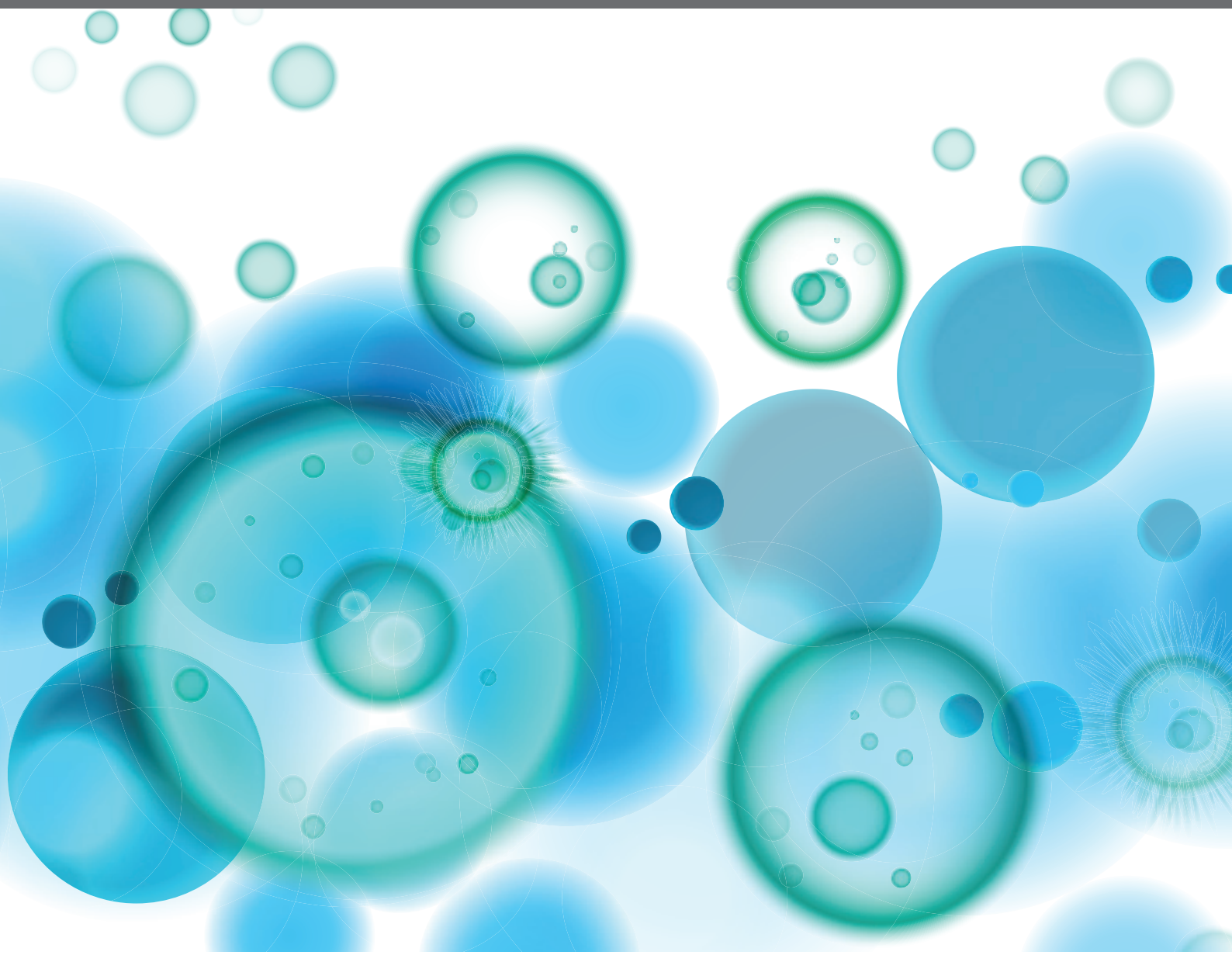
COMPLEMENT AND COVID-19 DISEASE

EDITED BY: Nicolas Stephane Merle and Zoltán Prohászka
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-662-8
DOI 10.3389/978-2-88976-662-8

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

COMPLEMENT AND COVID-19 DISEASE

Topic Editors:
Nicolas Stephane Merle, National Heart, Lung, and Blood Institute (NIH), United States
Zoltán Prohászka, Semmelweis University, Hungary

Citation: Merle, N. S., Prohászka, Z., eds. (2022). Complement and COVID-19 disease. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-662-8

Table of Contents

frontiers | Frontiers in Immunology

EDITORIAL
published: 01 July 2022
doi: 10.3389/fimmu.2022.960809

Editorial: Complement and COVID-19 Disease

Zoltán Prohászka[1,2*] *and Nicolas S. Merle*[3]

[1] Research Group for Immunology and Haematology, Semmelweis University- Eotvos Lorand Research Network (Office for Supported Research Groups), Department of Internal Medicine and Haematology, Semmelweis University, Budapest, Hungary, [2] Department of Internal Medicine and Haematology, Semmelweis University, Budapest, Hungary, [3] Complement and Inflammation Research Section (CIRS), National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, MD, United States

Keywords: COVID-19, SARS – CoV – 2, complement system, thromboinflammation, kinin-kallikrein system

Editorial on the Research Topic

Complement and COVID-19 Disease

Since December 2019 and the first confirmed case of SARS-CoV-2 in Wuhan, China, the world has faced an unprecedent global health crisis. Within 2.5 years, more than six million and counting individuals have died from COVID-19 disease, and at least half a billion cases have been reported. The COVID-19 crisis has been answered with an unprecedent effort of the entire Scientific community that was accompanied by worldwide safety measures and next-generation of mRNA vaccines. By inducing the production of specific neutralizing antibodies against the spike protein of SARS-CoV-2 to prevent virus entry in targeted cells, large-scale vaccination dramatically helped reducing the quick propagation of the virus. Unfortunately, despite all those efforts, individuals may still suffer from many complications that would require oxygen supply and lead to death from SARS-CoV-2.

Early on in the pandemic, many co-morbidities and factors – such as age, genetics, pre-existing disease condition– were identified as increasing risks of severe COVID-19 disease and related death. While it helped improving patients' management and saved many lives, deciphering the biological reaction leading patient's complications is necessary to provide better care and prevent death. Evidences quickly pointed towards an inappropriate immune response as being, at least in part, responsible for COVID-19 complications. Indeed, many studies conducted throughout the pandemic led to the current understanding of a 'bipolar' immune response. If an early inflammation is beneficial to prevent virus entry and its spread throughout the body, it becomes detrimental for the host when the whole system is not properly controlled and shutdown. When happening, overactivation of innate immune system and the subsequent cytokine storm escalates to severe lung injuries and generalized inflammation as observed in Multisystem Inflammation Syndrome (MIS). Based on this understanding, clinical trials and drug-repurposing established that anti-inflammatory treatment (e.g. dexamethasone and tocilizumab) would help lower down escalation of the inflammatory response. However, further studies are still necessary to acquire deeper understanding of the mechanisms at play to develop better targeted therapies and improve our knowledge of SARS-CoV-2 virus.

The complement system is part of the innate immunity. It is implicated in many pathological processes, particularly in hemolytic disorders associated with thrombotic microangiopathy (TMA) where monoclonal anti-C5 therapy eculizumab has revolutionized life prognosis (1). This ancient cascade has established shared activation and regulation at least in part, with many systems, ranging from chemotaxis activities to intricate relationships with coagulation pathways and kallikrein-kinin

OPEN ACCESS

Edited and reviewed by:
Francesca Granucci,
University of Milano-Bicocca, Italy

***Correspondence:**
Zoltán Prohászka
prohaszka.zoltan@med.semmelweis-univ.hu

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *03 June 2022*
Accepted: *14 June 2022*
Published: *01 July 2022*

Citation:
Prohászka Z and Merle NS (2022) Editorial: Complement and COVID-19 Disease. Front. Immunol. 13:960809. doi: 10.3389/fimmu.2022.960809

system. In COVID-19, besides inflammation, thrombosis is a clinical feature shared among patients suffering from severe symptoms. Furthermore, the hijacking of ACE2 protein by SARS-CoV-2 virus to enter cells that impairs its function led to speculation of an imbalanced kallikrein-kinin system and was soon after demonstrated to participate in the thromboinflammation caused by COVID-19 (2, 3). Finally, a sustained inflammation and high levels of complement activation were found in SARS-CoV-2 infected patients observing an unusual long course of the disease with late complications, also known as long COVID-19. Altogether, these evidences suggested a key role of the complement system in the clinical complications faced by those patients.

In this Research Topic, we aimed to gather Original Research articles, Reviews and Case Reports which have improved our understanding about the relation between complement system and COVID-19 disease severity, long-COVID-19, and outcomes. A total of fourteen articles were published under this collection addressing how complement system is activated by SARS-CoV-2, but also how complement proteins could be used as biomarkers for COVID-19 disease.

Complement system impairment has been documented as directly or indirectly involved in pathophysiological mechanisms of many diseases, including autoimmune and infection diseases (4). Easy access to complement proteins by serology make them markers of choice to identify pathologies in patients. In more recent years, complement deposits and circulating complement protein levels have been increasingly reported as predictive markers in different disease courses (5–8). In a cohort of 128 patients with COVID-19, Sinkovits et al. described that overactivation and consumption of C3 correlated with other markers of inflammation – such as IL6 and CRP – and can be used as predictive marker of mortality of SARS-CoV-2 infection. This result echoed the meta-analysis performed on 19 studies and more than 3700 COVID-19 patients by Zinellu and Mangoni and determining C3 and C4 as potential predictive factors of COVID-19 severity and mortality at the peak of the disease. Moreover, the study from Senent et al. suggested that complement activation is linked to long-term COVID-19. By analyzing samples from a total of 50 patients over the course of 3 months, authors were able to correlate high levels of circulating C5a with the presence of long-term respiratory symptoms suggesting the use of C5a as a potential biomarker for long-term SARS-CoV-2 infection.

Considering the length and complexity of the inflammation caused by COVID-19, complement system activation is likely driven by many different signals leading to activation of the three pathways rather than an exclusive one. Nonetheless, it would be expected that implication of each pathway to be time- and context-dependent.

For instance, lectin pathway importance in COVID-19 disease was challenged by Panteleimon Charitos et al. but authors rather found an association between classical and alternative pathways activities and critically ill patients. Noteworthy, SARS-CoV-2 receptor-binding domain drives IgG1 and IgG3 subclasses response (9–11). Considering their ability to bind C1q and to activate the classical pathway, Jarlhelt et al. demonstrated that IgG levels and disease severity directly correlate with classical pathway activation, indicating that elevated IgG levels and/or severe disease might be associated with prominent complement activation during viral infection.

However, by analyzing deposition of complement proteins in lungs and kidneys from patients who died from SARS-CoV-2 infection, Niederreiter et al. showed significant increase of MASP-2, C3d and C5b-9, suggesting that the lectin pathway is involved in worsening systemic inflammatory response. This was further supported by a study on a cohort of 74 hospitalized patients due to COVID-19, where Defendi et al. revealed that high levels of lectin pathway activation constituted the higher proportion of patients who required oxygen support or ICU care and died. Besides, the *in vitro* investigation conducted by Ali et al. incriminates the lectin pathway for inducing C3b deposition on cell surface and corroborated the encouraging performance of MASP-2 inhibitor Narsoplimabin a clinical trial conducted on severe COVID-19 patients (12).

Crosstalk between intravascular cascades like complement, coagulation and kallikrein-kinin systems can collectively contribute to cytokine storm, general inflammation and acute respiratory distress syndrome observed in SARS-CoV-2 infection. Savitt et al. showed that SARS-CoV-2 proteins – Envelope, Spike, Nucleocapside and Membrane proteins – can directly activate the classical complement, the coagulation and the kallikrein-kinin systems by binding to C1q, FXII and high molecular weight kininogen, respectively. Furthermore, they showed that viral proteins turn globular C1q Receptor as a platform for complement and kallikrein-kinin pathways, indicating a direct cross-reactivity between the 2 systems.

Pre-existing disease may be an aggravating factor of COVID-19 disease. Accordingly, Peerschke et al. established that thromboinflammation as evidenced by increased plasma D-dimer levels in cancer patients was associated with elevated complement activation. This was further linked with increased mortality in the setting of COVID-19, emphasizing the potential of complement system as a predictive marker in COVID-19 survival.

Thromboinflammation being a key feature associated with COVID-19 death, it is fair to assume that pre-existing hemolytic disease condition would inflate SARS-CoV-2 infection and worsen patients' complications. This hypothesis is particularly relevant here, considering that complement is usually involved in organ injuries occurring in hemolytic disorders. In this context, vaccination would be critical to prevent exacerbation of pre-existing hemolytic sensitivity. Fattizzo et al. reported 4 cases – supported by a review of the literature –with complement-mediated hemolytic anemia pre-condition who experienced hemolytic event within 10 days upon infection with SARS-CoV-2. Overall, patients were experiencing more complications and more fatalities compared to groups that received vaccination. Moreover, other therapies like complement inhibitors are especially to be considered in these cases to prevent death. Boudhabhay et al. reported a case of MIS associated with renal Thrombotic Micro-Angiopathy (TMA) and Acute Kidney Injury (AKI) in a 46-year-old patient with hypertension and obesity personal history. In this case report,

authors successfully resolved AKI and dramatically improved renal function upon treatment with eculizumab, strengthening the therapeutic potential of anti-complement therapies in COVID-19 patients.

Interestingly, Ali et al. described a cohort of 217 patients with severe COVID-19 disease in which defect in circulating complement proteins induced by the infection seems to predispose to bacterial infections, most frequently *K. pneumoniae*.

Overall, clinical and basic studies published in this Research Topic and elsewhere revealed that complement system is a key partner of the thromboinflammatory reaction occurring in COVID-19-associated organ injuries. Nilsson et al. reviewed the complexity of the inflammatory response and the intricate relationships between the complement, the coagulation and the kallikrein-kinin systems. Authors elegantly summarized how life-threatening COVID-19 ARDS is associated with a strong activation of the intravascular innate immune system (IIIS), and with the recognition of this link future clinical trials involving the use of complement inhibitors may be fueled with observational data.

Deciphering the mechanisms and understanding the subtilities of the immune response is a long way to go. However, we hope that the variety of articles published in this Research Topic have provided insights on the link between complement, inflammation, and COVID-19 disease, with promising outcomes and future perspectives. As Editors, we would like to thank all the contributing authors and the people in Frontiers in Immunology for their contribution and excellent editing support.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

1. Legendre CM, Licht C, Muus P, Greenbaum LA, Babu S, Bedrosian C, et al. Terminal Complement Inhibitor Eculizumab in Atypical Hemolytic-Uremic Syndrome. *New Engl J Med* (2013) 368(23):2169–81. doi: 10.1056/NEJMoa1208981
2. Sidarta-Oliveira D, Jara CP, Ferruzzi AJ, Skaf MS, Velander WH, Araujo EP, et al. SARS-CoV-2 Receptor is Co-Expressed With Elements of the Kinin-Kallikrein, Renin-Angiotensin and Coagulation Systems in Alveolar Cells. *Sci Rep* (2020) 10(1):19522. doi: 10.1038/s41598-020-76488-2
3. Sodhi CP, Wohlford-Lenane C, Yamaguchi Y, Prindle T, Fulton WB, Wang S, et al. Attenuation of Pulmonary ACE2 Activity Impairs Inactivation of Des-Arg(9) Bradykinin/BKB1R Axis and Facilitates LPS-Induced Neutrophil Infiltration. *Am J Physiol Lung Cell Mol Physiol* (2018) 314(1):L17–31. doi: 10.1152/ajplung.00498.2016
4. Ekdahl KN, Persson B, Mohlin C, Sandholm K, Skattum L, Nilsson B. Interpretation of Serological Complement Biomarkers in Disease. *Front Immunol* (2018) 9. doi: 10.3389/fimmu.2018.02237
5. Daugan MV, Revel M, Russick J, Dragon-Durey MA, Gaboriaud C, Robe-Rybkine T, et al. Complement C1s and C4d as Prognostic Biomarkers in Renal Cancer: Emergence of Noncanonical Functions of C1s. *Cancer Immunol Res* (2021) 9(8):891–908. doi: 10.1158/2326-6066.CIR-20-0532
6. Reese B, Silwal A, Daugherity E, Daugherity M, Arabi M, Daly P, et al. Complement as Prognostic Biomarker and Potential Therapeutic Target in Renal Cell Carcinoma. *J Immunol* (2020) 205(11):3218–29. doi: 10.4049/jimmunol.2000511
7. Martin M, Smolag KI, Bjork A, Gullstrand B, Okroj M, Leffler J, et al. Plasma C4d as Marker for Lupus Nephritis in Systemic Lupus Erythematosus. *Arthritis Res Ther* (2017) 19(1):266. doi: 10.1186/s13075-017-1470-2
8. Crnogorac M, Horvatic I, Kacinari P, Ljubanovic DG, Galesic K. Serum C3 Complement Levels in ANCA Associated Vasculitis at Diagnosis Is a Predictor of Patient and Renal Outcome. *J Nephrol* (2018) 31(2):257–62. doi: 10.1007/s40620-017-0445-3
9. Kallolimath S, Sun L, Palt R, Stiasny K, Mayrhofer P, Gruber C, et al. Highly Active Engineered IgG3 Antibodies Against SARS-CoV-2. *Proc Natl Acad Sci USA* (2021) 118(42):e2107249118. doi: 10.1073/pnas.2107249118
10. Luo H, Jia T, Chen J, Zeng S, Qiu Z, Wu S, et al. The Characterization of Disease Severity Associated IgG Subclasses Response in COVID-19 Patients. *Front Immunol* (2021) 12. doi: 10.3389/fimmu.2021.632814
11. Faustini SE, Jossi SE, Perez-Toledo M, Shields AM, Allen JD, Watanabe Y, et al. Development of a High-Sensitivity ELISA Detecting IgG, IgA and IgM Antibodies to the SARS-CoV-2 Spike Glycoprotein in Serum and Saliva. *Immunology* (2021) 164(1):135–47. doi: 10.1111/imm.13349
12. Rambaldi A, Gritti G, Mico MC, Frigeni M, Borleri G, Salvi A, et al. Endothelial Injury and Thrombotic Microangiopathy in COVID-19: Treatment With the Lectin-Pathway Inhibitor Narsoplimab. *Immunobiology* (2020) 225(6):152001. doi: 10.1016/j.imbio.2020.152001

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 25 March 2021
doi: 10.3389/fimmu.2021.663187

Complement Overactivation and Consumption Predicts In-Hospital Mortality in SARS-CoV-2 Infection

György Sinkovits[1†], Blanka Mező[1,2†], Marienn Réti[3†], Veronika Müller[4], Zsolt Iványi[5], János Gál[5], László Gopcsa[3], Péter Reményi[3], Beáta Szathmáry[6], Botond Lakatos[6], János Szlávik[6], Ilona Bobek[7], Zita Z. Prohászka[1], Zsolt Förhécz[1], Dorottya Csuka[1], Lisa Hurler[1], Erika Kajdácsi[1], László Cervenak[1], Petra Kiszel[2], Tamás Masszi[1], István Vályi-Nagy[6,7‡] and Zoltán Prohászka[1,2‡]*

[1] Department of Internal Medicine and Haematology, Semmelweis University, Budapest, Hungary, [2] Research Group for Immunology and Haematology, Semmelweis University–Eötvös Loránd Research Network (Office for Supported Research Groups), Budapest, Hungary, [3] Department of Haematology and Stem Cell Transplantation, Central Hospital of Southern Pest National Institute of Haematology and Infectious Diseases, Budapest, Hungary, [4] Department of Pulmonology, Semmelweis University, Budapest, Hungary, [5] Department of Anaesthesiology and Intensive Therapy, Semmelweis University, Budapest, Hungary, [6] Department of Infectology, Central Hospital of Southern Pest National Institute of Haematology and Infectious Diseases, Budapest, Hungary, [7] Department of Anaesthesiology and Intensive Therapy, Central Hospital of Southern Pest National Institute of Haematology and Infectious Diseases, Budapest, Hungary

Objectives: Uncontrolled thromboinflammation plays an important role in the pathogenesis of coronavirus disease (COVID-19) caused by SARS-CoV-2 virus. Complement was implicated as key contributor to this process, therefore we hypothesized that markers of the complement profile, indicative for the activation state of the system, may be related to the severity and mortality of COVID-19.

Methods: In this prospective cohort study samples of 102 hospitalized and 26 outpatients with PCR-confirmed COVID-19 were analyzed. Primary outcome was in-hospital, COVID-19 related mortality, and secondary outcome was COVID-19 severity as assessed by the WHO ordinal scale. Complement activity of alternative and classical pathways, its factors, regulators, and activation products were measured by hemolytic titration, turbidimetry, or enzyme-immunoassays. Clinical covariates and markers of inflammation were extracted from hospital records.

Results: Increased complement activation was characteristic for hospitalized COVID-19 patients. Complement activation was significantly associated with markers of inflammation, such as interleukin-6, C-reactive protein, and ferritin. Twenty-five patients died during hospital stay due to COVID-19 related illness. Patients with uncontrolled complement activation leading to consumption of C3 and decrease of complement activity were more likely to die, than those who had complement activation without consumption. Cox models identified anaphylatoxin C3a, and C3 overactivation and consumption (ratio of C3a/C3) as predictors of in-hospital mortality [HR of 3.63 (1.55–8.45, 95% CI) and 6.1 (2.1–17.8), respectively].

OPEN ACCESS

Edited by:
Cordula M. Stover, University of Leicester, United Kingdom

Reviewed by:
Marcin Okrój, Intercollegiate Faculty of Biotechnology of University of Gdańsk and Medical University of Gdańsk, Poland
Vinod Kumar, Radboud University Nijmegen Medical Centre, Netherlands

***Correspondence:**
Zoltán Prohászka
prohaszka.zoltan@med.semmelweis-univ.hu

[†]*These authors have contributed equally to this work and share first authorship*

[‡]*These authors have contributed equally to this work and share last authorship*

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *02 February 2021*
Accepted: *05 March 2021*
Published: *25 March 2021*

Citation:
Sinkovits G, Mező B, Réti M, Müller V, Iványi Z, Gál J, Gopcsa L, Reményi P, Szathmáry B, Lakatos B, Szlávik J, Bobek I, Prohászka ZZ, Förhécz Z, Csuka D, Hurler L, Kajdácsi E, Cervenak L, Kiszel P, Masszi T, Vályi-Nagy I and Prohászka Z (2021) Complement Overactivation and Consumption Predicts In-Hospital Mortality in SARS-CoV-2 Infection. Front. Immunol. 12:663187. doi: 10.3389/fimmu.2021.663187

Conclusion: Increased complement activation is associated with advanced disease severity of COVID-19. Patients with SARS-CoV-2 infection are more likely to die when the disease is accompanied by overactivation and consumption of C3. These results may provide observational evidence and further support to studies on complement inhibitory drugs for the treatment of COVID-19.

Keywords: SARS-CoV-2 infection, mortality, severity, complement system, coronavirus disease (COVID-19), complement activation and consumption

INTRODUCTION

Three highly pathogenic coronaviruses for human populations appeared in the past two decades. First, severe acute respiratory syndrome (SARS) was described in 2003 when the emergence of a human pathogen coronavirus (SARS-CoV) was noted (1–3). Second, in September 2012, Middle East Respiratory Syndrome Coronavirus (MERS-CoV) was reported to cause human infection (4). Third, in December, 2019, a novel coronavirus (SARS-CoV-2) causing coronavirus disease (COVID-19) was initially identified in Wuhan, China, and caused later a pandemic starting in early March, 2020 (5). The rapid, global spread of the virus is presently ongoing, by late January, 2021, over 4.0 million cases per week are being reported worldwide, with around 70.000 deaths per week (6). Organ dysfunction, particularly complex coagulopathy and respiratory failure often in the context of acute respiratory distress syndrome (ARDS), and multiple organ failure are associated with highest mortality, especially in the elderly (7).

The complement system is generally considered as part of the first line defense against pathogens, including viral infections, and this fact is marked by the multiple mechanisms by which viruses avoid complement attack (8). On the other hand, complement (with or without the contribution of antibodies) may also play an enhancing role in viral infections, as described for HIV (9–11). Furthermore, complement activation, especially generation of anaphylatoxin C5a, is involved in the development of acute lung injury caused by influenza A (12). Animal experiments also supported the involvement of complement activation in the development of SARS caused by CoV infection (13). Accordingly, complement inhibitors are considered as promising therapeutic options during the ongoing SARS-CoV-2 pandemic (14, 15), and the first COVID-19 cases treated by the C5 inhibitor monoclonal antibody eculizumab (16–18), and by the C3-targeting small peptide inhibitor AMY-101 (19) have recently been reported. Further in line with these considerations, Annane et al. reported promising survival results in a proof-of-concept, non-randomized study of 80 eculizumab treated intensive care unit patients with COVID-19 (20).

Complement system is a sensitive enzymatic cascade responsive through its lectin pathway to carbohydrate patterns of pathogens, or through its classical pathway to antibodies (21, 22). The alternative pathway (AP) represents an amplification loop on the level of the central component C3, with generation of powerful biological mediators including anaphylatoxins, opsonins, and lytic complexes (23). Patients with various infections may benefit from laboratory evaluation of the complex system, which can show the presence of complement deficiency, activation state, or consumption of the whole system, or its dedicated pathways (24, 25). Two recent studies have shown a clear association between respiratory failure, COVID-19 outcome, and systemic complement activation (26, 27) in COVID-19 patients. Furthermore, a large-scale multi-omic analysis of COVID-19 severity mapped several molecular features including the complement system, and identified strong associations between complement activation and severe COVID-19 phenotype (28). In a recent comprehensive review Perico and colleagues described multiple mechanisms of how endothelial cells, and complement system particularly, may contribute to severe thromboinflammation in COVID-19 (29). Finally, Eriksson et al. have identified complement activation through the MBL pathway as a novel amplification mechanism that contributes to pathological thrombosis in critically ill COVID-19 patients (30).

