5a produced in smoke-exposed tissues can stimulate neutrophils and monocytes to release tissue factor-rich microvesicles, which accelerates coagulation. Conversely, thrombin generated in smokers may further cleave complement components, thereby creating a vicious cycle. A recent proteomic study in a mouse smoking model illustrates this interplay: cigarette smoke led to a prothrombotic shift in lung lymphatic fluid with concurrent upregulation of coagulation and multiple complement proteins (95). These findings clearly underscore the notion that smoking can simultaneously engage the complement cascade and the clotting cascade, creating a compounded inflammatory-coagulatory response. Nevertheless, more studies will be needed to solidify this relationship in the context of chronic diseases development in both smokers and vapers, and determine the possible toxic ingredient(s) that are responsible for such an effect.

Complement activation by smoking: possible suspects

Concerning the potential tobacco chemicals that might activate the complement system, while the data available thus far are limited, activation of the classical pathway by tobacco glycoprotein (TGP)-which is derived from tobacco leaves- and a similar substance from cigarette smoke condensate- referred to as TGP-S- was explored (96). The data showed that these substances activate the complement system through direct interaction with the C1q component, which was indicative of a mechanism for classical pathway activation. A key finding is that TGP and TGP-S, by binding to C1q, can initiate complement activation, albeit TGP-S does not lead to the formation of a C3 cleaving enzyme, suggesting only partial activation. Additionally, the study provides evidence that polyphenols associated with tobacco, such as chlorogenic acid and rutin, can mimic the activation effects of TGP and TGP-S, further implicating/highlighting a role for tobacco's chemical constituents in complement activation (96). Consistent with the TGP effect on immunity, other studies have also shown that TGP is in fact capable of altering the immune system, through stimulation of B cell and T cell proliferation (97). However, this notion (TGP effects) was challenged by others, as it was shown that it is the TGP isolation protocol (98) that yielded contaminants- mainly polyacrylate- that are responsible for this activity; rather than

TGP itself. Nevertheless, this area of research is understudied, and more work needs to be done to clearly understand/uncover the role of TGP in modulating the complement system (99).

Smoldering tobacco products produce particulate matter (PM), which is a major indoor air pollutant that has been linked to multiple diseases, including cancer (100–102). To this end, PM, which is a mixture of different-sized liquid and solid particles, varies in source and composition (103). Traditionally, PM is classified according to particle size, which correlates with the penetration into the respiratory tract. The smaller the particles, the deeper they penetrate and the more severe the associated health effects are (104, 105). Consequently, PM could be another suspect that can activate the complement system. Indeed, there is data showing that the development of airway hyperresponsiveness (AHE) is a complement-mediated process (20). In this connection, PM exposure of wild-type mice resulted in significant increases in AHR, whereas it did not significantly increase airway reactivity in (C3^{-/-}) mice (20). Similarly, it was also shown that PM is capable of activating the complement system as a part of a “pan-activation” that also involves coagulation and the Kallikrein-Kinin systems. This specific activation was observed both *ex vivo* and *in vivo* through the rise in both C3a and C5a, which was found to be mainly mediated by fine particulate matter PM_{2.5} (106). PM was also shown to activate complement through membrane attack complex (MAC) on the surface of endothelial cells, which led to the promotion of inflammation (107).

Tobacco smoking was also linked to a profile of toxic metals such as arsenic (As), cadmium (Cd), chromium (Cr), nickel (Ni), and lead (Pb) (108). These metals were shown to be associated with negative health effects, including cancer (109), cardiovascular, and renal diseases as well as other health issues (110, 111). Thus, the association between exposure to metals such as copper, zinc, and arsenic, and serum levels of the complement components C3 and C4 was explored (112). In a recent study (with a sample size of (n=2,977)), 17 plasma metals, serum C3 and C4 levels, and genetic risk scores (GRSs) derived from single nucleotide polymorphisms that are associated with C3 or C4, were investigated. A positive association between plasma copper levels and both C3 and C4 levels, and between plasma zinc levels and C3 levels was found. Additionally, a significant interaction was observed between arsenic exposure and C3 genetic predisposition in relation to serum C3 level, suggesting that arsenic exposure could modify the association between genetic predisposition to C3 levels and actual serum C3 levels (112). Collectively, data from this study provides insight regarding the impact of metal exposure on the human immune system, particularly the complement system.