Based on the multiple potential interactions between viral pathogens and complement, and principally on the above described associations we hypothesized that complement activation may be an important factor in the pathogenesis of SARS-CoV-2 infection. Accordingly, the aim of our work was to describe activities of the alternative and classical complement pathways, levels of their components, regulators and activation products in COVID-19 patients, and to search for associations between severity and outcome of COVID-19 and complement activation, dysregulation, and consumption.

PATIENTS AND METHODS

Patient Selection

We enrolled an adult (age >18 years) cohort of SARS-CoV-2 infected hospitalized patients. One hundred and ten subjects, who received care for suspected COVID-19 in two tertiary hospitals in Budapest, were sampled and screened for inclusion into this study. Finally, to form groups as homogenous as possible, 102 hospitalized patients were included, as presented on the study flow chart (**Figure 1**). Inclusion criteria were: 1, COVID-19 disease confirmed by at least 1 positive SARS-CoV-2 RT-PCR test result from a nasopharyngeal swab specimen, 2, Available sample for complement analysis taken when the patient was hospitalized due to acute SARS-CoV-2 infection, 3,

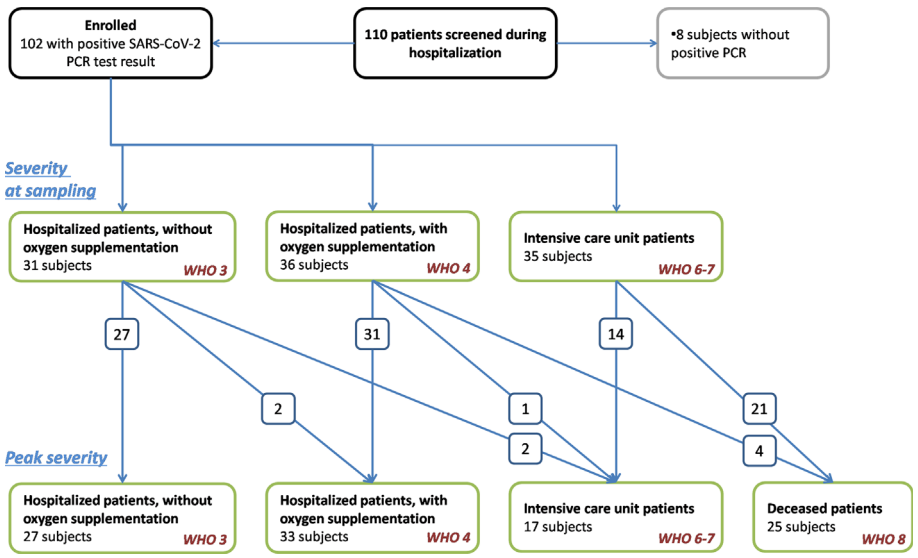


FIGURE 1 | Study flow chart. Screening and enrollment of patients with SARS-CoV-2 infection. Only hospitalized patients with PCR-confirmed SARS-CoV-2 infection were enrolled into the study. Clinical and treatment data indicating severity of COVID-19 were extracted from electronic hospital records, and patients were stratified according to severity in two time-points: first, at the time point when sampling for complement analysis was done, and second, according to the worst clinical condition (or death). Arrows and numbers indicate the number of patients whose COVID-19 progressed, i.e. these patients progressed to an advanced severity group (or died) after sampling. Definitions for severity groups were based on WHO protocol (31). Note, that extra-corporeal membrane oxygenator treatment, non-invasive ventilation, or high-flow oxygen therapy was not used for patients in this study, therefore, there is no "WHO 5" severity group in this study. Patients, who were registered to donate convalescent plasma in a clinical trial (32), and who had evidence of past SARS-CoV-2 infection but did not require hospital treatment were sampled in convalescent phase formed the patient control group.

Accessible digital hospital record to extract clinical data. Patients, who were registered to donate convalescent plasma in a clinical trial (32), and who had evidence of past SARS-CoV-2 infection but did not require hospital treatment were sampled in convalescent phase, and formed the patient control group (CONTR). The study was approved by the Hungarian Ethical Review Agency (ETT-TUKEB; No. IV/4403-2/2020/EKU). Written informed consent was obtained from the patients, or from the closest relative available, if the patient was unable to give consent. The Declaration of Helsinki and its subsequent revisions were followed.

Outcomes, Definitions

The primary outcome of this study was in-hospital, all-cause mortality, and the secondary outcome was severity of COVID-19. Assessment of disease severity was based on an eight-point ordinal scale as outlined by the WHO (31), using the following definitions: Hospitalized, but not critical patients were divided into two categories based on the requirement of supportive oxygen therapy [WHO category 3, "In-hospital patients without oxygen support" (HOSP); or WHO 4, "In-hospital patients with nasal oxygen support" (HOSP+O_2)]. Critical patients included all patients who required intensive care unit (ICU) treatment for any organ support (WHO category 6 and 7, "critical") and/or died (WHO 8, FATAL). Severity was first assessed when sampling was performed, and peak in-hospital severity was also registered (**Figure 1**).

Samples

Blood samples (native- and EDTA-anticoagulated blood) were taken after fasting from the antecubital vein, or from a central venous catheter. Samples were transferred to the processing laboratory immediately after the sample was taken, cells and serum/plasma were separated by centrifugation, and aliquots were stored at −70°C until measurements.

Laboratory Determinations

Fasting blood samples were used to measure standard clinical chemistry and inflammatory parameters and complete blood counts. Concentrations of C3 and C4 were measured by turbidimetry (Beckman Coulter, Brea, CA, USA). Total activity of alternative and lectin pathway was measured by a commercially available kit (Wieslab AP and LP ELISA KITs, KOMPL AP330 and KOMPL MBL320, Svar Life Science, Malmö, Sweden), according to the manufacturer's instructions. Total classical pathway activity was measured by hemolytic titration test based on Mayer's method (33). Radial immunodiffusion was performed to measure the antigenic concentrations of Factor I and Factor B, using specific polyclonal antibodies (34). Levels of Factor H and C1q were determined by homemade ELISA (34, 35). Complement activation markers such as sC5b-9 and C3a were detected by commercially available ELISA kits (MicroVue C3a-desArgEIA, A032; MicroVue sC5b-9 Plus EIA A029) in EDTA plasma sample.

Statistical Analysis

Categorical data are reported as frequencies (%). As most of the variables showed skewed distributions, data are presented as medians and interquartile (IQ) ranges, and non-parametric statistical tests were used (Spearman rank correlation test, Mann–Whitney test) for two-, and Kruskal-Wallis test (with Dunn's post test) for multiple independent groups). Uni- and multivariate Cox proportional hazards models were fitted to assess the effect of complement activation on primary outcome events. Survival times were measured from hospitalization until discharge of the patients from the hospital (including transmission to rehabilitation care) or death (all-cause, in-hospital mortality). The results of the Cox regression models are presented as hazard ratios (HR), the corresponding 95% confidence intervals (CIs), and the chi-squares and p values of likelihood ratio tests. Findings with p-values below 0.05 were considered statistically significant. Statistical calculations were performed with the GraphPad Prism 5 software (GraphPadSoftwares Inc., La Jolla, CA, USA) or by Statistica 13.5 (TIBCO Softwares Inc, Palo Alto, CA USA).

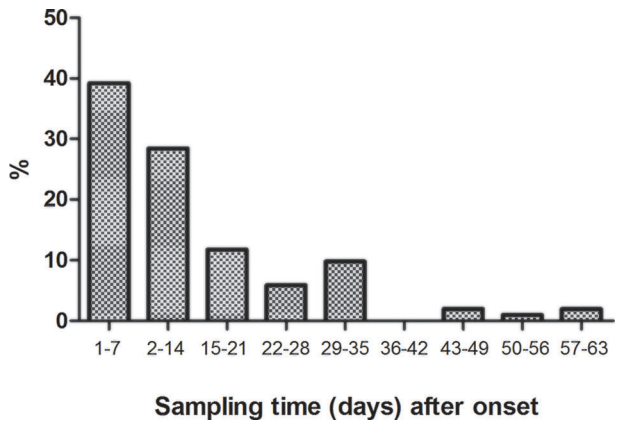


FIGURE 2 | Histogram (fraction of patients) showing time delay (days) between onset of disease (first symptoms) and sampling.

RESULTS

One hundred and two SARS-CoV-2 positive hospitalized patients were enrolled into this study (**Figure 1**). Twenty-six patients who were all outpatients when infected with SARS-CoV-2, and were sampled in convalescent phase, formed the patient control group [sampled at mean 53 (SD 13), min: 26, max: 74 days after onset of symptoms].

In-hospital patients (n = 102) were divided into three severity subgroups (without or with nasal oxygen support, or necessitating intensive care unit treatment) when sampled for this study. Sampling was done when the patient was first hospitalized in Central Hospital of Southern Pest National Institute of Haematology and Infectious Diseases or in Semmelweis University, or when the patient was transferred from a different hospital to our centers. Therefore, the elapsed time between onset of symptoms and sampling varied between 1 day and 63 days, as shown on **Figure 2**. Typically, sampling was done on day 8 (median) after onset of symptoms [this is day 6 (median) after first PCR positivity]. Sixty-seven percent of patients were sampled on the first 2 weeks after onset, and the remaining one-third on weeks 3–9 (**Figure 2**). Extra-corporeal membrane oxygenator treatment, non-invasive ventilation, or high-flow oxygen therapy was not used for patients in this study, therefore, no patients fall into WHO category 5.

Severity of COVID-19 changed over time during the hospital stay (as presented on **Figure 1**), therefore, severity was assessed at a second time when "peak" was reached. Thirty-three in-hospital patients required nasal oxygen support during the hospital stay. During the observation period 25 patients died, 17 survivor patients were discharged from ICU. Baseline characteristics revealed significant differences across various severity categories in comorbidities, complications, and inflammatory markers of COVID-19 (**Table 1**). Severe COVID-19 disease was associated with frequent occurrence of diabetes mellitus and malignant diseases ($p < 0.0001$). Patients, who died later, had four comorbidities (median), whereas survivors had only two (**Table 1**). Increased numbers of in-hospital complications, such as pneumonia, respiratory failure, sepsis, thromboembolic events, and acute kidney injury, were more common among those who required ICU treatment or died (**Table 1**, $p < 0.0001$). Lymphopenia, increased neutrophil count, and gradually increasing IL-6, C-reactive protein (CRP), troponin, and ferritin levels were all present in patients with more severe forms of COVID-19 (**Table 1**).

Complement Profile in Relation to Severity of COVID-19

Results of complement parameters are shown in **Table 2** according to the patient's actual severity stage when sampling was done (see above, and **Figure 2**). Levels of C3, C4, Factor B, and alternative pathway activity showed significant association with COVID-19 severity. Based on the results of post-tests C4 and Factor B levels were highest in hospitalized patients without ICU treatment. Lowest C3 concentration and AP activity were measured in patients in critical stage. The activity of lectin pathway showed no difference between severity groups. Complement activation products C3a and sC5b-9 showed increasing levels across severity groups with a highly significant association, and highest levels in critical patients. Complement parameters were associated with multiple markers of inflammation and coagulation, as shown in **Table 3**. Strong associations were noted between CRP and Factor B, C3a, sC5b-9; ferritin and C3a, sC5b-9; and haptoglobin and C3, C4, Factor B. D-dimer levels were inversely associated with concentrations of C3, C4, and activities of alternative and classical pathways, whereas positively associated with C3a. Among the complement factors, Factor B showed the highest level correlation with complement activation products (for C3a $r = 0.286$, $p = 0.003$; for sC5b-9 $r = 0.328$ and $p = 0.0008$).

TABLE 1 | Basic characteristics of SARS-CoV-2 infected patients, comparison according to peak severity.

Variables	Total, n = 128	Outpatients, n = 26	Hospitalized, no oxygen support, n = 27	Hospitalized, with nasal oxygen support, n = 33	Critical, n = 17	Death n = 25	p-value*
Male sex, n (%)	71 (55.5)	15 (57.7)	17 (63.0)	20 (60.6)	8 (47.1)	11 (44.0)	0.429
Mean age ± SD	60.5 ± 16.5	44.5 ± 10.1	57.0 ± 16.1	68.3 ± 11.3	56.5 ± 15.2	75.3 ± 9.4	<0.0001
Total number of comorbidities (median, IQR)	2 (1–3)	0 (0–1)	2 (1–3)	2 (2–3)	2 (1–3)	4 (2–4)	0.016
Hypertension, n (%)	73 (57.0)	7 (26.9)	13 (48.2)	22 (66.7)	11 (64.7)	20 (80.0)	0.118
Chronic pulmonary disease, n (%)	22 (17.2)	0 (0)	3 (11.1)	6 (18.2)	4 (23.6)	9 (36.0)	0.165
Diabetes mellitus, n (%)	26 (20.3)	1 (3.8)	4 (14.8)	8 (24.2)	2 (11.8)	11 (44.0)	0.046
Chronic heart disease, n (%)	34 (26.6)	0 (0)	6 (22.2)	14 (42.4)	3 (17.7)	11 (44.0)	0.117
Malignant disease, n (%)	23 (18.0)	0 (0)	4 (14.8)	2 (6.1)	8 (47.1)	9 (36.0)	0.003
Other comorbidity, n (%)	89 (69.5)	1 (3.8)	26 (96.3)	28 (84.8)	11 (64.7)	23 (92.0)	0.885
Presenting symptoms							
Fever, n (%)	72 (56.7)	15 (57.7)	9 (33.3)	19 (57.6)	16 (94.1)	13 (52.0)	0.0033
Cough, n (%)	70 (54.7)	14 (53.8)	11 (40.7)	21 (63.6)	11 (64.7)	13 (52.0)	0.3480
Dyspnea, n (%)	57 (44.5)	3 (11.5)	7 (25.9)	16 (48.5)	12 (70.6)	19 (76.0)	<0.0001
Transfer to ICU, n (%)	38 (29.7)	0 (0)	0 (0)	0 (0)	17 (100)	21 (84.0)	<0.0001
Delay between first symptom and sampling, days (median, IQR)	9 (5–20)	–	12.5 (8–28)	8.5 (6–15)	10 (7–28)	6 (3–16)	0.136
Complications							
Pneumonia, n (%)	81 (63.3)	1 (3.8)	14 (51.9)	28 (84.8)	16 (94.1)	22 (88.0)	<0.0001
Respiratory failure necessitating mechanical ventilation, n (%)	30 (23.4)	0 (0)	0 (0)	0 (0)	10 (58.8)	20 (80.0)	<0.0001
Sepsis, n (%)	18 (14.1)	0 (0)	1 (3.7)	1 (3.0)	5 (29.4)	11 (44.0)	<0.0001
Thromboembolic complications, n (%)	14 (10.9)	0 (0)	3 (11.1)	0 (0)	7 (41.2)	4 (16.0)	<0.0001
Acute kidney injury, n (%)	13 (10.2)	0 (0)	0 (0)	2 (6.1)	2 (11.8)	9 (36.0)	0.002
Other complication, n (%)	36 (28.1)	0 (0)	7 (25.9)	10 (30.3)	6 (35.3)	13 (52.0)	0.0013
Death, n (%)	25 (19.5)	0 (0)	0 (0)	0 (0)	0 (0)	25 (100)	<0.0001
Total number of in-hospital complications (median, IQR)	1 (0–2)	0 (0–0)	0 (0–1)	1 (1–2)	2 (1–3)	2 (1–4)	<0.0001
Laboratory findings (median, IQR)							
Neutrophil granulocyte count (2–7.5 G/L)	4.1 (3.0–5.9)	3.9 (3.0–4.6)	3.8 (2.8–5.1)	3.8 (2.9–5.9)	5.0 (3.2–6.1)	6.1 (2.1–10.0)	0.0100
Lymphocyte count (1.5–4 G/L)	1.4 (0.9–1.9)	2.0 (1.8–2.4)	1.6 (1.0–2.2)	1.5 (1.0–1.9)	0.9 (0.8–1.3)	0.8 (0.5–1.1)	<0.0001
Interleukin 6 (2–4.4 pg/mL)	24.2 (7.1–67.9)	1.7 (1.1–2.5)	12.5 (5.6–24.5)	27.8 (9.5–63.8)	40.1 (14.3–51.3)	90.4 (34.6–267.3)	<0.0001
C-reactive protein (<10 mg/L)	29.4 (3.7–107.6)	1.3 (0.3–2.5)	11.6 (5.6–41.0)	36.8 (17.5–88.6)	111 (61.3–169.1)	149.1 (54.9–196.8)	<0.0001
D-dimers (<500 ng/mL)	1,130 (580–1,924)	207 (158–453)	1,460 (610–2,210)	851 (530–1,526)	1,658 (912–3,080)	1,430 (1,106–4,380)	0.009
Ferritin (15–300 ng/mL)	536 (261–1,146)	NA	320 (163–547)	379 (230–710)	1,321 (929–1,784)	702 (423–2,080)	<0.0001
Troponin (<34.0 ng/mL)	20.5 (5.0–51.0)	NA	13 (5–26)	13 (4–31)	15 (4–40)	51 (31–89)	0.0054

**p-values were obtained for nominal variables by the chi-square test, for continuous variables by the Kruskal-Wallis test. Results of outpatients are shown for reference only, this group was not included in the statistical analysis. NA, not applicable/not available.*

Other comorbidities included: Acute myocardial infarction, stroke, chronic renal failure, chronic psychiatric diseases, dementia, epilepsy, sclerosis multiplex, Alzheimer's disease, acute myeloid leukemia, chronic lymphoid leukemia, HIV infection.

Other complications included: pneumothorax, acute atrial fibrillation, urinary tract infection, C. difficile infection/enterocolitis, stroke, ileus, peripheral gangrene.

For laboratory markers reference ranges are indicated in brackets.

TABLE 2 | Complement profile of the SARS-CoV-2 infected patients, comparison according to severity when sampling.

Complement parameter*	Reference range	Severity when sampling				
		Outpatients, n = 26	Hospitalized, no oxygen support, n = 31	Hospitalized, with nasal oxygen support, n = 36	Critical, n = 35	p-value**
Alternative pathway activity, %	70–130	99 (88–104)	94 (82–103)	88 (67–108)	80 (54–96)$^{\#}$	0.0136
Classical pathway activity, CH50/mL	48–103	69 (58–79)	71 (67–86)	83 (63–93)	71 (45–85)	0.1082
Lectin pathway activity, %	25–125	53 (1–130)	55 (1–120)	113 (23–143)	46 (1–120)	0.069
C3, g/L	0.9–1.8	1.26 (1.10–1.42)	1.31 (1.11–1.49)	1.29 (1.10–1.45)	1.11 (0.73–1.37)	0.0443
C4, g/L	0.15–0.55	0.28 (0.20–0.33)	0.37 (0.26–0.56)$^{\#}$	0.36 (0.27–0.47)$^{\#}$	0.30 (0.18–0.51)	0.0108
C1q, mg/L	60–180	99 (90–121)	109 (82–128)	103 (87–134)	114 (85–145)	0.737
Factor B, %	70–130	90 (74–111)	110 (97–139)$^{\#\#}$	129 (90–154)$^{\#\#}$	118 (97–138)	0.0075
Factor H, mg/L	250–880	762 (485–856)	821 (484–1,056)	739 (535–1,051)	640 (337–865)	0.167
Factor I, %	70–130	95 (72–111)	102 (84–119)	109 (91–118)	95 (80–119)	0.483
C3a, ng/mL	70–270	122 (95–171)	235 (117–292)$^{\#}$	210 (140–310)$^{\#\#}$	398 (230–574)$^{\#\#\#}$	<0.0001
sC5b-9, ng/mL	110–252	183 (143–254)	245 (168–374)	307 (220–395)$^{\#\#}$	365 (251–556)$^{\#\#\#}$	0.0001

** All data presented as median (IQR). **p-value based on Kruskal-Wallis test; hashtags indicate results of Dunn's post test ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$), when compared to outpatients.*

TABLE 3 | Correlation between complement parameters and markers of inflammation and fibrinolysis.

	Interleukin 6 (pg/mL)	C-reactive protein (mg/L)	Haptoglobin (g/L)	Ferritin (ng/mL)	D-dimers (ng/mL)
Alternative pathway activity, %	–0.172 (0.087)*	0.023 (0.817)	**0.215 (0.031)**	–0.068 (0.507)	**–0.287 (0.011)**
Classical pathway activity, CH50/mL	–0.074 (0.462)	0.118 (0.237)	**0.345 (0.0003)**	–0.006 (0.953)	–0.209 (0.066)
Lectin pathway activity, %	0.094 (0.393)	0.016 (0.882)	0.152 (0.150)	0.069 (0.545)	**–0.224 (0.049)**
C3, g/L	–0.114 (0.256)	0.069 (0.492)	**0.508 (<0.0001)**	–0.041 (0.693)	**–0.234 (0.039)**
C4, g/L	0.053 (0.599)	0.191 (0.056)	**0.285 (0.004)**	0.062 (0.548)	**–0.282 (0.012)**
C1q, mg/L	0.129 (0.202)	0.211 (0.036)	0.004 (0.966)	0.119 (0.252)	–0.054 (0.639)
Factor B, %	0.081 (0.421)	**0.423 (<0.0001)**	**0.478 (<0.0001)**	0.061 (0.556)	–0.038 (0.740)
Factor H, mg/L	0.014 (0.889)	**0.208 (0.037)**	**0.424 (<0.0001)**	0.123 (0.231)	–0.178 (0.118)
Factor I, %	0.025 (0.802)	0.152 (0.128)	**0.391 (0.0002)**	0.016 (0.876)	–0.041 (0.720)
C3a, ng/mL	**0.313 (0.001)**	**0.623 (<0.0001)**	0.154 (0.126)	**0.387 (0.0001)**	**0.227 (0.048)**
sC5b-9, ng/mL	0.136 (0.182)	**0.409 (<0.0001)**	**0.223 (0.025)**	**0.227 (0.027)**	0.114 (0.326)

**Spearman correlation coefficients and p-values are presented.*
Bold indicates significant associations ($p < 0.05$).

Figures 3 and **4** show individual results of complement profiles in patient groups according to peak severity. The most remarkable difference in complement parameters is in the group of critical patients: declining C3, C4, Factor B levels, alternative and classical pathway activities are all characteristic for non-survivor patients, when compared to survivors. Remarkably, deceased patients had the highest level of C1q, although C1q levels were not increased in the less severe groups of patients. Among complement parameters measured, complement activation products (anaphylatoxin C3a and terminal pathway activation marker sC5b-9) showed the strongest associations with severity of COVID-19. Significantly elevated C3a and sC5b-9 levels were observed in groups requiring oxygen support, ICU-treatment, and in the group of deceased patients, when compared to controls (**Figures 3** and **4**).

In order to further analyze the role of overactivation and consumption of complement in COVID-19, the ratio of C3a/C3 was calculated. **Figure 5** shows individual C3a/C3 in various severity groups of hospitalized COVID-19 patients, and showed strong association with severity (Kruskal-Wallis ANOVA $p < 0.001$). Significant elevation of C3a/C3 ratio was characteristic for non-survivor critical patients, when compared to non-critical patients (**Figure 5**). To analyze if C3a, sC5b-9, and C3a/C3 ratio are appropriate markers to differentiate between survivor *vs.* non-survivor patients, receiver-operator characteristics analysis was done (**Figure 6**). C-statistics were significantly higher than 0.5 for C3a (0.674) and C3a/C3 (0.788) while sC5b-9 marker was not significant in this analysis. Based on the ROC analysis, cut points of 324 ng/mL (for C3a) and 200 (for C3a/C3) were selected for further multivariable survival analysis.