As was highlighted earlier in this review, tobacco smoking is linked/contains a host of different chemicals, some of which are known to be highly toxic (113). In this connection, the toxic profile of tobacco mainstream smoke was characterized in different studies (114, 115), which makes it possible to investigate the impact of some of these chemicals on the complement system, using curated databases such as the comparative toxicogenomic database (CTD) to delineate possible impacted pathways (116). Hence, we first used

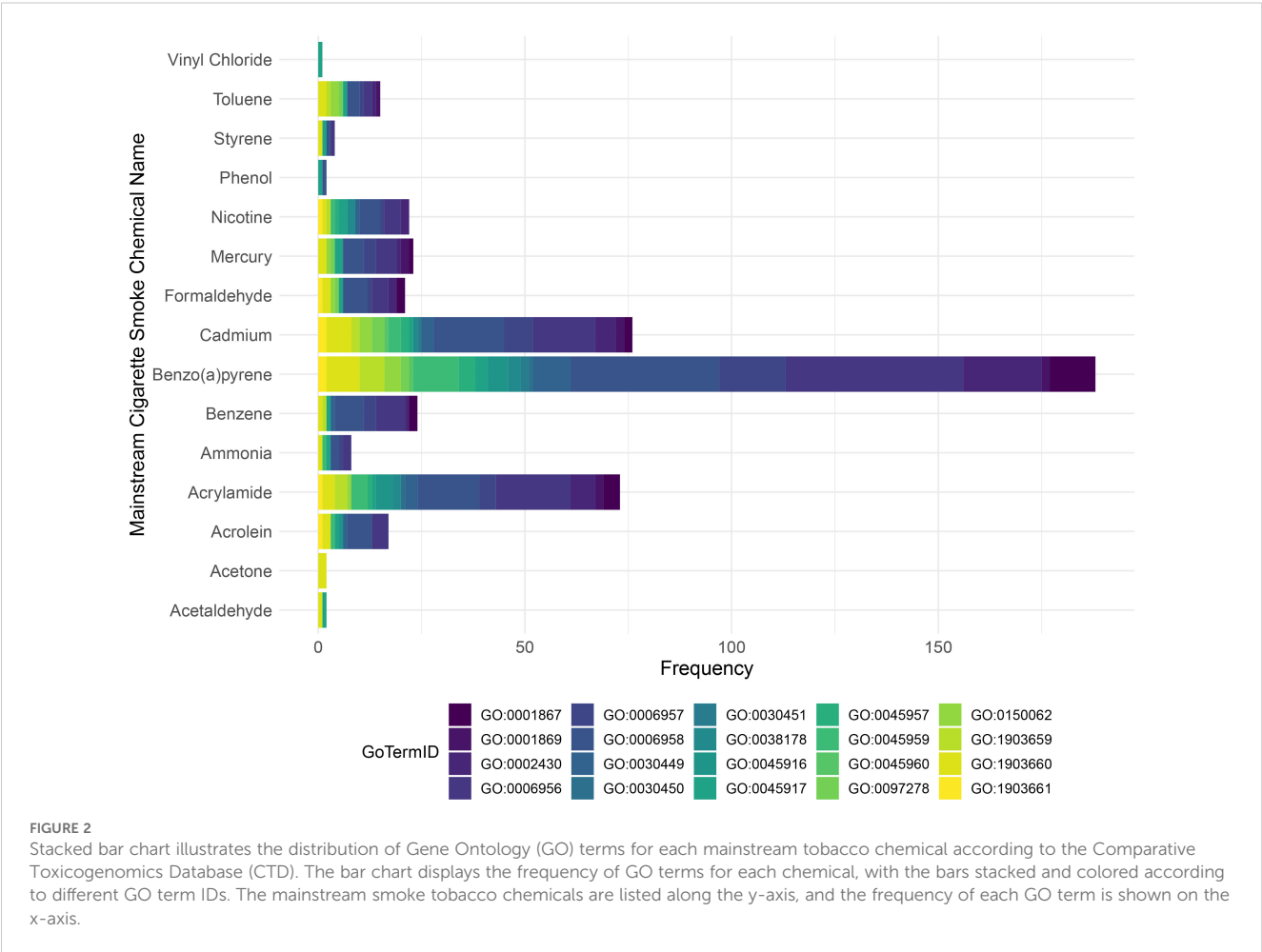
these studies to extract the mainstream cigarette smoke chemical profile (list of chemicals “produced” by mainstream cigarette smoke). We next utilized the CTD to enrich for complement biological processes to determine if there are any interactions between these chemicals and the complement system (Figure 2). Our analysis highlights the different complement processes affected by each mainstream cigarette smoke chemical, with Cadmium and Benzo(a)pyrene showing notably high frequencies of association with complement gene ontology (GO). Specifically, complement activation (GO:0006956) showed highest frequency in benzo(a)pyrene, acrylamide, and cadmium (Figure 2). Benzo(a)pyrene also showed high frequency for complement receptor mediated signaling pathway (GO:0002430) as well as complement activation-alternative pathway (GO:0006957) (Figure 2). These data- although predictive in nature- do provide new and important insight as to the possible culprit chemicals of tobacco smoke that might be engaged in activating the complement system and participating in the development of various disease states.