Prediction of In-Hospital Mortality by Complement Overactivation and Consumption

Twenty-five (24.5%) of the 102 hospitalized patients of this study died during hospital stay. First, we analyzed whether anaphylatoxin C3a, or the ratio of C3a/C3 were associated with mortality of hospitalized COVID-19 patients. The median (interquartile range) of C3a, and C3a/C3 ratio among survivors was 236 ng/mL (140–337) and 179 (123–270), *versus* 375ng/mL (195–459, $p = 0.009$) and 337 (250–644, $p < 0.0001$, Mann-Whitney test) among non-survivors, respectively. Next, the groups of patients with high *versus* low levels of C3a, and complement overactivation and consumption (C3a/C3, both as categorized variables) were compared regarding in-hospital

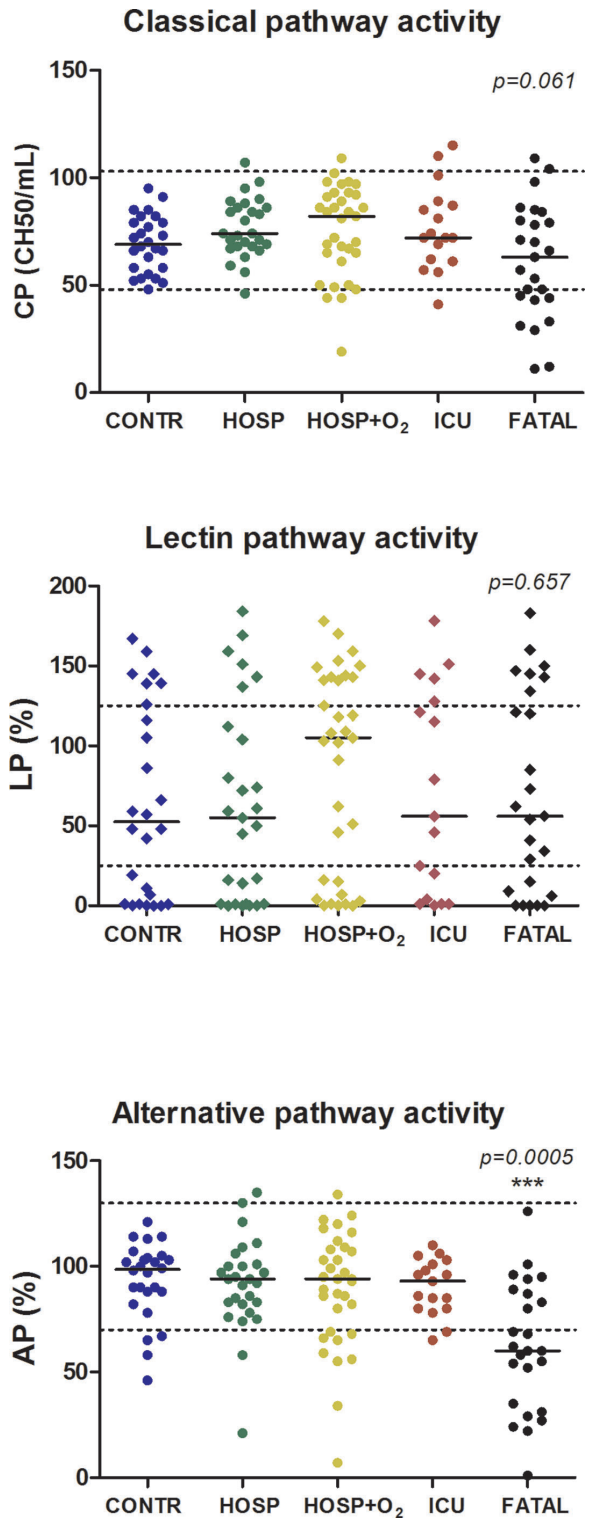


FIGURE 3 | Association between complement functional activity and peak severity of SARS-CoV-2 infection. The horizontal solid lines indicate group median, horizontal dashed lines indicate reference range limits, p-values were obtained by Kruskal-Wallis test; asterisks indicate results of Dunn's post test (***p < 0.001), when compared to control.

mortality. In the group of low (<324 ng/mL) C3a or low (<200) C3a/C3 mortality was 13.0 and 8.2%, respectively, whereas for high C3a or high overactivation and consumption groups mortality was 43.6 and 40.8%, respectively. Further, Cox proportional-hazard survival models were generated to analyze the effects of complement overactivation and consumption on in-hospital mortality. Univariate models (**Table 4**) showed significantly higher hazard ratios for those who have high levels of C3a, or high complement overactivation and consumption (C3a/C3), when compared to those with low levels. In particular, having C3a/C3 above 200 translates into 6.1 times (2.1–17.8, 95% CI) higher risk of death, when compared to those with <200 ratio. **Figures 7** and **8** show univariate Cox survival curves of COVID-19 patients with or without complement overactivation and consumption. Importantly, in separate multivariable models, after adjustment for total number of comorbidities, or total number of complications, or for CRP, the prototype inflammatory marker, high level of C3 overactivation and consumption remained significant predictors of in-hospital mortality in COVID-19 patients (**Table 4**). Due to the variance of delay time between onset of symptoms and sampling (**Figure 2**), the Cox models were adjusted for this confounder, too. The association between complement overactivation and consumption (C3a/C3) and mortality remained significant in these adjusted multivariate models (**Table 4**). Finally, as no standard observation time was used in this study, logistic regression analysis with dead or alive, as binary outcome, was also done to describe the associations between complement overactivation. Patients with C3a/C3 ratio above 200 had 12.58-times higher chance for fatal outcome (95% CI 3.391–40.47), when compared to patients with <200. If this association was adjusted for age, total number of comorbidities, or total number of complications (in separate models), the association remained significant (data not shown).

DISCUSSION

Our study provides the first observational proof that biomarkers of complement overactivation and consumption are useful tools for the prediction of in-hospital mortality of COVID-19 patients. We observed a continuous increase of complement activation markers C3a and sC5b-9 across WHO categories of COVID-19 severity (**Figure 4** and **Table 2**), with highest levels in patients presenting with critical illness. C3 concentrations were remarkably decreased in the group of non-survivors (**Figure 4**), with significantly elevated C3a/C3 ratios, reflecting overactivation and consumption (**Figure 5**). This difference translated to significant prediction of mortality in Cox proportional-hazards survival models, with hazard ratios of 6.1 (2.1–17.8, 95% CI) for those having increased ratio of C3a/C3. The model remained significant after adjustment for important covariates of critical illness, including comorbidities, in-hospital complications, or CRP.

The demonstration of complement C3 overactivation and consumption, and its strong association with COVID-19 severity

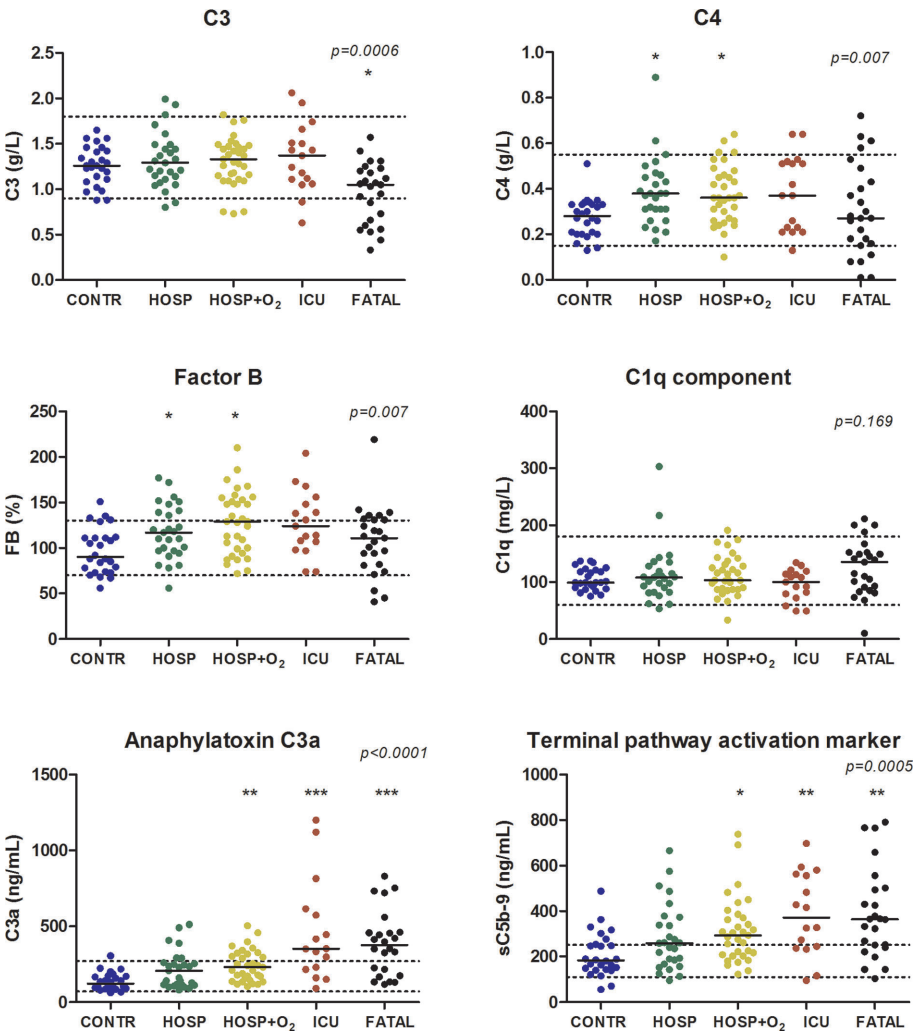


FIGURE 4 | Association between complement parameters and peak severity of SARS-CoV-2 infection. The horizontal solid lines indicate group median, horizontal dashed lines indicate reference range limits, p-values were obtained by Kruskal-Wallis test; asterisks indicate results of Dunn's post test (*p < 0.05, **p < 0.01, and ***p < 0.001), when compared to control.

and outcome, is intriguing and confirms recent observations. First, the RCI-COVID-19 group demonstrated activation of complement with elevated C3a, sC5b-9, and C3c levels in severe COVID-19 (27). High levels of complement activation markers were associated with mortality and thromboembolic complications, however, prediction of mortality was not formally evaluated in survival models. Recently, the activation through the MBL pathway and pathological thrombosis in critically ill COVID-19 patients was described (30), highlighting the critical role of complement activation in important complications of SARS-CoV2 infection. In addition, Fang et al. (19) reported low serum C3 levels in association with COVID-19 related mortality. Specifically, their study identified approximately seven times higher risk of death, per 1 g/L decrease in serum C3 level. In the same study complement activation has not been analyzed, hence whether decreased C3 was due to overactivation and consumption, or protein loss/decreased production, remained elusive. Of note, complement activation was shown to predict COVID-19 progression in chronic hemodialysis patients (36), and C5a was considered as an earlier marker, than C3a. Furthermore, we also observed decreased C4 levels in the group of non-survivors, indicating a potential involvement of the lectin or classical pathways behind complement activation and consumption. It is of note, that levels of C1q antigen [a protein produced mainly by monocytes and macrophages (37)] were the highest in deceased COVID-19 patients, suggesting the potential role macrophage overactivation in the fatal outcome of the disease (38). These observations require detailed molecular analysis in future studies.

Importantly, our results provide observational support to the milestone paper of Yu et al. published in the journal Blood. The group at Johns Hopkins University reported that SARS-CoV-2 spike proteins bind heparan sulfate on cell surface and overactivate mainly the alternative pathway by interfering with its main soluble

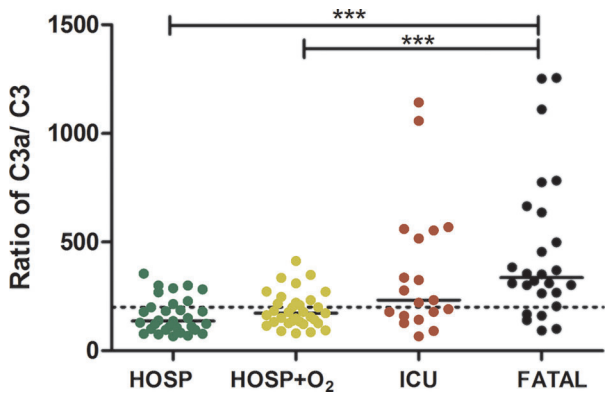


FIGURE 5 | Association of complement C3 overactivation and consumption, and peak severity of COVID-19. Ratios of complement anaphylatoxin C3a/C3 (Kruskal-Wallis test $p < 0.0001$, ***Dunn's post test $p < 0.001$) are presented in patient groups stratified according to peak severity. Cut-point (200) used in the survival analysis is indicated by horizontal, dashed line.

regulator, Factor H. Moreover, ACH145951, a small molecule Factor D inhibitor, blocked SARS-CoV-2 spike protein induced AP activation (39). Our clinical observations on the strong prediction of in-hospital mortality by the complement C3 overactivation and consumption provide independent observational support to the conclusion of the authors, who suggested that SARS-CoV-2 spike protein induced AP activation may have profound implications in the multiorgan dysfunction, coagulopathy, and endothelial injury, all characteristic of COVID-19.

Finally, in their elegant retrospective study Ramlall et al. (40) reported that history of age-related macular degeneration (AMD)—a condition known to be linked to complement dysregulation—is a risk factor for COVID-19 morbidity and mortality. In addition, with a candidate-driven genetic association study authors identified multiple putative risk loci in genes of complement regulators and components that were associated with clinical outcome of SARS-CoV-2 infection (40). Authors concluded that history of AMD predisposes patients to poor clinical outcome following SARS-CoV-2 infection, and variants in critical regulators of complement are associated with this inferior outcome. By showing the strong association between complement dysregulation (overactivation and consumption, as expressed by the C3a/C3 ratio, **Figure 5**) and severity and mortality of COVID-19, our current results corroborate the findings of Ramlall et al. Lastly, the C3 S/F polymorphism has been reported as a potential confounder of COVID-19 related mortality (41). C3 S/F polymorphism is functionally active (42), the F allele, being reported as a

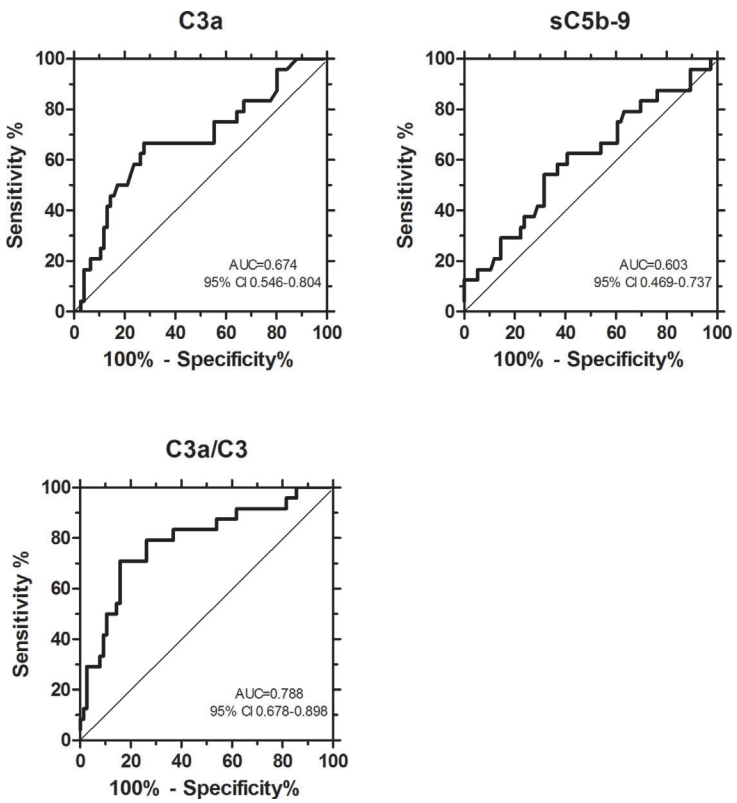


FIGURE 6 | Receiver-operator characteristics (ROC) analysis to evaluate the relationship between complement markers and mortality, and to obtain optimum cut-points differentiating survivor and non-survivor patients. ROC curves with C-statistics and 95% confidence interval are shown.

TABLE 4 | Results of univariate and multivariable Cox proportional-hazards regression models analyzing effects of complement overactivation in-hospital mortality.

Model	HR	95% CI	Chi-square	p-value
		C3a*		
Univariate	3.636	1.556–8.499	9.606	0.002
Adjusted for age	3.093	1.322–7.235	7.315	0.007
Adjusted for total number of comorbidities	2.978	1.237–7.168	6.303	0.012
Adjusted for total number of complications	2.150	0.813–5.688	2.472	0.116
Adjusted for C-reactive protein	2.346	0.898–6.127	3.155	0.076
Adjusted for delay between onset of symptoms and sampling	2.895	1.151–7.281	5.421	0.020
		C3a/C3 ratio*		
Univariate	6.1	2.08–17.87	15.01	<0.0001
Adjusted for age	3.90	1.31–11.58	7.71	0.005
Adjusted for total number of comorbidities	4.98	1.66–14.89	10.78	0.001
Adjusted for total number of complications	4.06	1.29–12.85	6.96	0.008
Adjusted for C-reactive protein	4.430	1.434–13.684	8.298	0.004
Adjusted for delay between onset of symptoms and sampling	4.909	1.638–14.709	10.325	0.001

**C3a level and C3a/C3 ratio was analyzed as categorical variable, differentiating patients with high or low level of complement activation (cut points were 324 ng/mL for C3a; and 200 for C3a/C3).*

HR, Hazard ratio; CI, confidence interval.

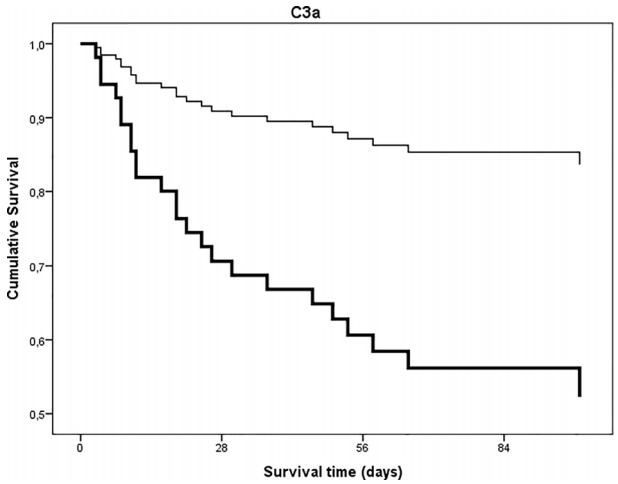


FIGURE 7 | Univariate time to in-hospital, all-cause mortality Cox proportional-hazards regression analysis in patients with COVID-19 as stratified by level of anaphylatoxin C3a. Patients were stratified according to C3a levels (cut point 324 ng/mL p = 0.002).

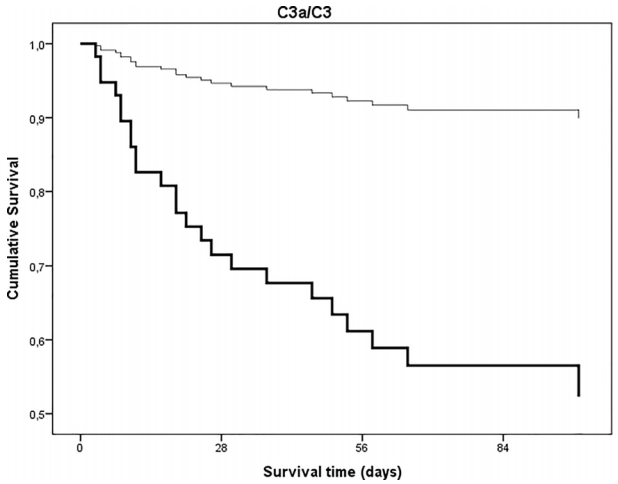


FIGURE 8 | Univariate time to in-hospital, all-cause mortality Cox proportional-hazards regression analysis in patients with COVID-19 as stratified by extent of complement C3 overactivation and consumption. Patients were stratified according to ratios indicating complement C3 overactivation and consumption (C3a/C3 ratio, cut point 200, p < 0.0001).

confounder of increased mortality, has also been associated with increased tendency to activate the alternative pathway. Therefore, inherited factors determining the activity and regulation of complement activation might indeed play important roles in the pathogenesis of COVID-19.

Inflammatory markers were closely related to COVID-19 severity (**Table 1**), which is an observation already reported at the beginning of the pandemic (43). Among the complement factors, Factor B, the enzymatic component of alternative pathway C3-convertase, was most closely related to inflammatory markers (**Table 3**) and showed the highest elevation across severity categories. Complement activation markers C3a and sC5b-9 showed significant, constant increase across severity groups of COVID-19 patients (**Table 2**, **Figure 4**). It is tempting to speculate that the particular tendency for complement overactivation and the "cytokine storm" after SARS-CoV-2 infection is based on the strong induction of hepatic acute phase reaction (including for example Factor B). Due to the high level and availability of alternative pathway components C3 and Factor B, complement activation with overproduction of inflammatory and cell damaging activation products is constant and increasing across all severity categories of COVID-19, and it is most probably the exhausting regulatory capacity that makes individuals prone to complement overactivation and consumption, and finally to death. Results of genetic association studies seem to support this assumption (40).

In COVID-19 viral pneumonia, acute respiratory distress syndrome and respiratory failure are frequent, life-threatening

complications often linked to thromboinflammation and vascular damage. Analyzing pulmonary biopsy and autopsy samples of SARS-CoV-2 infected patients with immunochemistry, Magro and coworkers were the first to describe septal capillary injury accompanied by extensive deposits of MASP-2, C4d, and terminal complement complex (44). Authors concluded that COVID-19 associated pauci-immune, complement mediated lung injury is distinct from the typical ARDS. Supporting the contribution of systemic complement activation to the development of COVID-19 associated respiratory failure are the recent observations of Holter et al. (26). Authors observed that increased admission C4d and sC5b-9 levels were associated with the development of respiratory failure and higher need for oxygen therapy, and that complement activation correlated to anti-viral antibody and ferritin levels, but not to viral load. Our observations are in line with these results, since there were significant increases in C3a and sC5b-9 levels in correlation with COVID-19 severity, with highest levels in critical patients often also having respiratory failure (**Figure 4** and **Table 2**). Furthermore, complement factor and activation product levels were associated with CRP and ferritin concentrations (**Table 3**), reflecting the relationship between the extent of inflammation and complement dysregulation.

An important novel observation of the current study is the association of mortality in COVID-19 patients with markers of complement overactivation and consumption, independent of comorbidities and in-hospital complications. A prospective cohort study from New York City identified higher age, decreased oxygen saturation at presentation, comorbidities (chronic heart and renal failure and malignant disease), and increased C-reactive protein, D-dimer, procalcitonin, and troponin levels as strong predictors of in-hospital mortality (45). Since in-hospital complications, including development of respiratory failure, sepsis, thromboembolic complications, and renal failure, are strong determinants of progression to critical illness and intensive care unit treatment, we adjusted the Cox survival models for the total number of in-hospital complications. Prediction of mortality by markers of complement overactivation and consumption remained still significant after adjustment for in-hospital complications (**Table 4**). Adjustment (in separate models) for age and C-reactive protein or delay between disease onset and sampling, did not change the results, either (**Table 4**).

Current evidences support that SARS-CoV-2 infection leads to immune dysfunction, widespread endothelial injury, complement associated coagulopathy, and systemic microangiopathy (29). Our data reinforce the importance of clinical trials with complement inhibitors to limit complement mediated tissue damage and to decrease mortality in COVID-19. Multiple available inhibitors of complement cascade, including anti-C5 monoclonals eculizumab (17, 18, 46) and ravulizumab (19, 47), narsoplimab, a monoclonal antibody against MASP-2 (48), the compstatin-based complement C3 inhibitor AMY-101 (49), C1-inhibitor (50), and the anaphylatoxin C5a blocking antibody IFX-1 (vilobelimab) (51) are currently being evaluated for COVID-19. Preliminary observations on safety and efficacy results are promising (20), but it needs more time to make firm conclusions on the utility of such drugs for COVID-19.

This study was limited by its relatively small size enrolling 102 in-hospital patients (25 non-survivors) with confirmed COVID-19 in two tertiary care centers. However, the severity groups were nearly equally sized and follow-up was complete for all of the patients, allowing for reliable statistical analysis. *Post-hoc* power analysis yield $P > 0.8$ for both C3a and sC5b-9, when comparing the 25 non-survivors and the 27 in-hospital patients without need for oxygen support (**Figure 4**). The low number of non-survivors in the study allowed adjustment for only one confounder, but since our study collected all of the relevant clinical data about comorbidities and in-hospital complications, we were able to adjust for the most important confounders (**Table 4**). One of the strengths of our study was the detailed characterization of large panel complement profile by methods regularly evaluated in external proficiency testing (25). In addition, our strategy to measure complement activity and factor levels together with activation markers allowed us to identify the clinical relevance of complement overactivation together with signs of consumption, a phenomenon indicating the presence of complement dysregulation. Results on the relationship between activity of lectin pathway and disease severity are heavily influenced by genetic factors strongly regulating mannose-binding lectin levels, but due to space limitations, the results of detailed analysis will be presented separately (manuscript in preparation).

Conclusion

Patients with SARS-CoV-2 infection are more likely to die, when the diseases is accompanied by overactivation and consumption of C3. These results may provide observational evidence and further support to studies on complement inhibitory drugs for the treatment of COVID-19.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hungarian Ethical Review Agency (ETT-TUKEB). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

BM, ZF, DC, LH, EK, LC, and PK designed and performed laboratory determinations, interpreted data, and drafted the manuscript. GS, ZZP, and ZP conceptualized research,

collected and analyzed clinical information and laboratory data, conducted statistical analysis, interpreted data, and wrote the manuscript. MR, VM, ZI, JG, LG, PR, BS, BL, JS, IB, TM, and IV-N conceptualization, collected and analyzed clinical information, interpreted and supervised data, and drafted the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The research was financed by the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the molecular biology thematic program of the Semmelweis University, by the National Office for Innovation and Research [KH130355, and "Befektetés a jövőbe" (2020-1-1-6-JÖVŐ-2021-00013) to ZP]. The study was performed in frame of the Premium Postdoctoral Fellowship Program of the Hungarian Academy of Sciences (PPD2018-016/2018 to DC). ZP and LH are supported by funds of the EU MSCA project CORVOS 860044.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Márta Kókai, Éva Zsuzsanna Szendrei, Lászlóné Kertész, Edina Szabó, and Beáta Takács, and the critical reading of the manuscript by János Sinkó (Central Hospital of Southern Pest National Institute of Hematology and Infectious Diseases), by Brandon M. Henry and Stefanie Benoit (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA) with many thanks.

REFERENCES

1. Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, et al. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* (2003) 300(5624):1394–9. doi: 10.1126/science.1085952
2. Peiris JS, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, et al. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* (2003) 361 (9366):1319–25. doi: 10.1016/S0140-6736(03)13077-2
3. Lee N, Hui D, Wu A, Chan P, Cameron P, Joynt GM, et al. A major outbreak of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* (2003) 348 (20):1986–94. doi: 10.1056/NEJMoa030685
4. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* (2012) 367(19):1814–20. doi: 10.1056/NEJMoa1211721
5. https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19—11-march-2020.
6. https://www.who.int/publications/m/item/weekly-epidemiological-update—12-january-2021.
7. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA* (2020) 323(11):1061–9. doi: 10.1001/jama.2020.1585
8. Lee SH, Jung JU, Means RE. 'Complementing' viral infection: mechanisms for evading innate immunity. *Trends Microbiol* (2003) 11(10):449–52. doi: 10.1016/j.tim.2003.08.004
9. Beck Z, Prohaszka Z, Fust G. Traitors of the immune system-enhancing antibodies in HIV infection: their possible implication in HIV vaccine development. *Vaccine* (2008) 26(24):3078–85. doi: 10.1016/j.vaccine.2007.12.028
10. Banhegyi D, Bacsi A, Toth FD, Prohaszka Z, Horvath A, Beck Z, et al. Significant decrease of the enhancement/neutralization index in HIV patients during highly active antiretroviral therapy (HAART). *Immunol Lett* (2003) 89 (1):25–30. doi: 10.1016/s0165-2478(03)00106-8
11. Prohaszka Z, Nemes J, Hidvegi T, Toth FD, Kerekes K, Erdei A, et al. Two parallel routes of the complement-mediated antibody-dependent enhancement of HIV-1 infection. *AIDS* (1997) 11(8):949–58. doi: 10.1097/00002030-199708000-00002
12. Bjornson AB, Mellencamp MA, Schiff GM. Complement is activated in the upper respiratory tract during influenza virus infection. *Am Rev Respir Dis* (1991) 143(5 Pt 1):1062–6. doi: 10.1164/ajrccm/143.5_Pt_1.1062
13. Gralinski LE, Sheahan TP, Morrison TE, Menachery VD, Jensen K, Leist SR, et al. Complement Activation Contributes to Severe Acute Respiratory Syndrome Coronavirus Pathogenesis. *mBio* (2018) 9(5):1–15. doi: 10.1128/mBio.01753-18
14. Risitano AM, Mastellos DC, Huber-Lang M, Yancopoulou D, Garlanda C, Ciceri F, et al. Complement as a target in COVID-19? *Nat Rev Immunol* (2020) 20(6):343–4. doi: 10.1038/s41577-020-0320-7
15. Campbell CM, Kahwash R. Will Complement Inhibition Be the New Target in Treating COVID-19-Related Systemic Thrombosis? *Circulation* (2020) 141 (22):1739–41. doi: 10.1161/CIRCULATIONAHA.120.047419
16. Mahajan R, Lipton M, Broglie L, Jain NG, Uy NS. Eculizumab treatment for renal failure in a pediatric patient with COVID-19. *J Nephrol* (2020) 33 (6):1373–6. doi: 10.1007/s40620-020-00858-2
17. Laurence J, Mulvey JJ, Seshadri M, Racanelli A, Harp J, Schenck EJ, et al. Anti-complement C5 therapy with eculizumab in three cases of critical COVID-19. *Clin Immunol* (2020) 219:108555. doi: 10.1016/j.clim.2020.108555
18. Diurno F, Numis FG, Porta G, Cirillo F, Maddaluno S, Ragozzino A, et al. Eculizumab treatment in patients with COVID-19: preliminary results from real life ASL Napoli 2 Nord experience. *Eur Rev Med Pharmacol Sci* (2020) 24 (7):4040–7. doi: 10.26355/eurrev_202004_20875
19. Fang S, Wang H, Lu L, Jia Y, Xia Z. Decreased complement C3 levels are associated with poor prognosis in patients with COVID-19: A retrospective cohort study. *Int Immunopharmacol* (2020) 89(Pt A):107070. doi: 10.1016/j.intimp.2020.107070
20. Annane D, Heming N, Grimaldi-Bensouda L, Fremeaux-Bacchi V, Vigan M, Roux AL, et al. Eculizumab as an emergency treatment for adult patients with severe COVID-19 in the intensive care unit: A proof-of-concept study. *EClinicalMedicine* (2020) 28:100590. doi: 10.1016/j.eclinm.2020.100590
21. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol* (2015) 6:262. doi: 10.3389/fimmu.2015.00262
22. Walport MJ. Complement. First of two parts. *N Engl J Med* (2001) 344 (14):1058–66. doi: 10.1056/NEJM200104053441406
23. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement System Part II: Role in Immunity. *Front Immunol* (2015) 6:257. doi: 10.3389/fimmu.2015.00257
24. Prohaszka Z, Kirschfink M, Frazer-Abel A. Complement analysis in the era of targeted therapeutics. *Mol Immunol* (2018) 102:84–8. doi: 10.1016/j.molimm.2018.06.001
25. Prohaszka Z, Nilsson B, Frazer-Abel A, Kirschfink M. Complement analysis 2016: Clinical indications, laboratory diagnostics and quality control. *Immunobiology* (2016) 221(11):1247–58. doi: 10.1016/j.imbio.2016.06.008
26. Holter JC, Pischke SE, de Boer E, Lind A, Jenum S, Holten AR, et al. Systemic complement activation is associated with respiratory failure in COVID-19 hospitalized patients. *Proc Natl Acad Sci U S A* (2020) 117(40):25018–25. doi: 10.1073/pnas.2010540117
27. de Nooijer AH, Grondman I, Janssen NAF, Netea MG, Willems L, van de Veerdonk FL, et al. Complement activation in the disease course of COVID-19 and its effects on clinical outcomes. *J Infect Dis* (2021) 223(2):214–24. doi: 10.1093/infdis/jiaa646
28. Overmyer KA, Shishkova E, Miller IJ, Balnis J, Bernstein MN, Peters-Clarke TM, et al. Large-Scale Multi-omic Analysis of COVID-19 Severity. *Cell Syst* (2021). 12(1):23–40.e7. doi: 10.1016/j.cels.2020.10.003

29. Perico L, Benigni A, Casiraghi F, Ng LFP, Renia L, Remuzzi G. Immunity, endothelial injury and complement-induced coagulopathy in COVID-19. *Nat Rev Nephrol* (2020) 17(1):46–64. doi: 10.1038/s41581-020-00357-4
30. Eriksson O, Hultstrom M, Persson B, Lipcsey M, Ekdahl KN, Nilsson B, et al. Mannose-Binding Lectin is Associated with Thrombosis and Coagulopathy in Critically Ill COVID-19 Patients. *Thromb Haemostasis* (2020) 120(12):1720–4. doi: 10.1055/s-0040-1715835
31. https://www.who.int/blueprint/priority-diseases/key-action/COVID-19_Treatment_Trial_Design_Master_Protocol_synopsis_Final_18022020.pdf.
32. Bobek I, Gopcsa L, Reti M, Beko G, Hancz L, Lakatos B, et al. Successful administration of convalescent plasma in critically ill COVID-19 patients in Hungary: the first two cases. *Orvosi Hetilap* (2020) 161(27):1111–21. doi: 10.1556/650.2020.31901
33. Fetterhoff TJ, McCarthy RC. A micromodification of the CH50 test for the classical pathway of complement. *J Clin Lab Immunol* (1984) 14(4):205–8.
34. Reti M, Farkas P, Csuka D, Razso K, Schlammadinger A, Udvardy ML, et al. Complement activation in thrombotic thrombocytopenic purpura. *J Thromb Haemostasis JTH* (2012) 10(5):791–8. doi: 10.1111/j.1538-7836.2012.04674.x
35. Delamarche C, Berger F, Pouplard A, Emile J. An ELISA technique for the measurement of C1q in cerebrospinal fluid. *J Immunol Methods* (1988) 114(1-2):101–6. doi: 10.1016/0022-1759(88)90160-3
36. Prendecki M, Clarke C, Medjeral-Thomas N, McAdoo SP, Sandhu E, Peters JE, et al. Temporal changes in complement activation in haemodialysis patients with COVID-19 as a predictor of disease progression. *Clin Kidney J* (2020) 13(5):889–96. doi: 10.1093/ckj/sfaa192
37. Gulati P, Lemercier C, Guc D, Lappin D, Whaley K. Regulation of the synthesis of C1 subcomponents and C1-inhibitor. *Behring Inst Mitt* (1993)(93):196–203.
38. McGonagle D, Sharif K, O'Regan A, Bridgewood C. The Role of Cytokines including Interleukin-6 in COVID-19 induced Pneumonia and Macrophage Activation Syndrome-Like Disease. *Autoimmun Rev* (2020) 19(6):102537. doi: 10.1016/j.autrev.2020.102537
39. Yu J, Yuan X, Chen H, Chaturvedi S, Braunstein EM, Brodsky RA. Direct activation of the alternative complement pathway by SARS-CoV-2 spike proteins is blocked by factor D inhibition. *Blood* (2020) 136(18):2080–9. doi: 10.1182/blood.2020008248
40. Ramlall V, Thangaraj PM, Meydan C, Foox J, Butler D, Kim J, et al. Immune complement and coagulation dysfunction in adverse outcomes of SARS-CoV-2 infection. *Nat Med* (2020) 26(10):1609–15. doi: 10.1038/s41591-020-1021-2
41. Delanghe JR, De Buyzere ML, Speeckaert MM. C3 and ACE1 polymorphisms are more important confounders in the spread and outcome of COVID-19 in comparison with ABO polymorphism. *Eur J Prev Cardiol* (2020) 27(12):1331–2. doi: 10.1177/2047487320931305
42. Heurich M, Martinez-Barricarte R, Francis NJ, Roberts DL, Rodriguez de Cordoba S, Morgan BP, et al. Common polymorphisms in C3, factor B, and factor H collaborate to determine systemic complement activity and disease risk. *Proc Natl Acad Sci U S A* (2011) 108(21):8761–6. doi: 10.1073/pnas.1019338108
43. Fu Y, Cheng Y, Wu Y. Understanding SARS-CoV-2-Mediated Inflammatory Responses: From Mechanisms to Potential Therapeutic Tools. *Virol Sin* (2020) 35(3):266–71. doi: 10.1007/s12250-020-00207-4
44. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement associated microvascular injury and thrombosis in the pathogenesis of severe COVID-19 infection: A report of five cases. *Trans Res J Lab Clin Med* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
45. Petrilli CM, Jones SA, Yang J, Rajagopalan H, O'Donnell L, Chernyak Y, et al. Factors associated with hospital admission and critical illness among 5279 people with coronavirus disease 2019 in New York City: prospective cohort study. *BMJ* (2020) 369:m1966. doi: 10.1136/bmj.m1966
46. Giudice V, Pagliano P, Vatrella A, Masullo A, Poto S, Polverino BM, et al. Combination of Ruxolitinib and Eculizumab for Treatment of Severe SARS-CoV-2-Related Acute Respiratory Distress Syndrome: A Controlled Study. *Front Pharmacol* (2020) 11:857. doi: 10.3389/fphar.2020.00857
47. Smith K, Pace A, Ortiz S, Kazani S, Rottinghaus S. A Phase 3 Open-label, Randomized, Controlled Study to Evaluate the Efficacy and Safety of Intravenously Administered Ravulizumab Compared with Best Supportive Care in Patients with COVID-19 Severe Pneumonia, Acute Lung Injury, or Acute Respiratory Distress Syndrome: A structured summary of a study protocol for a randomised controlled trial. *Trials* (2020) 21(1):639. doi: 10.1186/s13063-020-04548-z
48. Rambaldi A, Gritti G, Mico MC, Frigeni M, Borleri G, Salvi A, et al. Endothelial injury and thrombotic microangiopathy in COVID-19: Treatment with the lectin-pathway inhibitor narsoplimab. *Immunobiology* (2020) 225(6)152001. doi: 10.1016/j.imbio.2020.152001
49. Mastaglio S, Ruggeri A, Risitano AM, Angelillo P, Yancopoulou D, Mastellos DC, et al. The first case of COVID-19 treated with the complement C3 inhibitor AMY-101. *Clin Immunol* (2020) 215:108450. doi: 10.1016/j.clim.2020.108450
50. Urwyler P, Moser S, Charitos P, Heijnen I, Rudin M, Sommer G, et al. Treatment of COVID-19 With Conestat Alfa, a Regulator of the Complement, Contact Activation and Kallikrein-Kinin System. *Front Immunol* (2020) 11:2072. doi: 10.3389/fimmu.2020.02072
51. Vlaar APJ, de Bruin S, Busch M, Timmermans S, van Zeggeren IE, Koning R, et al. Anti-C5a antibody IFX-1 (vilobelimab) treatment versus best supportive care for patients with severe COVID-19 (PANAMO): an exploratory, open-label, phase 2 randomised controlled trial. *Lancet Rheumatol* (2020) 2(12):e764–73. doi: 10.1016/S2665-9913(20)30341-6

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.

frontiers in Immunology

SYSTEMATIC REVIEW
published: 07 June 2021
doi: 10.3389/fimmu.2021.696085

Serum Complement C3 and C4 and COVID-19 Severity and Mortality: A Systematic Review and Meta-Analysis With Meta-Regression

Angelo Zinellu[1] *and Arduino A. Mangoni*[2,3]*

[1] Department of Biomedical Sciences, University of Sassari, Sassari, Italy, [2] Discipline of Clinical Pharmacology, College of Medicine and Public Health, Flinders University, Adelaide, SA, Australia, [3] Department of Clinical Pharmacology, Flinders Medical Centre, Southern Adelaide Local Health Network, Adelaide, SA, Australia

Activation of the complement system has been observed in coronavirus disease 19 (COVID-19). We conducted a systematic review and meta-analysis with meta-regression to investigate possible differences in the serum concentrations of two routinely measured complement components, C3 and C4, in COVID-19 patients with different severity and survival status. We searched PubMed, Web of Science and Scopus, between January 2020 and February 2021, for studies reporting serum complement C3 and C4, measures of COVID-19 severity, and survival. Eligibility criteria were a) reporting continuous data on serum C3 and C4 concentrations in COVID-19 patients, -b) investigating COVID-19 patients with different disease severity and/or survival status, c) adult patients, d) English language, e) ≥10 patients, and f) full-text available. Using a random-effects model, standardized mean differences (SMD) with 95% confidence intervals (CIs) were calculated to evaluate differences in serum C3 and C4 concentrations between COVID-19 patients with low vs. high severity or survivor vs. non-survivor status. Risk of bias was assessed using the Newcastle-Ottawa scale whereas publication bias was assessed with the Begg's and Egger's tests. Certainty of evidence was assessed using GRADE. Nineteen studies in 3,764 COVID-19 patients were included in the meta-analysis. Both C3 and C4 concentrations were significantly lower in patients with high disease severity or non-survivor status than patients with low severity or survivor status (C3 SMD=-0.40, 95% CI -0.60 to -0.21, p<0.001; C4 SMD=-0.29, 95% CI -0.49 to -0.09, p=0.005; moderate certainty of evidence). Extreme between-study heterogeneity was observed (C3, I^2 = 82.1%; C4, I^2 = 84.4%). Sensitivity analysis, performed by sequentially removing each study and re-assessing the pooled estimates, showed that the magnitude and direction of the effect size was not modified. There was no publication bias. In meta-regression, the SMD of C3 was significantly associated with white blood cell count, C-reactive protein (CRP), and pro-thrombin time, whereas the SMD of C4 was significantly associated with CRP, pro-thrombin time, D-dimer, and albumin. In conclusion, lower concentrations of C3 and C4, indicating complement activation, were significantly associated with higher COVID-19 severity

OPEN ACCESS

Edited by:
Zoltan Prohaszka, Semmelweis University, Hungary

Reviewed by:
Morteza Arab-Zozani, Birjand University of Medical Sciences, Iran
Giuseppe Ricci, University of Trieste, Italy

***Correspondence:**
Arduino A. Mangoni
arduino.mangoni@flinders.edu.au

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *16 April 2021*
Accepted: *21 May 2021*
Published: *07 June 2021*

Citation:
Zinellu A and Mangoni AA (2021) Serum Complement C3 and C4 and COVID-19 Severity and Mortality: A Systematic Review and Meta-Analysis With Meta-Regression. Front. Immunol. 12:696085. doi: 10.3389/fimmu.2021.696085

andmortality. C3 and C4 might be useful to predict adverse clinical consequences in these patients.

Systematic Review Registration: PROSPERO, Registration number: CRD42021239634.

Keywords: complement system, C3, C4, COVID-19 severity, mortality

INTRODUCTION

The complement system exerts several protective effects against infectious agents following activation during innate, through the alternative and lectin pathways, and acquired, through the classical pathway, immunity (1). The activation of the classical, lectin, and alternative pathways ultimately leads to the cleavage of the central component 3, C3, by convertases. This, in turn, initiates a sequence of events that include phagocytosis, leucocyte attraction and activation, mast cell and basophil degranulation with the release of several mediators of inflammation, activation of the inflammasome complex and specific cytokines, and B lymphocyte activation with the consequent secretion of specific antibodies (2). In the setting of viral infections, additional effects mediated by the activation of the complement system include virus aggregation-mediated neutralization, phagocytosis, and lysis of viruses and virus-infected cells (3). While these processes suggest an overall beneficial effect against viruses, complement activation might also increase the risk of adverse clinical outcomes, in virtue of the sustained release of pro-inflammatory mediators with additional toxic effects at the cellular and tissue level (4).

The potential opposite nature of the effects mediated by the complement system has been investigated during the last three pandemics, caused by the viral agents, severe acute respiratory syndrome coronavirus, Middle East respiratory syndrome coronavirus, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), respectively. In particular, the clinical presentation and progress of coronavirus disease 19 (COVID-19), caused by SARS-CoV-2 and responsible for the current global pandemic, might significantly depend on the fine balance between different degrees of complement activation. For example, an excessive and unrestrained complement activation might favour the development of a systemic pro-inflammatory, pro-oxidant, and pro-coagulant state with multi-organ dysfunction and increased risk of adverse clinical outcomes (4–6). The measurement of specific components, e.g., the complement proteins C3 and C4, using immunoassays is routinely used in clinical practice to determine and monitor complement activation. The latter is reflected by a reduction in serum concentrations of C3 and/or C4 due to increased product consumption (2, 3). The assessment of C3 and C4 in COVID-19 patients might provide useful information regarding the balance between 'physiological' *vs.* 'abnormal' complement activation and overall clinical risk.

We sought to investigate the clinical role of complement activation in COVID-19 by conducting a systematic review and meta-analysis of studies reporting serum complement C3 and C4 concentrations in patients with different disease severity and survival status during follow-up. We speculated that patients with severe disease and/or reduced survival had lower complement C3 and C4 when compared to those with milder disease and favourable outcomes, reflecting a state of unrestrained complement activation in the former. Furthermore, a meta-regression analysis was performed to investigate possible associations between the effect size of the between-group differences in C3 and C4 concentrations and several clinical and demographic factors and markers of organ damage, inflammation, and coagulation.

MATERIALS & METHODS

Search Strategy, Eligibility Criteria & Study Selection

We conducted a systematic literature search, using the terms "complement C3" or "complement component 3" or "complement C4" or "complement component 4" and "coronavirus disease 19" or "COVID-19", in the electronic databases PubMed, Web of Science and Scopus, from January 2020 to February 2021, to identify peer-reviewed studies reporting serum complement C3 and C4 concentrations in COVID-19 patients according to disease severity and/or survival status (PROSPERO registration number: CRD42021239634). The references of the retrieved articles were also reviewed to identify additional studies. Eligibility criteria for inclusion were a) reporting continuous data on serum C3 and C4 concentrations in COVID-19 patients, b) investigating COVID-19 patients with different degree of disease severity and/or survival status, c) adult patients, d) English language, e) ≥10 patients, and f) full-text available (7). Two investigators independently screened the abstracts. If relevant, the full-text articles were independently reviewed. A third investigator was involved in case of disagreement. Data extracted from each article included country where the study was conducted, endpoint, study design, number of participants, age, sex, serum C3 and C4 concentrations, and parameters used in meta-regression analysis (see specific details under Statistical analysis). The Newcastle-Ottawa scale was used to assess the risk of bias of each study, with a score ≥6 indicating low risk, 4-5 moderate risk, and <4 high risk (7–9). Certainty of evidence was assessed using the Grades of Recommendation, Assessment, Development and Evaluation (GRADE) Working Group system, which considers the following criteria: study design (randomized vs. observational), risk of bias (Newcastle-Ottawa scale), unexplained heterogeneity, indirectness of evidence, imprecision of results (sample size, 95% confidence interval width and threshold crossing), effect size (small, SMD <0.5, medium, SMD 0.5-0.8, and large, SMD >0.8) (10), and high probability of publication bias (11–13).

Statistical Analysis

Standardized mean differences (SMD) with 95% confidence intervals (CIs) were calculated to build forest plots of continuous data and evaluate differences in serum C3 and C4

concentrations between COVID-19 patients with low *vs.* high severity or survivor *vs.* non-survivor status, with a p-level of significance set at < 0.05. Means and standard deviations were extrapolated from medians and interquartile ranges (14), or from graph data using the Graph Data Extractor software (7). The Q-statistic was used to test the heterogeneity of SMD across studies (p<0.10). Inconsistency across studies was evaluated through the I^2 statistic, with I^2<25% indicating no heterogeneity, I^2 25-50% moderate heterogeneity, I^2 50-75% large heterogeneity, and I^2>75% extreme heterogeneity (7, 15, 16). A random-effects model was used to calculate the pooled SMD and 95% CIs in the presence of significant heterogeneity. Sensitivity analyses were performed to evaluate the influence of individual studies on the overall effect size using the leave-one-out method (7, 17). The presence of publication bias was assessed with the Begg's adjusted rank correlation test and the Egger's regression asymmetry test (p<0.05 for both) (18, 19). The Duval and Tweedie "trim and fill" procedure, a funnel-plot-based method of testing and adjusting for publication bias, was also used (7, 20). This is a nonparametric (rank-based) data augmentation technique that increases the observed data, so that the funnel plot is more symmetric, and recalculates the pooled SMD based on the complete data. Univariate meta-regression analysis was used to identify possible contributors to between-study variance. In particular, we investigated associations between the SMD and biologically and/or clinically plausible factors, including age, gender, clinical endpoint, diabetes, hypertension and cardiovascular disease, biomarkers of inflammation (C-reactive protein, CRP, white blood cell count, WBC, neutrophils, lymphocytes), liver damage (aspartate aminotransferase, AST, alanine aminotransferase, ALT, albumin), renal damage (serum creatinine), tissue damage (lactate dehydrogenase, LDH), and pro-thrombotic tendency (D-dimer, pro-thrombin time). Statistical analyses were performed using Stata 14 (STATA Corp., College Station, TX, USA). The study was compliant with the PRISMA 2020 statement regarding the reporting of systematic reviews and meta-analyses (21).

RESULTS

Study Selection

We initially identified 585 studies. A total of 564 studies were excluded because they were either duplicates or irrelevant. After a full-text review of the remaining 21 articles, two were excluded because they did not meet the inclusion criteria. Thus, 19 studies were included in the meta-analysis (**Figure 1**) (22–40). These studies enrolled 3,764 COVID-19 patients, 2,643 (48% males, mean age 54 years) with low disease severity or survivor status and 1,121 (58% males, mean age 65 years) with high severity or non-survivor status during follow up.

Study Characteristics

One study was conducted in Turkey (23), one in Spain (33), and the remaining 17 in China (22, 24–32, 34–40). Of the 17 studies conducted in China, 10 were from the Renmin Hospital, Wuhan (24–29, 35, 38–40) (**Supplementary Table**). One study was prospective (29), 16 retrospective (22–26, 28, 30–35, 37–40), whereas the remaining two did not provide information regarding the study design (27, 36). Eleven studies assessed disease severity based on current clinical guidelines (26–29, 31–34, 37, 38, 40), one on clinical progress (36), one on ICU transfer (23), one on hospital length of stay (30), whereas the remaining six assessed survival status (**Table 1**) (22, 24, 25, 35, 39, 40). Seventeen studies reported C3 and C4 concentrations measured within the first 24-48 h from admission (22–27, 30–40), whilst the remaining two did not specify the collection time (28, 29).

Risk of Bias

The risk of bias was considered low in nine studies (22, 24–26, 30, 35, 36, 39, 40), moderate in nine (27–29, 31–34, 37, 38) and high in the remaining one (23).

Results of Individual Studies and Syntheses

Complement C3

The overall SMD in complement C3 concentrations between COVID-19 patients with low *vs.* high severity or survivor vs. non-survivor status in the 19 studies is shown in **Figure 2**. In 15 studies, patients with high severity or non-survivor status had lower C3 concentrations when compared to those with low severity or survivor status (mean difference range, -0.37 to -0.15) (22–25, 27, 29, 32, 33, 35–40), with a significant difference in 11 (22, 23, 27, 29, 32, 33, 35–40). No between-group difference was reported in one study (mean difference 0.00) (31). By contrast, in the remaining four studies, the C3 concentration was lower in patients with low severity or survivor status (mean difference range, 0.06 to 0.54) (26, 28, 30, 33), although only one study reported a significant difference (28). The pooled results confirmed that C3 concentrations were significantly lower in patients with high disease severity or non-survivor status during follow up (SMD -0.40, 95% CI -0.60 to -0.21, p<0.001) (**Figure 2**). Extreme heterogeneity between studies was observed (I^2 = 82.1%, p<0.001).