Taken together, these data provide evidence supporting the notion that tobacco smoking- through its constituents- can trigger activation of the complement system, which in turn participates in initiating an inflammatory state and creates/contributes to a host of disease conditions.

Conclusion

This review underscores the profound and multifaceted impact of smoking on the complement system and highlights the intricate mechanisms through which tobacco use exacerbates the pathogenesis of a wide range of diseases. The evidence presented highlights the dual nature of the complement system—on one hand, it serves as a critical player/defender against invaders, whereas on the other hand, it appears to also be a potential perpetrator of tissue damage, inflammation, and diseases when dysregulated by external factors such as smoking. Through detailed exploration of the literature, we have delineated how smoking influences complement activation, and contributes to the genesis of cardiovascular, respiratory, and a host of inflammatory and degenerative conditions.

Crucially, the interaction between smoking and the complement system is not merely a linear process, but rather involves a complex network of genetic, biochemical, and environmental factors. To this end, genetic predispositions, such as polymorphisms in complement-related genes, can significantly modulate the impact of smoking on disease risk and progression, emphasizing the importance of a personalized approach in understanding and managing these risks. Moreover, through work by us and others, the specific components of tobacco smoke, including chemical constituents like TGP and PM, as



well as toxic metals, and potentially other mainstream smoking chemicals have been identified as key players in complement activation. The implications of these findings are at least two folds: firstly, they provide a compelling argument reaffirming the need for public health initiatives aimed at reducing tobacco and tobacco-related products use, given the clear link between smoking and disease development; and secondly, they highlight the urgent need for further research into targeted therapies that can mitigate the effects of complement activation in smokers, while also potentially offering new avenues for the treatment of smoking-related diseases. This can be achieved, for example, by targeting specific components of the complement system, such as anaphylatoxins (C5a and C3a) and their receptors that are known for their potent inflammatory effect. However, more work is needed in order to be able to fine-tune complement activation using a targeted approach. In conclusion, this review not only adds to the body of evidence on the harmful effects of smoking, but also importantly- opens new research directions for (mechanistic) understanding of the complex interactions between smoking, the complement system, and the pathogenesis of a host of diseases. It also calls for a holistic research approach that combines public health measures, individual genetic risk assessment, and innovative therapeutic strategies to combat the global health crisis posed by tobacco use.

Author contributions

AA: Conceptualization, Writing – original draft, Formal Analysis, Writing – review & editing. FA: Conceptualization, Writing – review & editing. FK: Conceptualization, Funding acquisition, Writing – review & editing.

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Conflict of interest

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