Sensitivity analysis, performed by sequentially removing each study and re-assessing the pooled estimates, showed that the magnitude and direction of the effect size were not substantially modified (effect size range, between -0.45 and -0.37) (**Figure 3A**) (7). Furthermore, after removing the studies conducted at the Renmin Hospital, Wuhan, barring the largest ones for disease severity (28) and survival status (39), the SMD remained significant (SMD -0.32, 95% CI -0.59 to -0.05, p=0.02; I^2 = 87.3%, p<0.001) (**Figure 4A**).

Complement C3 concentrations remained significantly lower (SMD -0.42, 95% CI -0.65 to -0.20, p<0.001; I^2 = 80.9%, p<0.001) in patients with high severity or non-survivor status after removing three relatively large studies that accounted for nearly 36% of the total sample size (34, 37, 39).

In univariate meta-regression, the SMD was significantly associated with WBC (t=-2.39, p=0.03), CRP (t=3.08, p=0.008), and pro-thrombin time (t=3.95, p=0.004), with a further trend observed with neutrophils (t=-2.13, p=0.052). By contrast, no significant correlations were observed with age (t=0.78, p= 0.45), gender, (t=1.75, p=0.10), lymphocytes (t=0.60, p=0.56), AST (t=0.04, p=0.97), ALT (t=1.19, p=0.26), LDH (t=-0.14, p=0.89),

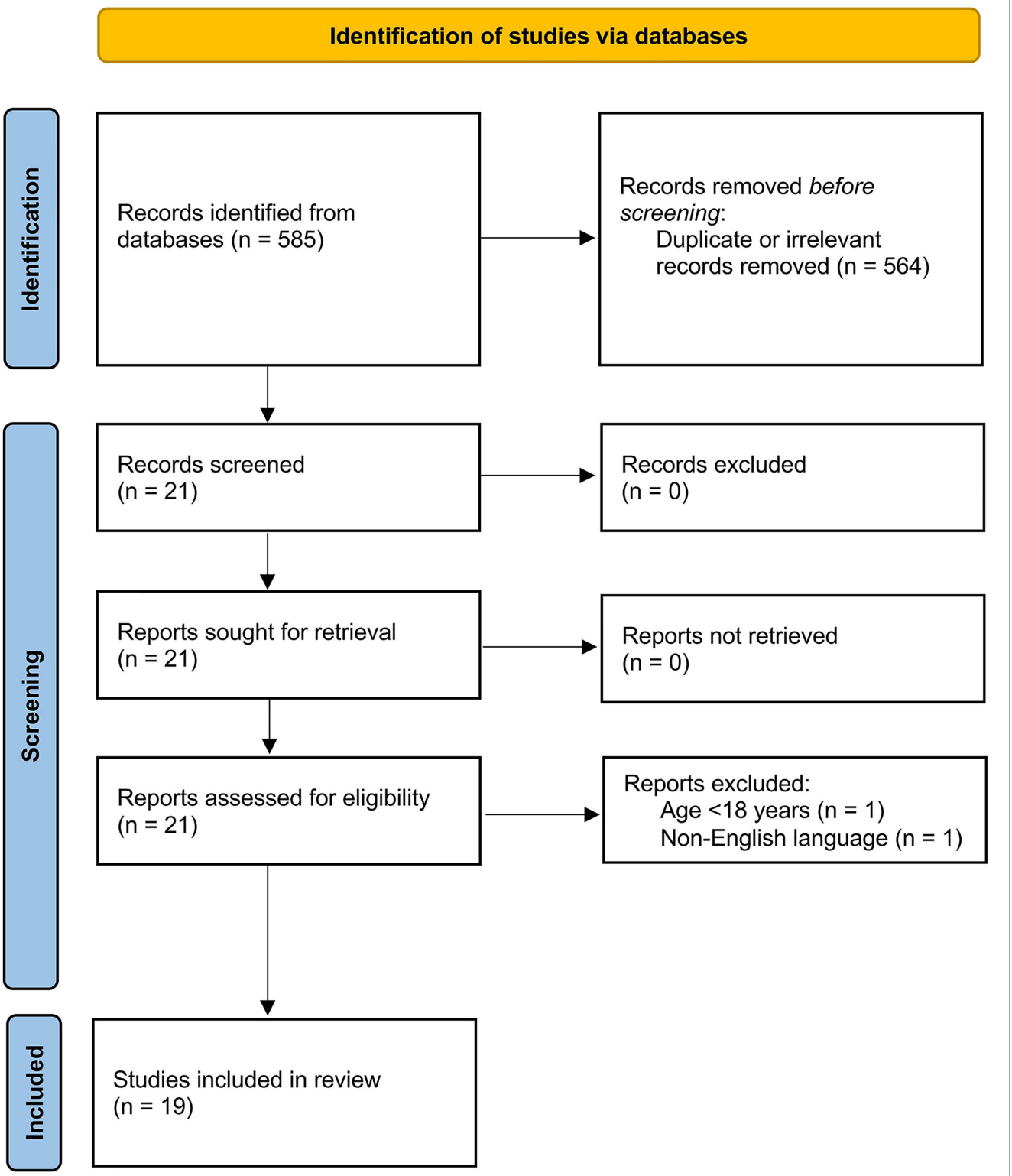


FIGURE 1 | PRISMA 2020 flow diagram.

TABLE 1 | Characteristics of the studies included in the meta-analysis.

First Author, Country (ref)	Endpoint	Design	NOS (stars)	Low severity or survivor					High severity or non-survivor				
				n	Age (Years)	Gender (M/F)	C3 g/L (Mean ± SD)	C4 g/L (Mean ± SD)	n	Age (Years)	Gender (M/F)	C3 g/L (Mean ± SD)	C4 g/L (Mean ± SD)
Chen T et al, China (22)	Survival	R	6	161	51	88/73	0.90 ± 0.15	0.27 ± 0.07	113	68	83/30	0.77 ± 0.22	0.23 ± 0.07
Dheir H et al, Turkey (23)	ICU transfer	R	3	28	NR	NR	1.48 ± 0.36	0.31 ± 0.12	29	NR	NR	1.15 ± 0.36	0.23 ± 0.16
Fang S et al, China (24)	Survival	R	7	169	51	71/98	1.02 ± 0.29	0.26 ± 0.15	67	72	42/25	0.94 ± 0.37	0.24 ± 0.19
Fu YQ et al, China (25)	Survival	R	7	71	62	38/33	1.03 ± 0.18	0.24 ± 0.09	14	67	11/3	0.95 ± 0.17	0.25 ± 0.14
Han Y et al, China (26)	Disease severity	R	7	59	61	29/30	1.03 ± 0.23	0.26 ± 0.15	48	67	31/17	1.07 ± 0.28	0.28 ± 0.08
He B et al, China (27)	Disease severity	NR	5	32	42	15/17	1.07 ± 0.22	0.27 ± 0.07	21	57	13/8	0.87 ± 0.22	0.17 ± 0.07
He R et al, China (28)	Disease severity	R	5	135	43	42/93	0.84 ± 0.17	0.23 ± 0.10	69	61	37/32	0.90 ± 0.16	0.26 ± 0.09
Li L et al, China (29)	Disease severity	P	5	60	45	32/28	0.83 ± 0.25	0.26 ± 0.14	12	52	7/5	0.51 ± 0.12	0.10 ± 0.03
Lin P et al, China (30)	Length of stay*	R	7	20	35	8/12	0.78 ± 0.14	0.18 ± 0.05	27	41	16/11	0.89 ± 0.24	0.26 ± 0.13
Liu J et al, China (31)	Disease severity	R	5	27	43	8/19	0.80 ± 0.20	0.30 ± 0.10	23	60	7/6	0.80 ± 0.10	0.30 ± 0.10
Liu SL et al, China (32)	Disease severity	R	5	194	43	91/103	1.17 ± 0.28	0.24 ± 0.04	31	64	17/14	1.13 ± 0.24	018 ± 0.06
Marcos-Jiménezet al, Spain (33)	Disease severity	R	5	235	62	132/103	1.22 ± 0.28	0.28 ± 0.10	41	68	31/10	0.96 ± 0.32	0.19 ± 0.12
Qin C et al, China (34)	Disease severity	R	5	166	53	80/86	0.88 ± 0.17	0.26 ± 0.08	286	61	155/131	0.89 ± 0.17	0.26 ± 0.08
Qin W et al, China (35)	Survival	R	7	239	63	113/126	1.01 ± 0.21	0.25 ± 0.08	23	69	10/13	0.96 ± 0.16	0.27 ± 0.1
Xie J et al, China (36)	Disease progression	NR	6	75	51	45/30	1.33 ± 0.24	0.37 ± 0.11	29	66	18/11	1.19 ± 0.18	0.35 ± 0.08
Xie L et al, China (37)	Disease severity	R	5	322	NR	168/154	1.20 ± 0.22	0.34 ± 0.33	51	NR	29/22	1.07 ± 0.24	0.28 ± 0.09
Yuan X et al, China (38)	Disease severity	R	5	60	66	30/30	0.92 ± 0.25	0.23 ± 0.08	56	68	26/30	0.84 ± 0.12	0.21 ± 0.11
Zhao Y et al, China (39)	Survival	R	7	414	54	184/230	0.99 ± 0.21	0.25 ± 0.10	125	70	71/54	0.89 ± 0.22	0.23 ± 0.10
Zou L et al. (a), China (40)	Disease severity	R	6	69	60	34/35	1.06 ± 0.18	0.27 ± 0.10	52	70	32/20	0.94 ± 0.16	0.26 ± 0.10
Zou L et al. (b), China (40)	Survival	R	6	107	64	57/50	1.03 ± 0.18	0.27 ± 0.10	14	68	9/5	0.88 ± 0.13	0.21 ± 0.08

*ICU, intensive care unit; NOS, Newcastle-Ottawa quality assessment scale for case-control studies; R, retrospective; P, prospective; NR, not reported; *, <21 vs. ≥21 days.*

D-dimer (t=-1.32, p=0.17), albumin (t=1.08, p=0.31), creatinine (t=0.31, p=0.76), diabetes (t=-0.23, p=0.82), hypertension (t=1.23, p=0.24) and cardiovascular disease (t=0.29, p=0.78).

In sub-group analysis, the pooled SMD in studies investigating disease severity (SMD -0.38, 95% CI -0.67 to -0.08, p<0.001; I^2 = 86.4, p=0.013) was non-significantly higher than that in studies investigating survival status (SMD -0.48, 95% CI -0.61 to -0.21, p<0.001; I^2 = 42.6, p=0.12; t=-0.54, p=0.60) (**Figure 5**). However, the between-study variance was substantially lower in studies investigating survival status (I^2 = 42.6 *vs.* I^2 = 86.4).

Complement C4

The overall SMD in complement C4 concentrations between COVID-19 patients with low vs. high severity or survivor vs. non-survivor status in the 19 studies is shown in **Figure 6**. In 13 studies, patients with high severity or non-survivor status had lower C4 concentrations when compared to those with low severity or survivor status (mean difference range, -1.43 to -0.10) (22–24, 27, 29, 32, 33, 36–40), with a significant difference in seven (22, 23, 27, 29, 32, 33, 40). No between-group difference was observed in two studies (mean difference 0.00) (31, 34), whereas in the remaining five the C4 concentration was lower in patients with low severity or survivor status (mean difference range, 0.10 to 0.77) (25, 26, 28, 30, 35), with a significant difference in two (28, 30). The pooled results confirmed that C4 concentrations were significantly lower in patients with high severity or non-survivor status during follow up (SMD -0.29, 95% CI -0.49 to -0.09, p=0.005) (**Figure 6**). Extreme heterogeneity between studies was observed (I^2 = 84.4%, p<0.001).

Sensitivity analysis, performed by sequentially removing each study and re-assessing the pooled estimates, showed that the

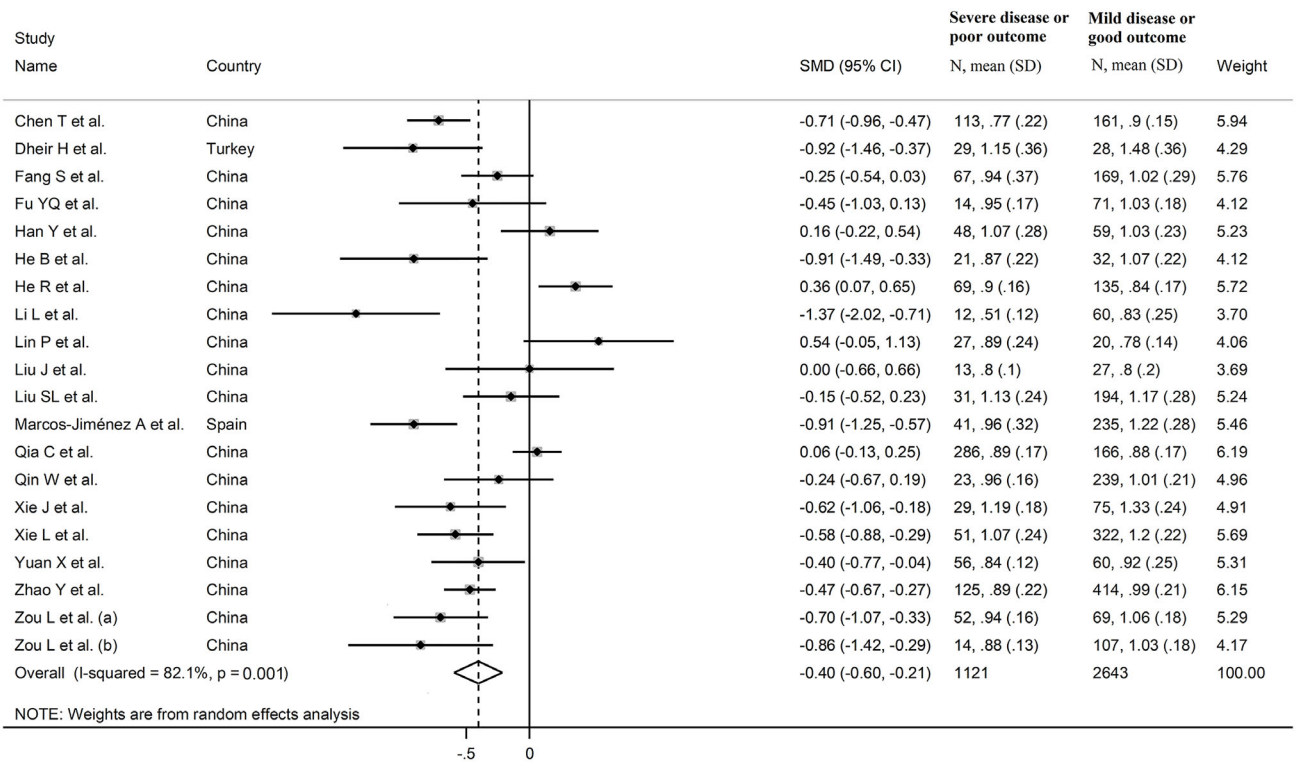


FIGURE 2 | Forest plot of studies reporting complement C3 concentrations in patients with COVID-19.

magnitude and direction of the effect size was not substantially altered (effect size range, between -0.31 and -0.23) (**Figures 7A, B**) (7). Furthermore, after removing the studies conducted at the Renmin Hospital, Wuhan, barring the largest ones for disease severity (28) and survival status (39), the SMD remained substantially unchanged, albeit borderline significant (SMD -0.28, 95% CI -0.56 to 0.00, p=0.05; I^2 = 88.2%, p<0.001) (**Figure 4B**).

Similar to C3, complement C4 concentrations remained significantly lower (SMD -0.33, 95% CI -0.59 to -0.06, p=0.015; I^2 = 85.9%, p<0.001) in patients with high severity or non-survivor status after removing three relatively large studies accounting for nearly 36% of the total sample size (34, 37, 39).

In meta-regression, CRP (t=2.58, p=0.02), pro-thrombin time (t=-2.53, p=0.03), D-dimer (t=-2.78, p=0.02), and albumin (t=3.66, p=0.006) were significantly associated with the pooled SMD. A trend toward a significant association was also observed between effect size and WBC (t=-1.93, p=0.07) and neutrophils (t=-2.07, p=0.06). By contrast, no significant correlations were observed between the SMD and age (t=-1.00, p=0.33), gender, (t=1.05, p=0.31), lymphocytes (t=0.72, p=0.49), AST (t=-0.16, p=0.87), ALT (t=-0.94, p=0.36), LDH (t=-0.08, p=0.93), creatinine (t=0.40, p=0.70), diabetes (t=-0.55, p=0.54), hypertension (t=0.61, p=0.55) and cardiovascular disease (t=-0.21, p=0.84).

In sub-group analysis, the pooled SMD in studies reporting disease severity (SMD -0.42, 95% CI -0.75 to -0.09, p<0.001; I^2 = 89.3, p=0.013) was non-significantly lower than that in studies reporting survival status (SMD -0.21, 95% CI -0.45 to 0.03, p=0.09; I^2 = 68.2, p=0.008; t=0.78, p=0.44) (**Figure 8**). However, the between-study variance was relatively lower in studies reporting survival (I^2 = 68.2 *vs.* I^2 = 89.3).

Publication Bias

Complement C3

There was no publication bias according to the Begg's (p=0.63) and Egger's (p=0.30) t-tests. Accordingly, the trim-and-fill analysis showed that no study was missing or should be added (**Figure 3B**) (7).

Complement C4

The Begg's (p=0.35) and Egger's (p=0.37) t-tests did not show publication bias. Accordingly, the trim-and-fill analysis showed that no study was missing or should be added (**Figure 7B**) (7).

Certainty of Evidence

The initial level of certainty for serum C3 and C4 SMD values was considered low because of the observational nature of the selected studies (rating 2, ⊕⊕⊖⊖). After considering the presence of a low-moderate risk of bias in 18 out of 19 studies (no rating change required), a generally extreme and unexplained heterogeneity (serious limitation, downgrade one level), lack of indirectness (no rating change required), the

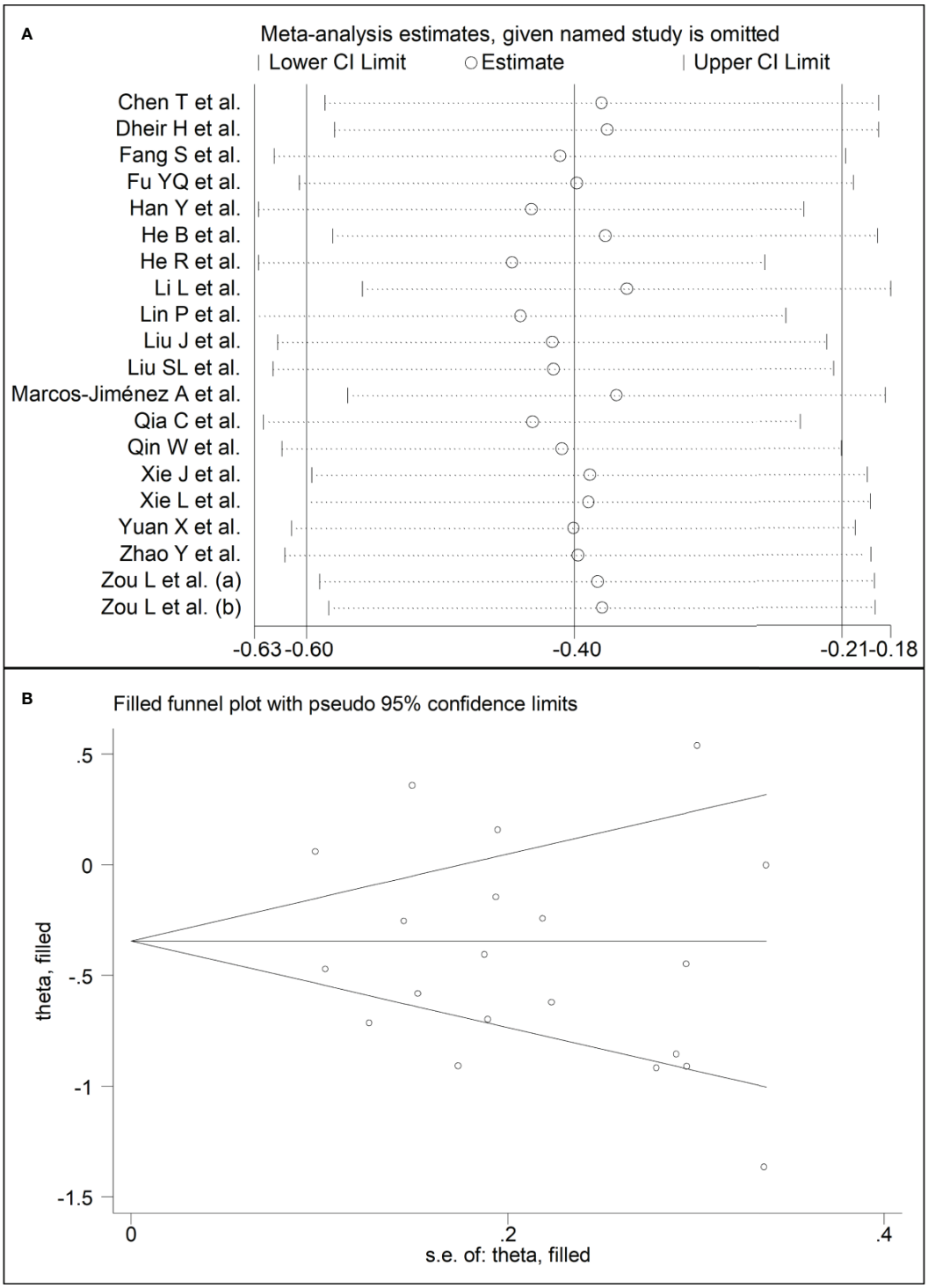


FIGURE 3 | **(A)** Sensitivity analysis of the association between complement C3 and COVID-19. The influence of individual studies on the overall standardized mean difference (SMD) is shown. The middle vertical axis indicates the overall SMD, and the two vertical axes indicate the 95% confidence intervals (CIs). The hollow circles represent the pooled SMD when the remaining study is omitted from the meta-analysis. The two ends of each broken line represent the 95% CI. **(B)** Funnel plot of studies investigating low *vs.* high severity or survivor *vs.* non-survivor status after trimming and filling. Dummy studies and genuine studies are represented by enclosed circles and free circles, respectively.

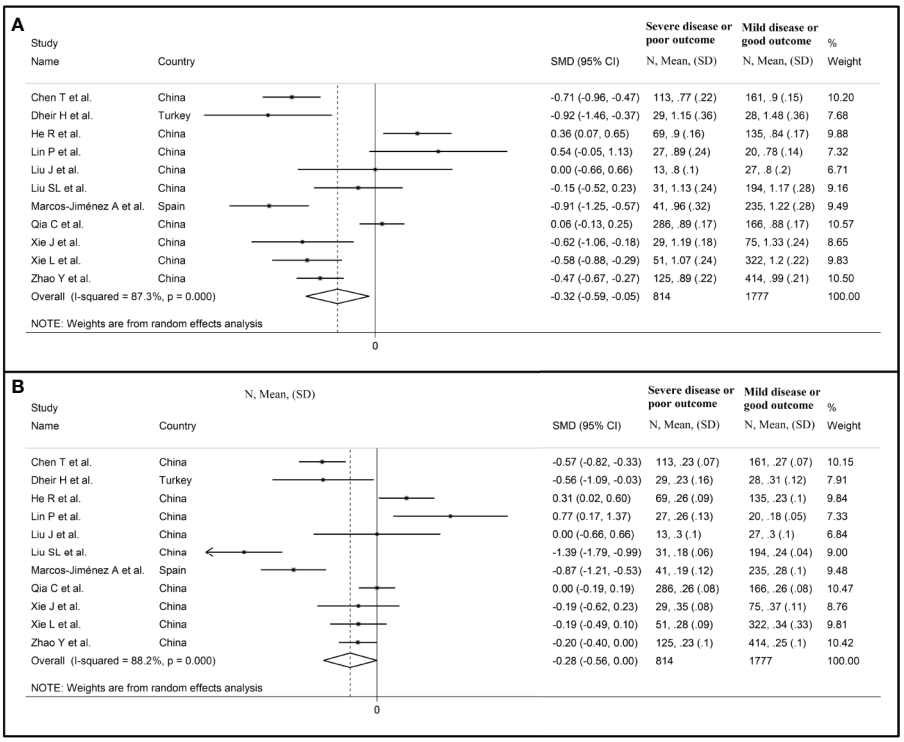


FIGURE 4 | Forest plot of studies reporting complement C3 **(A)** and C4 concentrations **(B)** after removing those conducted at the Renmin Hospital, Wuhan, barring the largest ones for disease severity (28) and survival status (39).

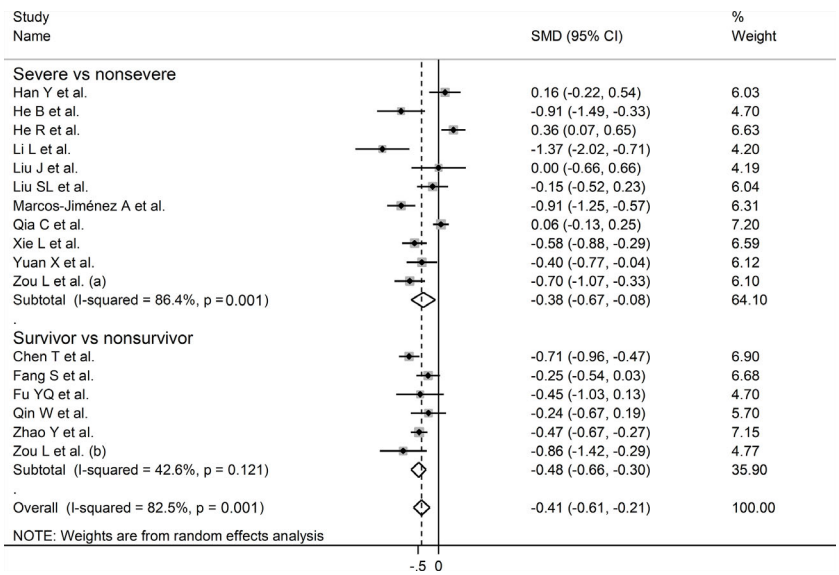


FIGURE 5 | Forest plot of studies reporting complement C3 concentrations in patients with COVID-19 according to disease severity or survival status. The diamond represents the point estimate and confidence intervals after combining and averaging the individual studies. The vertical line through the vertical points of the diamond represents the point estimate of the averaged studies.

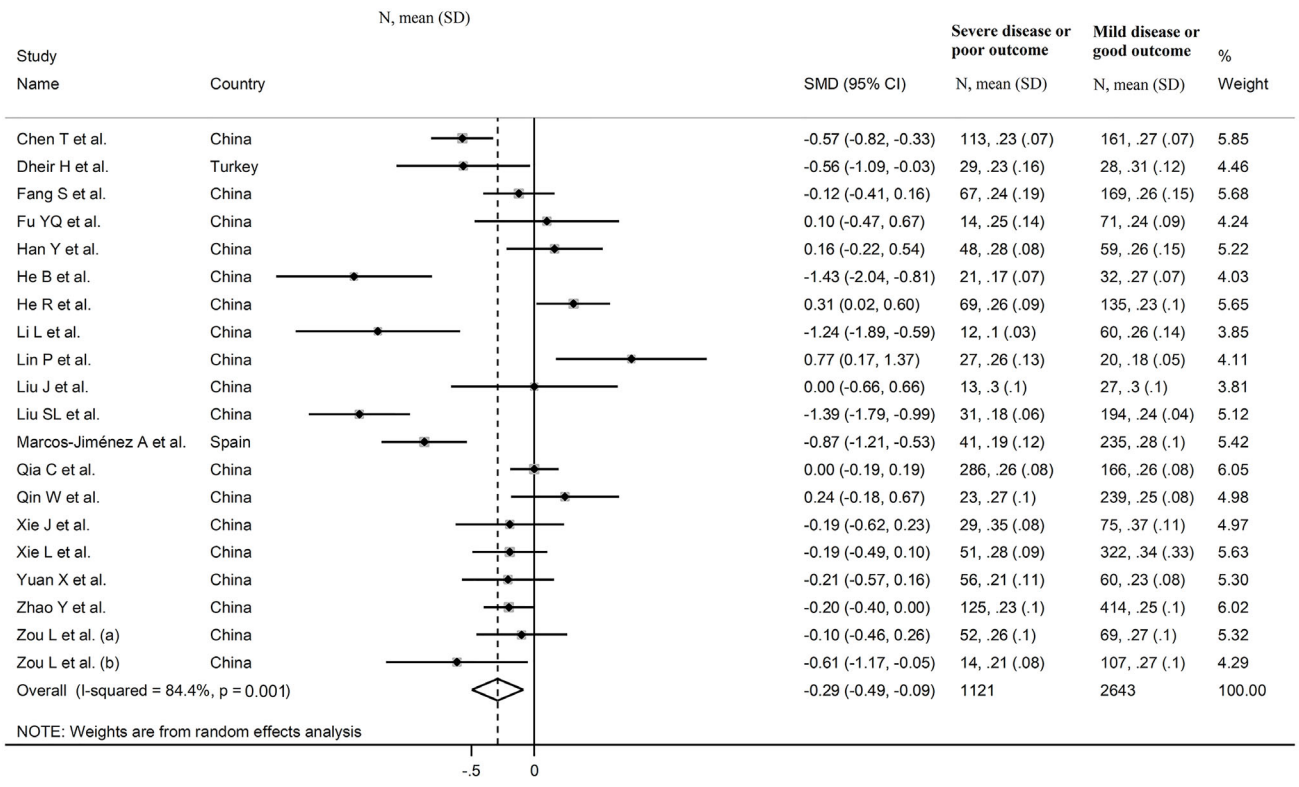


FIGURE 6 | Forest plot of studies reporting complement C4 concentrations in patients with COVID-19.

relatively low imprecision (relatively narrow confidence intervals without threshold crossing, upgrade one level), the relatively small effect size (SMD between -0.29, C4, and -0.40, C3, no rating change required) (10), and absence of publication bias (upgrade one level), the overall level of certainty was considered moderate (rating 3, ⊕⊕⊕⊖).

DISCUSSION

In this systematic review and meta-analysis, we observed that the serum concentrations of complement C3 and C4 were significantly lower in COVID-19 patients with more severe disease or who died during follow up when compared to those with milder disease or survivor status. The magnitude of the observed SMD values, -0.40 for C3 and -0.29 for C4, suggests that the between-group differences are significant either from a biological and a clinical point of view (10). The between-group heterogeneity was extreme however the sequential omission of individual studies did not exert tangible effects on the overall SMD value. Furthermore, there was no evidence of publication bias. Meta-regression analysis showed significant associations between the SMD of C3 and white blood cell count, CRP, and pro-thrombin time, and between the SMD of C4 and CRP, pro-thrombin time, D-dimer, and albumin.

The measurement of the serum concentrations of complement C3 and C4 is useful in the diagnosis and the monitoring of blood associated infectious diseases and immune complex diseases. By and large, C3 is often decreased through consumption during infections whereas a combined reduction in C3 and C4 is observed in immune complex disease (1, 2). The assessment the complement system during SARS-CoV-2 has gained considerable attention because of the potential adverse consequences of an unrestrained activation of the system on the structural and functional integrity of different organs and tissues. This proposition is supported by the results of studies reporting a beneficial effect of corticosteroid treatment in patients with COVID-19, which suggests that the organ and tissue injury is not directly caused by viral infection but, rather, is the consequence of an excessive host immune response. This, also reflected by the activation of the complement system, facilitates the release of pro-inflammatory cytokines and, consequently, a state of intra-vascular coagulation and cell death (5, 41). Autoptic studies in patients with COVID-19 have shown the accumulation of complement components in the lungs and kidneys, and concomitant evidence of tissue injury in these organs, confirming the detrimental role of excessive complement activation in this group (42, 43). The latter is likely to involve the contribution of C3b, a product of the C3 convertases, to the formation of complement C5 convertases.

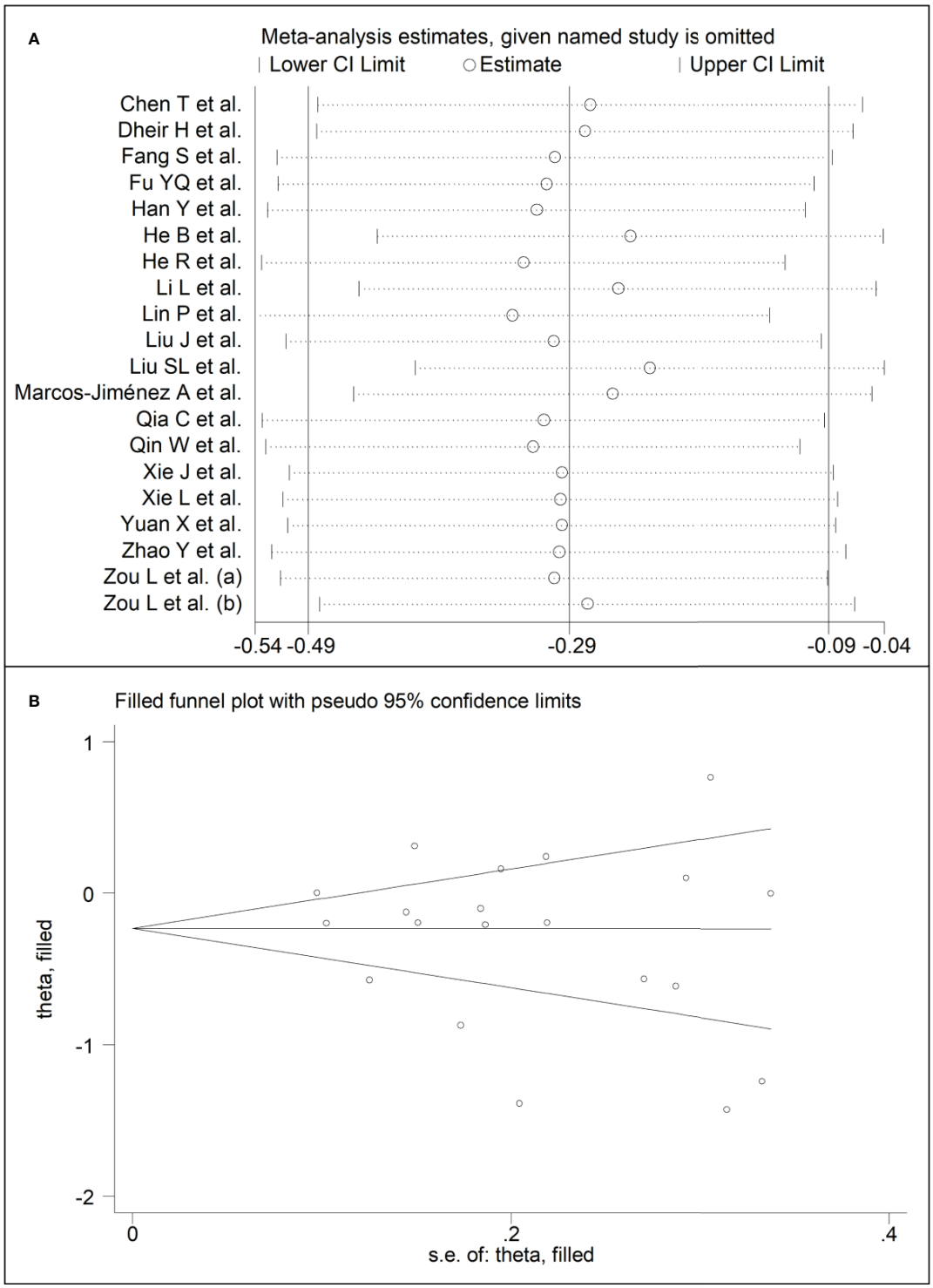


FIGURE 7 | **(A)** Sensitivity analysis of the association between complement C4 and COVID-19. The influence of individual studies on the overall standardized mean difference (SMD) is shown. The middle vertical axis indicates the overall SMD, and the two vertical axes indicate the 95% confidence intervals (CIs). The hollow circles represent the pooled SMD when the remaining study is omitted from the meta-analysis. The two ends of each broken line represent the 95% CI. **(B)** Funnel plot of studies investigating low *vs.* high severity or survivor *vs.* non-survivor status after trimming and filling. Dummy studies and genuine studies are represented by enclosed circles and free circles, respectively.

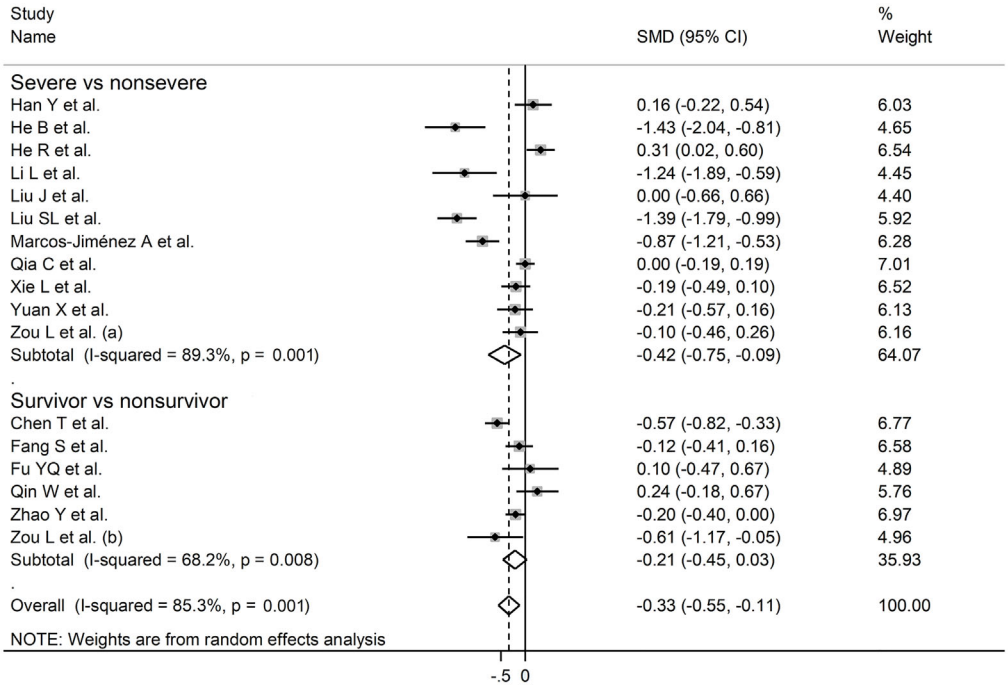


FIGURE 8 | Forest plot of studies reporting complement C4 concentrations in patients with COVID-19 according to disease severity or survival status. The diamond represents the point estimate and confidence intervals after combining and averaging the individual. The vertical line through the vertical points of the diamond represents the point estimate of the averaged studies.

These, in turn, cleave C5 into C5a, an anaphylatoxin that exacerbates the activity of pro-inflammatory pathways, and C5b, which triggers the downstream events of complement activation, i.e., the formation of the membrane-attack complex and, by forming C5b-9, the induction of cell injury that also involves the endothelium (6, 44). The observed associations, in meta-regression analysis, between the SMD values of C3 and C4 and CRP, pro-thrombin time, and D-dimer (C4 only) further support the presence of a complex, yet relevant from a pathophysiologic point of view, interplay between the activation of the complement system, inflammatory and pro-coagulant pathways, on one hand, and the degree of disease severity and its clinical consequences, on the other, in patients with COVID-19.

The extreme between-study heterogeneity observed in our analyses represents a significant limitation that reduces to a certain extent the generalizability of the results. It is possible that other, unreported factors might have contributed to the observed heterogeneity. At the same time, there was no evidence of publication bias and the overall effect size was not affected in sensitivity analysis. Another significant limitation is that 3,501 of the 3,764 patients were Chinese, and 10 studies were conducted in the same hospital (Renmin Hospital, Wuhan). While similar SMD values were observed after removing the studies from this hospital, barring the largest ones for disease severity (28) and survival status (39), the possibility of duplicate data cannot be completely ruled out. Furthermore, no selected study performed a serial measurement of complement component concentrations during hospitalization. This might provide additional information regarding possible clinical deterioration. In one study investigating serial concentrations of C3a and C5a, significant elevations of the latter, but not of the former, preceded the onset of clinical deterioration (45). Further studies are required to determine whether serial measurements of complement components, including C3 and C4, provide additional prognostic information to that of single measurements on admission.

The increasing evidence of an unrestrained complement activation in severe COVID-19 has also prompted the search for targeted therapies that suppress this phenomenon. Inhibitors targeting the early steps of complement activation have shown, in small studies, promising effects on inflammatory markers, respiratory function, and clinical outcomes (46). Larger, randomized controlled studies, are urgently required to explore the full potential of this treatment strategy in patients with different degrees of COVID-19 severity and complement activation.

In conclusion, our systematic review and meta-analysis with meta-regression has shown that lower serum concentrations of C3 and C4, indicating excessive complement activation and product consumption, are significantly associated with the presence of severe disease and increased mortality in patients with COVID-19. Additional studies are required to determine whether single or serial measurement of complement components, with or without other clinical, demographic, and biochemical characteristics, can further increase our capacity to predict COVID-19 severity and adverse clinical outcomes.

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

Initial idea: AZ and AM. Data collection and analysis: AZ. Data interpretation: AZ and AM. Writing - first draft: AM. Writing - Review & Editing: AZ and AM. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.696085/full#supplementary-material

REFERENCES

1. Holers VM. Complement and its Receptors: New Insights Into Human Disease. *Annu Rev Immunol* (2014) 32:433–59. doi: 10.1146/annurev-immunol-032713-120154
2. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement System Part II: Role in Immunity. *Front Immunol* (2015) 6:257. doi: 10.3389/fimmu.2015.00257
3. Bhukya PL, Bramhachari PV. Activation of Complement System During Viral Infections: Prospects and Future Challenges. In: PV Bramhachari, editor. *Dynamics of Immune Activation in Viral Diseases*. Singapore: Springer (2020).
4. Santiesteban-Lores LE, Amamura TA, da Silva TF, Midon LM, Carneiro MC, Isaac L, et al. A Double Edged-Sword - The Complement System During SARS-CoV-2 Infection. *Life Sci* (2021) 272:119245. doi: 10.1016/j.lfs.2021.119245
5. Fajgenbaum DC, June CH. Cytokine Storm. *N Engl J Med* (2020) 383 (23):2255–73. doi: 10.1056/NEJMra2026131
6. Noris M, Remuzzi G. Overview of Complement Activation and Regulation. *Semin Nephrol* (2013) 33(6):479–92. doi: 10.1016/j.semnephrol.2013.08.001
7. Zinellu A, Mangoni AA. Serum Prealbumin Concentrations, COVID-19 Severity, and Mortality: A Systematic Review and Meta-Analysis. *Front Med (Lausanne)* (2021) 8:638529. doi: 10.3389/fmed.2021.638529
8. Wells GA, Shea B, O'Connell D, Peterson J, Welch V, Losos M, et al. *The Newcastle-Ottawa Scale (NOS) for Assessing the Quality of Nonrandomised Studies in Meta-Analyses The Ottawa Hospital Research Institute* (2013). Available at: http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp.
9. Zinellu A, Paliogiannis P, Carru C, Mangoni AA. Serum Amyloid A Concentrations, COVID-19 Severity and Mortality: An Updated Systematic Review and Meta-Analysis. *Int J Infect Dis* (2021) 105:668–74. doi: 10.1016/j.ijid.2021.03.025
10. Cohen J. *Statistical Power Analysis for the Behavioral Sciences. 2nd*. Hillsdale, NJ, USA: Erlbaum (1988).
11. Balshem H, Helfand M, Schunemann HJ, Oxman AD, Kunz R, Brozek J, et al. GRADE Guidelines: 3. Rating the Quality of Evidence. *J Clin Epidemiol* (2011) 64(4):401–6. doi: 10.1016/j.jclinepi.2010.07.015
12. Hultcrantz M, Rind D, Akl EA, Treweek S, Mustafa RA, Iorio A, et al. The GRADE Working Group Clarifies the Construct of Certainty of Evidence. *J Clin Epidemiol* (2017) 87:4–13. doi: 10.1016/j.jclinepi.2017.05.006
13. Zhang Y, Coello PA, Guyatt GH, Yepes-Nunez JJ, Akl EA, Hazlewood G, et al. GRADE Guidelines: 20. Assessing the Certainty of Evidence in the Importance of Outcomes or Values and Preferences-Inconsistency, Imprecision, and Other Domains. *J Clin Epidemiol* (2019) 111:83–93. doi: 10.1016/j.jclinepi.2018.05.011
14. Wan X, Wang W, Liu J, Tong T. Estimating the Sample Mean and Standard Deviation From the Sample Size, Median, Range and/or Interquartile Range. *BMC Med Res Methodol* (2014) 14:135. doi: 10.1186/1471-2288-14-135
15. Bowden J, Tierney JF, Copas AJ, Burdett S. Quantifying, Displaying and Accounting for Heterogeneity in the Meta-Analysis of RCTs Using Standard and Generalised Q Statistics. *BMC Med Res Methodol* (2011) 11:41. doi: 10.1186/1471-2288-11-41
16. Higgins JP, Thompson SG. Quantifying Heterogeneity in a Meta-Analysis. *Stat Med* (2002) 21(11):1539–58. doi: 10.1002/sim.1186
17. Tobias A. Assessing the Influence of a Single Study in the Meta-Analysis Estimate. *Stata Tech Bull* (1999) 47:15–7.
18. Begg CB, Mazumdar M. Operating Characteristics of a Rank Correlation Test for Publication Bias. *Biometrics* (1994) 50(4):1088–101. doi: 10.2307/2533446
19. Sterne JA, Egger M. Funnel Plots for Detecting Bias in Meta-Analysis: Guidelines on Choice of Axis. *J Clin Epidemiol* (2001) 54(10):1046–55. doi: 10.1016/s0895-4356(01)00377-8
20. Duval S, Tweedie R. Trim and Fill: A Simple Funnel-Plot-Based Method of Testing and Adjusting for Publication Bias in Meta-Analysis. *Biometrics* (2000) 56(2):455–63. doi: 10.1111/j.0006-341x.2000.00455.x
21. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 Statement: An Updated Guideline for Reporting Systematic Reviews. *BMJ* (2021) 372:n71. doi: 10.1136/bmj.n71
22. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, et al. Clinical Characteristics of 113 Deceased Patients With Coronavirus Disease 2019: Retrospective Study. *BMJ* (2020) 368:m1091. doi: 10.1136/bmj.m1091
23. Dheir H, Sipahi S, Yaylaci S, Koroglu M, Erdem AF, Karabay O. Is There Relationship Between SARS-CoV-2 and the Complement C3 and C4? *Turk J Med Sci* (2020) 50(4):687–8. doi: 10.3906/sag-2004-336
24. Fang S, Wang H, Lu L, Jia Y, Xia Z. Decreased Complement C3 Levels are Associated With Poor Prognosis in Patients With COVID-19: A Retrospective Cohort Study. *Int Immunopharmacol* (2020) 89(Pt A):107070. doi: 10.1016/j.intimp.2020.107070
25. Fu YQ, Sun YL, Lu SW, Yang Y, Wang Y, Xu F. Effect of Blood Analysis and Immune Function on the Prognosis of Patients With COVID-19. *PloS One* (2020) 15(10):e0240751. doi: 10.1371/journal.pone.0240751
26. Han Y, Zhang H, Mu S, Wei W, Jin C, Tong C, et al. Lactate Dehydrogenase, an Independent Risk Factor of Severe COVID-19 Patients: A Retrospective and Observational Study. *Aging (Albany NY)* (2020) 12(12):11245–58. doi: 10.18632/aging.103372
27. He B, Wang J, Wang Y, Zhao J, Huang J, Tian Y, et al. The Metabolic Changes and Immune Profiles in Patients With Covid-19. *Front Immunol* (2020) 11:2075. doi: 10.3389/fimmu.2020.02075
28. He R, Lu Z, Zhang L, Fan T, Xiong R, Shen X, et al. The Clinical Course and its Correlated Immune Status in COVID-19 Pneumonia. *J Clin Virol* (2020) 127:104361. doi: 10.1016/j.jcv.2020.104361
29. Li L, Chen C. Contribution of Acute-Phase Reaction Proteins to the Diagnosis and Treatment of 2019 Novel Coronavirus Disease (COVID-19). *Epidemiol Infect* (2020) 148:e164. doi: 10.1017/S095026882000165X
30. Lin P, Chen W, Huang H, Lin Y, Cai M, Lin D, et al. Delayed Discharge is Associated With Higher Complement C3 Levels and a Longer Nucleic Acid-Negative Conversion Time in Patients With COVID-19. *Sci Rep* (2021) 11 (1):1233. doi: 10.1038/s41598-021-81010-3
31. Liu J, Li S, Liu J, Liang B, Wang X, Wang H, et al. Longitudinal Characteristics of Lymphocyte Responses and Cytokine Profiles in the Peripheral Blood of SARS-CoV-2 Infected Patients. *EBioMedicine* (2020) 55:102763. doi: 10.1016/j.ebiom.2020.102763
32. Liu SL, Wang SY, Sun YF, Jia QY, Yang CL, Cai PJ, et al. Expressions of SAA, CRP, and FERR in Different Severities of COVID-19. *Eur Rev Med Pharmacol Sci* (2020) 24(21):11386–94. doi: 10.26355/eurrev_202011_23631
33. Marcos-Jimenez A, Sanchez-Alonso S, Alcaraz-Serna A, Esparcia L, Lopez-Sanz C, Sampedro-Nunez M, et al. Deregulated Cellular Circuits Driving Immunoglobulins and Complement Consumption Associate With the Severity of COVID-19 Patients. *Eur J Immunol* (2021) 51:634–47. doi: 10.1002/eji.202048858

34. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, et al. Dysregulation of Immune Response in Patients With Coronavirus 2019 (Covid-19) in Wuhan, China. *Clin Infect Dis* (2020) 71(15):762–8. doi: 10.1093/cid/ciaa248
35. Qin WD, Bai W, Liu K, Liu Y, Meng X, Zhang K, et al. Clinical Course and Risk Factors of Disease Deterioration in Critically Ill Patients With COVID-19. *Hum Gene Ther* (2021) 32:310–5. doi: 10.1089/hum.2020.255
36. Xie J, Shi D, Bao M, Hu X, Wu W, Sheng J, et al. A Predictive Nomogram for Predicting Improved Clinical Outcome Probability in Patients With COVID-19 in Zhejiang Province, China. *Eng (Beijing)* (2020). doi: 10.1016/j.eng.2020.05.014
37. Xie L, Wu Q, Lin Q, Liu X, Lin W, Hao S, et al. Dysfunction of Adaptive Immunity Is Related to Severity of COVID-19: A Retrospective Study. *Ther Adv Respir Dis* (2020) 14:1753466620942129. doi: 10.1177/1753466620942129
38. Yuan X, Huang W, Ye B, Chen C, Huang R, Wu F, et al. Changes of Hematological and Immunological Parameters in COVID-19 Patients. *Int J Hematol* (2020) 112(4):553–9. doi: 10.1007/s12185-020-02930-w
39. Zhao Y, Nie HX, Hu K, Wu XJ, Zhang YT, Wang MM, et al. Abnormal Immunity of non-Survivors With COVID-19: Predictors for Mortality. *Infect Dis Poverty* (2020) 9(1):108. doi: 10.1186/s40249-020-00723-1
40. Zou L, Dai L, Zhang Y, Fu W, Gao Y, Zhang Z, et al. Clinical Characteristics and Risk Factors for Disease Severity and Death in Patients With Coronavirus Disease 2019 in Wuhan, China. *Front Med (Lausanne)* (2020) 7:532. doi: 10.3389/fmed.2020.00532
41. Group RC, Horby P, Lim WS, Emberson JR, Mafham M, Bell JL, et al. Dexamethasone in Hospitalized Patients With Covid-19 - Preliminary Report. *N Engl J Med* (2021) 384(8):693–704. doi: 10.1056/NEJMoa2021436
42. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement Associated Microvascular Injury and Thrombosis in the Pathogenesis of Severe COVID-19 Infection: A Report of Five Cases. *Transl Res* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
43. Pfister F, Vonbrunn E, Ries T, Jack HM, Uberla K, Lochnit G, et al. Complement Activation in Kidneys of Patients With Covid-19. *Front Immunol* (2020) 11:594849. doi: 10.3389/fimmu.2020.594849
44. Song WC, FitzGerald GA. Covid-19, Microangiopathy, Hemostatic Activation, and Complement. *J Clin Invest* (2020) 130(8):3950–3. doi: 10.1172/JCI140183
45. Prendecki M, Clarke C, Medjeral-Thomas N, McAdoo SP, Sandhu E, Peters JE, et al. Temporal Changes in Complement Activation in Haemodialysis Patients With COVID-19 as a Predictor of Disease Progression. *Clin Kidney J* (2020) 13(5):889–96. doi: 10.1093/ckj/sfaa192
46. Kurtovic L, Beeson JG. Complement Factors in COVID-19 Therapeutics and Vaccines. *Trends Immunol* (2021) 42(2):94–103. doi: 10.1016/j.it.2020.12.002

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.

frontiers in Immunology

CASE REPORT
published: 23 June 2021
doi: 10.3389/fimmu.2021.680567

Case Report: Adult Post-COVID-19 Multisystem Inflammatory Syndrome and Thrombotic Microangiopathy

*Idris Boudhabhay[1,2], Marion Rabant[2], Lubka T. Roumenina[3], Louis-Marie Coupry[1], Victoria Poillerat[3], Armance Marchal[4], Véronique Frémeaux-Bacchi[4], Khalil El Karoui[5], Mehran Monchi[1] and Franck Pourcine[1,6]**

[1] Groupe Hospitalier Sud Ile de France, Service de Réanimation, Melun, France, [2] Centre Hospitalo-Universitaire Necker, Service d'Anatomie Pathologique, Paris, France, [3] Centre de Recherche des Cordeliers, INSERM, Sorbonne Université, Université de Paris, Paris, France, [4] Hôpital Européen Georges Pompidou, Laboratoire d'Immunologie Biologique, Paris, France, [5] Centre Hospitalo-Universitaire Henri Mondor, Service de Néphrologie et Transplantation, Créteil, France, [6] Groupe Hospitalier Sud Ile de France, Service de Néphrologie, Melun, France

OPEN ACCESS

Edited by:
Nicolas Stephane Merle, National Heart, Lung, and Blood Institute (NHLBI), United States

Reviewed by:
Magdalena Riedl Khursigara, Hospital for Sick Children, Canada
Bradley Patton Dixon, Children's Hospital Colorado, United States

***Correspondence:**
Franck Pourcine
franck.pourcine@ghsif.fr

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: 14 March 2021
Accepted: 27 May 2021
Published: 23 June 2021

Citation:
Boudhabhay I, Rabant M, Roumenina LT, Coupry L-M, Poillerat V, Marchal A, Frémeaux-Bacchi V, El Karoui K, Monchi M and Pourcine F (2021) Case Report: Adult Post-COVID-19 Multisystem Inflammatory Syndrome and Thrombotic Microangiopathy. Front. Immunol. 12:680567. doi: 10.3389/fimmu.2021.680567

Background: The coronavirus disease 2019 (COVID-19) pandemic has affected millions of people worldwide. A clinical series of Kawasaki-like multisystem inflammatory syndrome (MIS), occurring after SARS-CoV-2 infection, have been described in children (MIS-C) and adults (MIS-A), but the pathophysiology remains unknown.

Case Presentation: We describe a case of post-COVID-19 MIS-A in a 46-year-old man with biopsy-proven renal thrombotic microangiopathy (TMA). Specific complement inhibition with eculizumab was initiated promptly and led to a dramatic improvement of renal function.

Conclusion: Our case suggests that that TMA could play a central role in the pathophysiology of post-COVID-19 MIS-A, making complement blockers an interesting therapeutic option.

Keywords: thrombotic microangiopathy, multisystem inflammatory syndrome, COVID-19, complement system, eculizumab, case report

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic caused by SARS-CoV-2 has affected millions of people worldwide. In adults, COVID-19 is typically characterised by severe interstitial pneumonia and hyperactivation of the inflammatory cascade (1). There is growing evidence that COVID-19 affects the endothelial system, leading to endothelial dysfunction characterised by a pro-inflammatory and pro-coagulative state (2–5). Clinical series of Kawasaki-like multisystem inflammatory syndrome (MIS), occurring after viral clearance, have been described in children (MIS-C) (6–9). Recently, similar case

Abbreviations: aHUS, atypical haemolytic uremic syndrome; AKI, acute kidney injury; BMI, body mass index; CFB, Complement factor B; CFH, Complement factor H; CFI, Complement factor I; MCP, Membrane Cofactor Protein; CRP, C-reactive protein; hsTroponin, high-sensitivity troponin; MAC, membrane attack complex; MIS-C, multisystem inflammatory syndrome in children; MIS-A, multisystem inflammatory syndrome in adults; PCR, polymerase chain reaction; RRT, renal replacement therapy; sCr, serum creatinine; sC5b-9, soluble C5b-9; TMA, thrombotic microangiopathy; THBD, Thrombomodulin.

series of MIS were described in adults (MIS-A) (10–15). However, the pathophysiology of MIS remains unknown. We report a case of MIS-A with biopsy-proven thrombotic microangiopathy (TMA) successfully treated with eculizumab.

CASE PRESENTATION

A 46-year-old patient of West African ancestry was admitted to our hospital for hypertensive emergency (189/123 mmHg) and fever. He had a personal history of arterial hypertension and obesity (BMI = 36 kg/m^2) and family history of arterial hypertension. No previous COVID-19 symptoms were reported, and the patient did not take any prescribed or over-the-counter medications. Physical examination was normal. SARS-CoV-2 PCR of nasopharyngeal swab was negative (repeated twice), but COVID-19 serology was positive for IgG (80 UA/mL, positive if > 12 UA/mL, Immunoassay YHLO iFlash 1800) and negative for IgM. Thoracoabdominopelvic CT scan was unremarkable. First investigations revealed an inflammatory state, anaemia, thrombocytopenia and acute kidney injury (AKI). The serum creatinine (sCr) level was 169 µmol/L and associated with 1g per day proteinuria, aseptic pyuria, no haematuria and low natriuresis (< 20 mmol/L). C-reactive protein (CRP) level was 312 mg/L and neutrophil count was 18.7 g/L (**Table 1**). On day 4, the patient presented with evanescent facial erythema and developed acute myocardial dysfunction with reduced left ventricular ejection fraction (40%), pericardial effusion and elevation of high-sensitivity troponin (hsTroponin). Taking into account the frequency of vascular thromboses related to COVID-19, therapeutic anticoagulation with heparin was started. On day 5, neurological impairment appeared with coma, leading to intubation and mechanical ventilation. Cerebrospinal fluid analysis was unremarkable. Abnormal supratentorial periventricular MRI signals responsible for a restriction of the diffusion testified to acute vasculitis. No immunosuppressive treatment was introduced because of concomitant tracheal aspiration positive for *Enterobacter aerogenes*, which was treated with trimethoprim-sulfamethoxazole. On day 7, myocardial and renal function worsened (sCr 660 µmol/L), requiring initiation of dobutamine and intermittent renal replacement therapy (RRT). A kidney biopsy was performed. Light microscopy revealed typical lesions of TMA, including fibrin thrombi within glomeruli and myxoid intimal alterations of arterioles and small-to-medium sized renal arteries. The remaining glomeruli were normal without hypercellularity. A significant interstitial infiltrate, mainly composed of neutrophils, was responsible for severe tubulitis and moderate acute tubular necrosis (**Figure 1A**). Immunofluorescence study showed isolated mesangial complement C3c-positive deposits without evidence for IgG, IgA, IgM, C1q or C4d deposits (**Figure 1B**). Immunochemistry study showed C5b-9 deposits at the same localisation (**Figure 1C**). Immunological work-up is shown in **Table 2**. ADAMTS13 activity was moderately decreased but did not reach the cut-off for a diagnosis of thrombotic thrombocytopenic purpura. Complement work-up evaluation found an elevated soluble C5b-9 (sC5b-9) with low C4 and normal C3 levels in the serum (**Table 2**). Cryoglobulinemia was negative. All coding sequences of *CFH*, *CFI, MCP, C3, CFB* and *THBD* genes were analysed by next-generation sequencing. We defined a variant as rare when its minor allele frequency was below 1% in the general population. No rare variants were detected in the six complement genes implicated in atypical haemolytic uremic syndrome (aHUS).

On day 8, specific complement inhibition with eculizumab (900 mg) was initiated. Three days later, cardiac function and neurological impairment improved, urine output increased, and blood creatinine decreased, allowing the withdrawal of dobutamine, RRT and mechanical ventilation (**Table 1**). On day 15, the patient received a second and last dose of eculizumab (900 mg). On day 30, the patient was discharged from the hospital, with a sCr 109 µmol/L and cardiac MRI showing no pericardial effusion, no sequelae of segmental hypokinesia and a left ventricular ejection fraction of 50%. Six months later, the patient resumed normal activities of daily living. Left ventricular function has normalised, despite persistent arterial hypertension. sCr is 82 µmol/L, without significant albuminuria (**Tables 1** and **2**).

DISCUSSION AND CONCLUSION

We describe the first case of post-COVID19 MIS-A associated with renal TMA successfully treated with eculizumab.

In this case, the IgG-positive serology, negative PCR swab and the absence of pulmonary involvement demonstrate the post-infectious nature of this syndrome, occurring after viral clearance.

Kidney involvement is frequent in COVID-19, as more than 40% of cases have abnormal proteinuria at hospital admission (16, 17). Scarce histological data are available, showing in most cases ATN, collapsing glomerulopathy or TMA in patients with acute COVID-19 infection (18–22). AKI is also common during MIS-C, ranging from 10% to 60% of the cases (6, 23, 24) while it was described in four adults in a case series of 20 MIS-A with cardiac involvement (12). Currently, the pathogenesis of AKI in MIS is thought to be mainly related to cytokine-mediated hypotension and cardiac dysfunction, leading to renal hypoperfusion (25).

Our case describes the first kidney biopsy performed in a patient with MIS-A. Scarce histological data are available on this syndrome. A first report showed intraepithelial collections of neutrophils with necrotic keratinocytes in skin biopsy (10). Likewise, in a fatal case of MIS-A, cardiac vasculitis composed of numerous neutrophils and CD4+ T cells was described (14). Similarly, in our case, renal biopsy revealed an aggressive interstitial infiltrate, mainly composed of neutrophils together with TMA.

TMA refers to pathological features of microvascular injury, including thrombi of platelets and fibrin in capillaries and arterioles (26, 27). These lesions are usually associated with peripheral thrombocytopenia and mechanical haemolytic anaemia, although some of these biological markers may be absent (27, 28). In our patient, the absence of a decreased haptoglobin level could be explained by the intensity of the inflammatory syndrome and predominant intrarenal TMA.

TABLE 1 | Clinical and laboratory findings.

		Days from hospital admission																				
Finding		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8***	**9**	**10**	**11**	**12**	**13**	**14**	**15***	**16**	**17**	**18**	**19**	**30**	**182**
Clinical																						
Respiratory status		SB	SB	SB	O2	MV	MV	MV	MV	MV	MV	MV	MV	SB	SB	SB	SB	SB	SB	SB	SB	SB
Dobutamine (gamma/kg/min)		–	–	–	–	–	–	5.75	10	10	10	5	–	–	–	–	–	–	–	–	–	–
Urine output (L/day)		–	–	–	0.3	0.3	0.5	0.7	0.8	0.9	3	7.8	6.9	7.4	5.2	6.3	4.2	2.8	2.7	2.2	1.5	–
Temperature (°C)		40.7	40.6	40	40.2	39.1	38.7	38.4	37.3	38.1	36.3	36.8	36.2	36.1	36.2	36	35.7	34.7	35.7	36	36	37
Laboratory	**Normal values**																					
Creatinine (µmol/L)	[59-104]	169	225	348	536	627	666	RRT	691	RRT	441	395	324	256	208	167	147	130	151	129	109	82
CRP (mg/L)	[<5]	312	469	528	621	551	462	319	292	213	153	87	58	45	32.9	25	19	12.9	11.4	8.8	3	3.7
Leucocyte count (G/L)	[4-10]	18.7	15.7	20	25	25.1	26.8	25.7	26.7	19.9	17	12.3	11.9	10.3	9.3	7.7	8	5.5	4.4	3.3	5.7	8.6
Haemoglobin level (g/dL)	[13-16.7]	12.3	9.8	8.3	7.5	7.1	6.6	7.3	7	6.2	6.7	7.6	7.8	8.7	8.7	9.2	9.7	8.8	9.7	9.3	12.6	13.3
Platelet count (G/L)	[150-450]	98	90	97	288	392	450	392	339	260	261	267	267	311	315	330	347	304	326	300	217	389
LDH (UI/L)	[135-225]	461	432	473	–	–	–	491	636	435	449	413	386	–	372	358	333	280	288	243	–	–
hsTroponin (ng/L)	[<14]	25	74	–	1006	1700	2791	2464	1669	–	1048	628	481	294	214	130	98.7	–	120	140	28	21.6
Haptoglobin (g/L)	[0.3-2]	–	–	–	2.91	2.91	–	–	–	–	–	–	–	–	–	–	–	2.67	2.41	2.71	–	–
Ferritin (µg/L)	[30-400]	–	3206	–	–	3538	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Schistocytes (%)	<1%	–	–	< 1	<1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<1	–
UPCR (mg/g)	<300	996	–	–	–	–	686	–	–	–	–	–	–	–	–	–	–	–	–	–	90	90
Others	HBV, HCV and HIV 1 serologies: negatives; glycosylated ferritin 12% (N > 20%);																					

HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency Virus; hsTroponin, High-sensitive Troponin; LDH, Lactate dehydrogenase; MV, mechanical ventilation; O2, oxygen therapy; RRT, renal replacement therapy; SB, spontaneous breathing; UPCR, urine protein creatinine ratio.

**eculizumab 900mg.*

Bold data on the first line correspond to the days from patient's hospital admission.

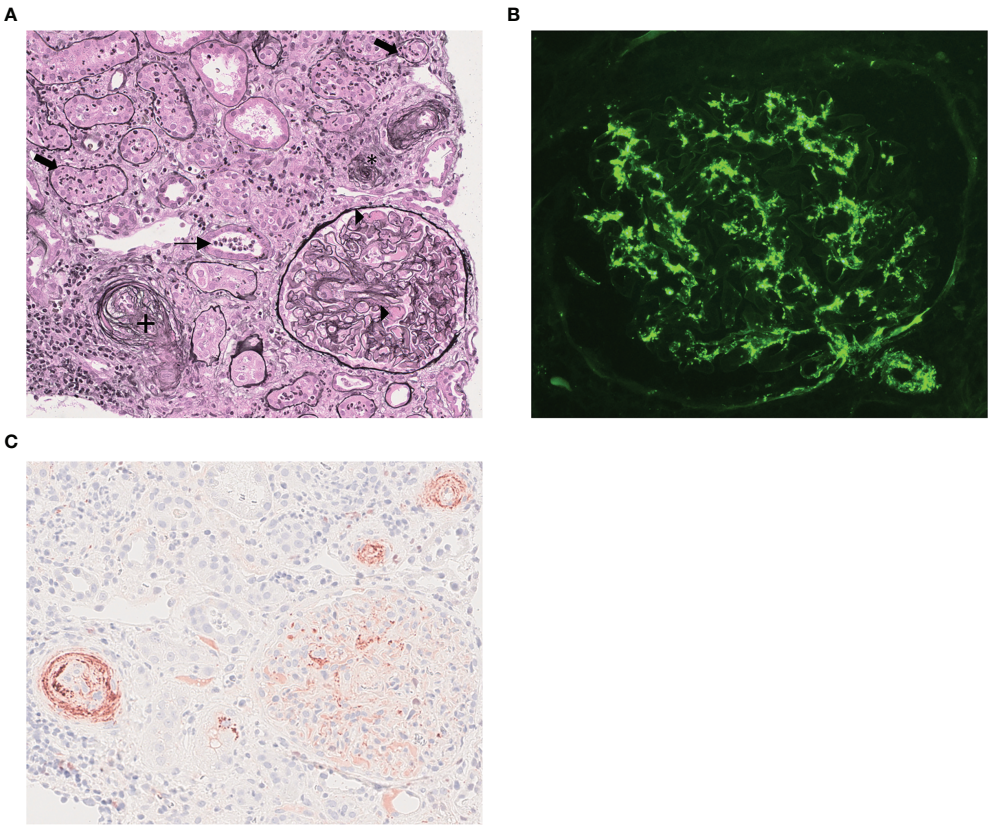


FIGURE 1 | Kidney biopsy findings. **(A)** Light microscopy analysis: Fibrin within glomerular capillary loops (black triangle). Small inter-lobular artery with intimal mucoid alterations and endothelial cells swelling (+). Arteriolar occlusion (*). Polymorphonuclear infiltration with tubulitis (thick arrows) and granular casts (thin arrow) (Jones methanamine silver staining, x200). **(B)** Immunofluorescence stucy showing predominant mesangial and sub-endothelial C3 deposits in glumeruli and in glomerular arteriole. (Anti-C3c fluorescein isothiocyanate-conjugate x400). **(C)** Immunochemistry study showing positive C5b-9 staining in arterioles, inter-lobular artery and glomeruli within the mesangium (Mouse IgG, B7 antineoepitope, [1]x40). [1]Kindly provided by Prof. P. Morgan (Cardiff Intitute of Infection&Immunity, UK).

TABLE 2 | Immunological work-up.

Laboratory	Normal values	Day 1	Day 182
ADAMTS-13 activity (%)	50-150	26	–
CH 50 (%)	70-130	64	106
C3 (mg/L)	660-1250	1030	1360
C4 (mg/L)	93-380	69	314
C1q (mg/L)	125-225	227	–
Factor H (%)	65-140	93	116
Factor I (%)	70-130	122	134
sC5b9 (ng/mL)	<420	469	–
Properdin (mg/L)	12-40	38	–
C1 inhibitor antigen (mg/L)	170-540	357	–
C1 inhibitor function (%)	70-130	>125	–
Anti-Factor H antibodies	–	negative	negative
Antinuclear antibodies	–	1/160	–
Anti-DNA antibodies	–	negative	–
Cryoglobulinemia	–	negative	–
Anti-cardiolipin antibodies	–	negative	–
Anti-B2GP1 antibodies	–	negative	–
Lupus anticoagulant	–	negative	–

ADAMTS13, a disintegrin and metalloprotease with thrombospondin type I repeats-13; sC5b9, soluble C5b-9 plasmatic level; DNA, desoxyribonucleic acid; B2GP1, beta 2 glycoprotein 1.

Diorio et al. recently proposed criteria for clinical TMA associated with MIS-C, including: schistocytes on blood smear, anaemia, elevated LDH, new thrombocytopenia, anaemia, proteinuria, hypertension and elevated sC5b9 (29). Our patient fulfilled five out of seven of the criteria, thus meeting their definition. aHUS is a form of TMA with predominant kidney involvement. The pathophysiology of aHUS involves multiple hits (30), but complement activation has a crucial role in this syndrome. Genetically determined or acquired dysregulation of the complement alternative pathway (CAP) has been found in up to 70% of patients with aHUS (27). Genetic screening was negative in our patient. Although we cannot exclude an unknown variant, it is likely that our patient presented with MIS-A complicated with TMA, rather than aHUS unmasked by SARS-CoV-2 infection.

The complement system (CS) seems to play a pivotal role in the pathophysiology of COVID-19, as few series have reported TMA injury in lungs and skin with sustained activation of CAP and lectin pathway during COVID-19 disease (31–33). Moreover, mice lacking complement component C3, display less

severe respiratory failure and inflammatory syndrome after *SARS-Cov* infection (34). Likewise, complement overactivation likely contributes to the renal injury during the course of COVID-19 infection, since a few studies have showed complement deposits in vascular beds and tubules (35). In this case, low serum C4 with normal C3 and mildly elevated sC5b-9 is suggestive of classical and/or lectin complement pathways activation. As MIS-A is a post-infectious immune-mediated phenomenon, anti-SARS-CoV-2 immune complexes could drive complement activation. However, Diorio et al. found no correlation between SARS-CoV-2 antibodies and sC5b-9 elevations (29). Moreover, in our case, histopathological analysis revealed evidence of TMA together with C3c deposits but without C4d or immunoglobulin deposits, which suggestive of alternative complement pathway activation. Lectin pathway triggering, though, cannot be excluded, since it can occur in a C4-bypass pathway and MASP-2 has been suggested to play a key role in the disease process of COVID-19 (33, 36, 37). In our patient, sC5b-9 levels were elevated and C5b-9 staining was positive in kidney biopsy, which is indicative of C5 cleavage by C5 convertase, as described in patients with COVID-19 (38). Likewise, Diorio et al. studied 50 hospitalised paediatric patients with acute SARS-CoV-2 infection (n=21 minimal COVID-19; n=11 severe COVID-19 and n=18 MIS-C) (29); 11 of 18 patients with MIS-C met clinical criteria for TMA. The median sC5b-9 was higher in the patients meeting TMA criteria and associated with AKI. None of the 18 patients needed RRT and no kidney biopsy was performed. Noteworthy, sC5b-9 was also elevated in patients with minimal COVID-19 disease. Eculizumab is a monoclonal anti-C5 antibody that blocks the formation of the membrane attack complex on the surface of endothelial cells and has revolutionised the prognosis of aHUS (39, 40). Small case series have suggested the potential benefits of eculizumab in COVID-19 (41–43). However, no randomized clinical trial has been published to date (32). In our patient, kidney function improved after eculizumab. However, fever, thrombocytopenia and troponin levels were already improving before using any complement blockade. Six months later and after only two courses of eculizumab, our patient's kidney function has normalised without albuminuria. Likewise, in the case series of MIS-C with TMA published by Diorio et al., all the children recovered (29). Therefore, we cannot exclude that improvement was due to the natural course of the disease rather than to eculizumab, as described in HUS caused by an infection from shiga toxin-producing Escherichia coli (STEC) (27). Complement blockers have never been tested in patients with MIS, except in a 14-year-old child with features of both acute COVID-19 infection and MIS-C, who developed TMA (44). In this case, a kidney biopsy could not be performed, but AKI resolved on eculizumab, as in our patient.

In conclusion, our case suggests that TMA could play a central role in the pathophysiology of post COVID-19 MIS, making complement blockers an interesting therapeutic option.

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

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

IB and FP wrote the manuscript. MR is the pathologist who made the diagnosis and took the photographs in **Figure 1**. KE-K, L-MC, LR, and MM participated in proofreading and collection of data. AM performed the biochemical analysis of the complement pathway. VP performed C5b-9 staining on the kidney biopsy. VF-B performed genetic analysis. FP is the corresponding author. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank all the team at the Melun Intensive Care Unit who were involved in the care of patients during the COVID-19 pandemic.

REFERENCES

1. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
2. Varga Z, Flammer AJ, Steiger P, Haberecker M, Andermatt R, Zinkernagel SA, et al. Endothelial Cell Infection and Endotheliitis in COVID-19. *Lancet* (2020) 395:1417–8. doi: 10.1016/S0140-6736(20)30937-5
3. Gupta A, Madhavan MV, Sehgal K, Nair N, Mahajan S, Sehrawat TS, et al. Extrapulmonary Manifestations of COVID-19. *Nat Med* (2020) 26:1017–32. doi: 10.1038/s41591-020-0968-3
4. Libby P, Lüscher T. Covid-19 Is, in the End, An Endothelial Disease. *Eur Heart J* (2020) 41:3038–44. doi: 10.1093/eurheartj/ehaa623
5. Huertas A, Montani D, Savale L, Pichon J, Tu L, Parent F, et al. Endothelial Cell Dysfunction: A Major Player in SARS-CoV-2 Infection (COVID-19)? *Eur Respir J* (2020) 56(1):2001634. doi: 10.1183/13993003.01634-2020
6. Basalely A, Gurusinghe S, Schneider J, Shah SS, Siegel LB, Pollack G, et al. Acute Kidney Injury in Pediatric Patients Hospitalized With Acute COVID-19 and Multisystem Inflammatory Syndrome in Children Associated With COVID-19. *Kidney Int* (2021)S0085–2538(21):00268–4. doi: 10.1016/j.kint.2021.02.026
7. Feldstein LR, Rose EB, Horwitz SM, Collins JP, Newhams MM, Son MBF, et al. Multisystem Inflammatory Syndrome in U.S. Children and Adolescents. *N Engl J Med* (2020) 383:334–46. doi: 10.1056/NEJMoa2021680
8. Chiotos K, Bassiri H, Behrens EM, Blatz AM, Chang J, Diorio C, et al. Multisystem Inflammatory Syndrome in Children During the Coronavirus

2019 Pandemic: A Case Series. *J Pediatr Infect Dis Soc* (2020) 9:393–8. doi: 10.1093/jpids/piaa069
9. Ahmed M, Advani S, Moreira A, Zoretic S, Martinez J, Chorath K, et al. Multisystem Inflammatory Syndrome in Children: A Systematic Review. *EClinicalMedicine* (2020) 26:100527. doi: 10.1016/j.eclinm.2020.100527
10. Shaigany S, Gnirke M, Guttmann A, Chong H, Meehan S, Raabe V, et al. An Adult With Kawasaki-Like Multisystem Inflammatory Syndrome Associated With COVID-19. *Lancet* (2020) 396(10246):e8–e10. doi: 10.1016/S0140-6736(20)31526-9
11. Sokolovsky S, Soni P, Hoffman T, Kahn P, Scheers-Masters J. Covid-19 Associated Kawasaki-Like Multisystem Inflammatory Disease in an Adult. *Am J Emerg Med* (2021) 39:253.e251–3.e252. doi: 10.1016/j.ajem.2020.06.053
12. Hékimian G, Kerneis M, Zeitouni M, Cohen-Aubart F, Chommeloux J, Bréchot N, et al. Coronavirus Disease 2019 Acute Myocarditis and Multisystem Inflammatory Syndrome in Adult Intensive and Cardiac Care Units. *Chest* (2021) 159:657–62. doi: 10.1016/j.chest.2020.08.2099
13. Chau VQ, Giustino G, Mahmood K, Oliveros E, Neibart E, Oloomi M, et al. Cardiogenic Shock and Hyperinflammatory Syndrome in Young Males With Covid-19. *Circ Heart Fail* (2020) 13:e007485. doi: 10.1161/CIRCHEARTFAILURE.120.007485
14. Fox SE, Lameira FS, Rinker EB, Vander Heide RS. Cardiac Endotheliitis and Multisystem Inflammatory Syndrome After Covid-19. *Ann Intern Med* (2020) 173:1025–7. doi: 10.7326/L20-0882
15. Newton-Cheh C, Zlotoff DA, Hung J, Rupasov A, Crowley JC, Funamoto M. Case 24-2020: A 44-Year-Old Woman With Chest Pain, Dyspnea, and Shock. *N Engl J Med* (2020) 383:475–84. doi: 10.1056/NEJMcpc2004975
16. Cheng Y, Luo R, Wang K, Wang Z, Dong L, Li J, et al. Kidney Disease Is Associated With In-Hospital Death of Patients With COVID-19. *Kidney Int* (2020) 97:829–38. doi: 10.1016/j.kint.2020.03.005
17. Batlle D, Soler MJ, Sparks MA, Hiremath S, South AM, Welling PA, et al. Acute Kidney Injury in COVID-19: Emerging Evidence of a Distinct Pathophysiology. *J Am Soc Nephrol* (2020) 31:1380–3. doi: 10.1681/ASN.2020040419
18. Su H, Yang M, Wan C, Yi LX, Tang F, Zhu HY, et al. Renal Histopathological Analysis of 26 Postmortem Findings of Patients With COVID-19 in China. *Kidney Int* (2020) 98(1):219–27. doi: 10.1016/j.kint.2020.04.003
19. Larsen CP, Bourne TD, Wilson JD, Saqqa O and Sharshir MA. Collapsing Glomerulopathy in a Patient With Coronavirus Disease 2019 (Covid-19). *Kidney Int Rep* (2020) 5(6):935–9. doi: 10.1016/j.ekir.2020.04.002
20. Ferlicot S, Jamme M, Gaillard F, Oniszczuk J, Couturier A, May O, et al. The Spectrum of Kidney Biopsies in Hospitalized Patients With COVID-19, Acute Kidney Injury, and/or Proteinuria. *Nephrol Dial Transplant* (2021) 12: gfab042. doi: 10.1093/ndt/gfab042
21. Kudose S, Batal I, Santoriello D, Xu K, Barasch J, Peleg Y, et al. Kidney Biopsy Findings in Patients With COVID-19. *J Am Soc Nephrol* (2020) 31:1959–68. doi: 10.1681/ASN.2020060802
22. Jhaveri KD, Meir LR, Flores Chang BS, Parikh R, Wanchoo R, Barilla-LaBarca ML, et al. Thrombotic Microangiopathy in a Patient With COVID-19. *Kidney Int* (2020) 98(2):509–12. doi: 10.1016/j.kint.2020.05.025
23. González-Dambrauskas S, Vásquez-Hoyos P, Camporesi A, Díaz-Rubio F, Piñeres-Olave BE, Fernández-Sarmiento J, et al. Pediatric Critical Care and COVID-19. *Pediatrics* (2020) 146(3):e20201766. doi: 10.1542/peds.2020-1766
24. Godfred-Cato S, Bryant B, Leung J, Oster ME, Conklin L, Abrams J, et al. Covid-19-Associated Multisystem Inflammatory Syndrome in Children - United States, March-July 2020. *MMWR Morbid Mortal Wkly Rep* (2020) 69:1074–80. doi: 10.15585/mmwr.mm6932e2
25. Whittaker E, Bamford A, Kenny J, Kaforou M, Jones CE, Shah P, et al. Clinical Characteristics of 58 Children With a Pediatric Inflammatory Multisystem Syndrome Temporally Associated With SARS-Cov-2. *JAMA* (2020) 324:259–69. doi: 10.1001/jama.2020.10369
26. George JN, Nester CM. Syndromes of Thrombotic Microangiopathy. *N Engl J Med* (2014) 371:654–66. doi: 10.1056/NEJMra1312353
27. Fakhouri F, Zuber J. Fremeaux-Bacchi V and Loirat C.Haemolytic Uraemic Syndrome. *Lancet (Lond Engl)* (2017) 390:681–96. doi: 10.1016/S0140-6736(17)30062-4
28. Sellier-Leclerc AL, Fremeaux-Bacchi V, Dragon-Durey MA, Macher MA, Niaudet P, Guest G, et al. Differential Impact of Complement Mutations on Clinical Characteristics in Atypical Hemolytic Uremic Syndrome. *J Am Soc Nephrol: JASN* (2007) 18:2392–400. doi: 10.1681/ASN.2006080811
29. Diorio C, McNerney KO, Lambert M, Paessler M, Anderson EM, Henrickson SE, et al. Evidence of Thrombotic Microangiopathy in Children With SARS-CoV-2 Across the Spectrum of Clinical Presentations. *Blood Adv* (2020) 4:6051–63. doi: 10.1182/bloodadvances.2020003471
30. Frimat M, Boudhabhay I, Roumenina LT. Hemolysis Derived Products Toxicity and Endothelium: Model of the Second Hit. *Toxins (Basel)* (2019) 11(11):660. doi: 10.3390/toxins11110660
31. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement Associated Microvascular Injury and Thrombosis in the Pathogenesis of Severe COVID-19 Infection: A Report of Five Cases. *Transl Res* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
32. Java A, Apicelli AJ, Liszewski MK, Coler-Reilly A, Atkinson JP, Kim AH, et al. The Complement System in COVID-19: Friend and Foe? *JCI Insight* (2020) 5 (15):e140711. doi: 10.1172/jci.insight.140711
33. Rambaldi A, Gritti G, Micò MC, Frigeni M, Borleri G, Salvi A, et al. Endothelial Injury and Thrombotic Microangiopathy in COVID-19: Treatment With the Lectin-Pathway Inhibitor Narsoplimab. *Immunobiology* (2020) 225:152001. doi: 10.1016/j.imbio.2020.152001
34. Gralinski LE, Sheahan TP, Morrison TE, Menachery VD, Jensen K, Leist SR, et al. Complement Activation Contributes to Severe Acute Respiratory Syndrome Coronavirus Pathogenesis. *mBio* (2018) 9(5):e01753–18. doi: 10.1128/mBio.01753-18
35. Pfister F, Vonbrunn E, Ries T, Jäck HM, Überla K, Lochnit G, et al. Complement Activation in Kidneys of Patients With Covid-19. *Front Immunol* (2020) 11:594849. doi: 10.3389/fimmu.2020.594849
36. Farrar CA, Tran D, Li K, Wu W, Peng Q, Schwaeble W, et al. Collectin-11 Detects Stress-Induced L-fucose Pattern to Trigger Renal Epithelial Injury. *J Clin Invest* (2016) 126:1911–25. doi: 10.1172/JCI83000
37. Boudhabhay I, Poillerat V, Grunenwald A, Torset C, Leon J, Daugan MV, et al. Complement Activation Is a Crucial Driver of Acute Kidney Injury in Rhabdomyolysis. *Kidney Int* (2021) 99:581–97. doi: 10.1016/j.kint.2020.09.033
38. Cugno M, Meroni PL, Gualtierotti R, Griffini S, Grovetti E, Torri A, et al. Complement Activation in Patients With COVID-19: A Novel Therapeutic Target. *J Allergy Clin Immunol* (2020) 146(1):215–7. doi: 10.1016/j.jaci.2020.05.006
39. Legendre CM, Licht C, Muus P, Greenbaum LA, Babu S, Bedrosian C, et al. Terminal Complement Inhibitor Eculizumab in Atypical Hemolytic-Uremic Syndrome. *N Engl J Med* (2013) 368:2169–81. doi: 10.1056/NEJMoa1208981
40. Zuber J, Frimat M, Caillard S, Kamar N, Gatault P, Petitprez F, et al. Use of Highly Individualized Complement Blockade Has Revolutionized Clinical Outcomes After Kidney Transplantation and Renal Epidemiology of Atypical Hemolytic Uremic Syndrome. *J Am Soc Nephrol* (2019) 30:2449–63. doi: 10.1681/ASN.2019040331
41. Diurno F, Numis FG, Porta G, Cirillo F, Maddaluno S, Ragozzino A, et al. Eculizumab Treatment in Patients With COVID-19: Preliminary Results From Real Life ASL Napoli 2 Nord Experience. *Eur Rev Med Pharmacol Sci* (2020) 24:4040–7. doi: 10.26355/eurrev_202004_20875
42. Peffault de Latour R, Bergeron A, Lengline E, Dupont T, Marchal A, Galicier L, et al. Complement C5 Inhibition in Patients With COVID-19 - A Promising Target? *Haematologica* (2020) 105:2847–50. doi: 10.3324/haematol.2020.260117
43. Annane D, Heming N, Grimaldi-Bensouda L, Frémeaux-Bacchi V, Vigan M, Roux AL, et al. Eculizumab as an Emergency Treatment for Adult Patients With Severe COVID-19 in the Intensive Care Unit: A Proof-of-Concept Study. *EClinicalMedicine* (2020) 28:100590. doi: 10.1016/j.eclinm.2020.100590
44. Mahajan R, Lipton M, Broglie L, Jain NG, Uy NS. Eculizumab Treatment for Renal Failure in a Pediatric Patient With COVID-19. *J Nephrol* (2020) 33:1373–6. doi: 10.1007/s40620-020-00858-2

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 05 July 2021
doi: 10.3389/fimmu.2021.714511

Lectin Pathway Mediates Complement Activation by SARS-CoV-2 Proteins

Youssif M. Ali[1,2], Matteo Ferrari[1], Nicholas J. Lynch[1], Sadam Yaseen[3], Thomas Dudler[3], Sasha Gragerov[3], Gregory Demopulos[3], Jonathan L. Heeney[1] and Wilhelm J. Schwaeble[1]*

[1] Department of Veterinary Medicine, School of Biological Sciences, University of Cambridge, Cambridge, United Kingdom, [2] Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt, [3] Omeros Corporation, Seattle, WA, United States

OPEN ACCESS

Edited by:
Zoltan Prohaszka, Semmelweis University, Hungary

Reviewed by:
Maciej Cedzynski, Institute for Medical Biology (PAN), Poland
Péter Gál, Hungarian Academy of Sciences (MTA), Hungary

***Correspondence:**
Youssif M. Ali
myima2@cam.ac.uk

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *25 May 2021*
Accepted: *18 June 2021*
Published: *05 July 2021*

Citation:
Ali YM, Ferrari M, Lynch NJ, Yaseen S, Dudler T, Gragerov S, Demopulos G, Heeney JL and Schwaeble WJ (2021) Lectin Pathway Mediates Complement Activation by SARS-CoV-2 Proteins. Front. Immunol. 12:714511. doi: 10.3389/fimmu.2021.714511

Early and persistent activation of complement is considered to play a key role in the pathogenesis of COVID-19. Complement activation products orchestrate a proinflammatory environment that might be critical for the induction and maintenance of a severe inflammatory response to SARS-CoV-2 by recruiting cells of the cellular immune system to the sites of infection and shifting their state of activation towards an inflammatory phenotype. It precedes pathophysiological milestone events like the cytokine storm, progressive endothelial injury triggering microangiopathy, and further complement activation, and causes an acute respiratory distress syndrome (ARDS). To date, the application of antiviral drugs and corticosteroids have shown efficacy in the early stages of SARS-CoV-2 infection, but failed to ameliorate disease severity in patients who progressed to severe COVID-19 pathology. This report demonstrates that lectin pathway (LP) recognition molecules of the complement system, such as MBL, FCN-2 and CL-11, bind to SARS-CoV-2 S- and N-proteins, with subsequent activation of LP-mediated C3b and C4b deposition. In addition, our results confirm and underline that the N-protein of SARS-CoV-2 binds directly to the LP- effector enzyme MASP-2 and activates complement. Inhibition of the LP using an inhibitory monoclonal antibody against MASP-2 effectively blocks LP-mediated complement activation. FACS analyses using transfected HEK-293 cells expressing SARS-CoV-2 S protein confirm a robust LP-dependent C3b deposition on the cell surface which is inhibited by the MASP-2 inhibitory antibody. In light of our present results, and the encouraging performance of our clinical candidate MASP-2 inhibitor Narsoplimab in recently published clinical trials, we suggest that the targeting of MASP-2 provides an unsurpassed window of therapeutic efficacy for the treatment of severe COVID-19.

Keywords: complement system, lectin pathway, SARS-CoV-2, COVID-19, innate immunity

INTRODUCTION

Coronaviruses (CoVs) are single-stranded RNA viruses causing life threatening respiratory infection in humans and other species. The CoV genome encodes four main structural proteins, spike (S), membrane (M), envelope (E), and nucleocapsid (N), as well as other accessory proteins that facilitate replication and entry into cells. The transmembrane bound spike protein (S), consists of two subunits S1 and S2 that cover the surface of CoVs and serve as receptor binding entry proteins for infection. The nucleocapsid protein complexes with the viral RNA and plays a major role in viral replication as well as viral pathogenesis. M and E are two transmembrane proteins, which are responsible for viral assembly (1). In 2019, a pandemic respiratory infection caused by corona virus was reported and identified as coronavirus disease 2019 (COVID-19), the etiological agent of which is a β-coronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The clinical manifestation of SARS-CoV-2 infection include fever, cough, fatigue, myalgia, and pneumonia, that may develop into acute respiratory distress syndrome (ARDS), necessitating respiratory support, as well as disseminated intravascular coagulopathy and kidney failure (2, 3).

The complement system (CS) is an integral part of the innate and the adaptive immune systems. The CS is composed of more than 30 plasma and cell-resident components that form a first defence-line against infection and provides an essential scavenger system to eliminate injured, apoptotic or aberrant cells. Complement activation products modulate inflammation and direct the innate and the adaptive immune response. The CS is activated *via* three pathways, which funnel into a shared terminal activation route. The classical pathway (CP) is initiated through the binding of a recognition subcomponent, specifically complement component 1q (C1q). Two C1q-associated serine protease zymogens, C1r and C1s, form a C1s-C1r-C1r-C1s hetero-tetramer, which sits within the calix of the C1q macromolecule. The C1r/C1s zymogen complex is converted into active form when at least two arms of the C1q macromolecule bind to the Fc region of immune complexes. Activation of C1s leads to the cleavage of C4 to C4a and C4b, with the latter binding to C2. C4b-bound C2 is then cleaved by C1s to create the C3 convertase C4b2a, which cleaves the abundant complement component C3 into the anaphylatoxin C3a and the major fragment C3b (4). The Lectin pathway (LP) is initiated by multimolecular pattern-recognition complexes that bind to immune complexes and pathogen-associated molecular patterns (PAMPs). Six different LP recognition subcomponents can form LP activation complexes by binding dimers of three different mannan-binding lectin-associated serine proteases (i.e., MASP-1, MASP-2 and MASP-3). The recognition subcomponents comprise multimers of homotrimeric chains, which can bind directly to their cognate ligands present on pathogens, or to aberrant glycosylation patterns on apoptotic, necrotic, malignant, or damaged host cells (5, 6). The LP recognition subcomponents in humans are: mannan-binding lectin-2 (MBL-2), collectin-11 (CL-11), heterocomplexes of CL-11 and CL-10 and three different ficolins (ficolins 1, 2, and 3), two of which can also form heterocomplexes (ficolins 2 and 3). MASP-2 is the key enzyme of the LP; only MASP-2 can cleave C4 efficiently, whereas both MASP-2 and MASP-1 can cleave C2. In the absence of MASP-2, complement can no longer be activated by the LP activation route because the C3 and C5 convertase complexes C4b2a and C4b2a (C3b)n cannot be formed (7, 8). In addition, MASP-2 was shown to cleave C3 directly, forming a novel C4-bypass activation route. This C4-bypass route was shown to be important in the innate immune defence (9). The alternative pathway (AP) fulfils its surveillance function through a constant low-rate activation (C3 tick-over) and provides an efficient amplification loop of C3 activation. The C3 activation product C3b can bind to zymogen complement factor B (FB), forming a complex that can in turn convert more C3 into C3a and C3b if C3b-bound FB is cleaved by a serine protease called FD (10).

Complement activation was reported to be associated with development of acute respiratory distress syndrome (ARDS) and respiratory failure during viral pneumonia (11, 12). A direct link between complement activation and pathogenesis of Corona virus infection was established using C3-deficient mice infected with SARS-CoV. C3-deficient mice showed significantly less severe respiratory inflammation, decreased infiltration of neutrophils and inflammatory monocytes, and lower levels of cytokines and chemokines in both the lungs and sera compared to wild-type control mice (13). The involvement of complement-mediated pathology and lung injury during SARS-CoV-2 infection was revealed by a histopathological study of post-mortem biopsies taken from COVID-19 patients. The presence of thrombotic microangiopathies (TMAs) and the deposition of complement activation products, including C5b-9, C3d, C4d and the LP effector enzyme MASP-2 implied the involvement of LP and CP activation in severe COVID-19 (14). In this study, we address the involvement of the lectin activation pathway of complement in the response against recombinant SARS-CoV-2 proteins and which can trigger activation of the complement system.

MATERIALS AND REAGENTS

Recombinant S and N proteins of SARS-CoV-2 expressed in mammalian cell lines were purchased from R & D systems, UK. The pEVAC plasmids expressing the coding sequences for S protein were kindly provided by DIOSynVax Ltd Cambridge, UK. Recombinant truncated MASP-2, containing the 2 CCP domains and the serine protease domain, was expressed previously described (9).

HG4, a monospecific fully humanised antibody against MASP-2 that inhibits LP-mediated C4 cleavage was kindly provided by Omeros Corporation, Seattle, USA. Pre-pandemic, non-immune NHS, was pooled from 4 healthy donors. The mean levels of key lectin pathway components in the pool were: MBL, 1.43μg/ml; FCN2, 2.9μg/ml; CL-11, 0.39μg/ml; and MASP-2, 0.4μg/ml.

Transfection of HEK 293T Cells

HEK 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal bovine serum albumin (FBS), 2 mM glutamine and 10 U/mL penicillin, 10 µg/mL streptomycin (Gibco). Cells were maintained in a CO_2 incubator at 37°C. HEK 293T cells were seeded in 6-well plates with cell density of 1×10^6 cells/mL. Next day, cells were transfected with 1 µg of plasmid DNA for each well using the Fugene transfection kit (Promega) according to the manufacturer's protocol. Cells transfected with empty pEVAC vector were used as a control. 48 hours after transfection, cells were harvested for flow cytometer analysis.

FACS Analysis

Transfected HEK 293T cells were washed twice using Hank's balanced salt solution with C^{2+} and Mg^{2+} ($HBSS^{++}$) and resuspended in $HBSS^{++}$ to a final concentration of 10^7 cell/mL. 10^6 cells were opsonised with 2.5% NHS in $HBSS^{++}$ for 30 minutes at 37°C with or without 100 nM of HG4. Cells transfected with empty vector were used as a negative control. After opsonization, cells were washed twice with $HBSS^{++}$ buffer, and bound C3b was detected using FITC-conjugated rabbit anti-human C3c (Dako). Fluorescence intensity was measured with The Attune NxT Flow Cytometer (Invitrogen).

Solid Phase Binding Assays

Nunc MaxiSorp microtiter ELISA plates were coated with 10 µg/mL of purified recombinant SARS-CoV-2 proteins S and N in coating buffer (10 mM Tris-HCl, 140 mM NaCl, pH 7.4). Control wells were coated with 10 µg/mL mannan (a control for MBL binding), 10 µg/mL zymosan (a control for CL-11 binding) or 10 µg/mL N-acetylated BSA (a control for L-ficolin binding). Immune complexes formed by incubation of BSA with rabbit anti-BSA were prepared. The ELISA plates were coated with 1 µg/mL BSA-anti-BSA immune complex as a control ligand for C1q binding. The following day, wells were blocked for 2 hours at room temperature with 250 µL of 1% (w/v) BSA in TBS buffer (10 mM Tris-HCl, 140 mM NaCl, pH 7.4), then washed three times with 250 µL of TBS with 0.05% Tween 20 and 5 mM $CaCl_2$ (wash buffer). Serial dilutions of serum in 100 µL of wash buffer were added to the wells and the plates were then incubated for 90 minutes at room temperature. Plates were washed as above and bound proteins were detected using rabbit anti-human L-ficolin, mouse anti-human CL-11 or mouse anti-human MBL mAbs. HRP conjugated goat anti-rabbit IgG followed by the colorimetric substrate (15).

Complement Deposition Assays

To measure C3 and C4 activation, Nunc MaxiSorp microtiter plates were coated with 100 µL of 10 µg/mL mannan (Promega), or 100 µL of 10 µg/mL SARS-CoV-2 proteins in coating buffer. After overnight incubation, wells were blocked with 1% BSA in TBS then washed with wash buffer. Serum samples were diluted in BBS (4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4), starting at 5%, then added to the plates and incubated for 1.5 hours at 37°C. The plates were washed again, and bound C3b or C4b were detected using rabbit anti-human C3c (Dako) or rabbit anti-human C4c (Dako) followed by HRP conjugated goat anti-rabbit IgG followed by the colorimetric substrate TMB (15).

Complement Inhibition Assay

The activity of HG4 against MASP-2 was tested using C4b deposition assay. 2.5% NHS containing different concentrations of Hg4 in BBS were added to an ELISA plate coated with mannan as previously described. Control wells received no antibodies. The plate was incubated at 37°C for 15 min, then washed. Bound C4b were detected using rabbit anti human C4c (Dako, Denmark) followed by an HRP conjugated goat anti- rabbit IgG (Sigma, USA). Bound antibody was detected using the Colorimetric substrate TMB.

MASP-2 Binding Assay With SARS-CoV-2 Proteins

An ELISA plate was coated with 100 µL of 10 µg/mL SARS-CoV-2 proteins in coating buffer. Wells were blocked with 1% BSA in TBS then washed with wash buffer. Wells coated with BSA only were used as a negative control. Serial concentrations of recombinant MASP-2 in BBS, starting from 1µg/mL, were added to the plate and incubated at room temperature. After 1 hour, the plate was washed and MASP-2 binding to SARS-CoV-2 proteins was detected using monoclonal antibodies against MASP-2 followed by HRP-conjugated rabbit anti-human IgG and the chromogenic substrate ELISA Colorimetric TMB Reagent (Sigma). In a parallel experiment, 1µg of rMASP-2 in 100 µL BBS was incubated with wells coated with SARS-CoV-2 proteins for 1 hour at 37°C. After three washing steps using wash buffer, 2.5 µg of purified C4 (Comptech, USA) in 100 µL BBS were added to each well. Purified C4 added to wells coated with BSA was used as a negative control. After 1-hour incubation at 37°C, supernatants were collected from each well and boiled with 4X SDS loading dye. C4 cleavage mediated *via* MASP-2 was detected using SDS-PAGE and Coomassie bule staining under reducing conditions.

RESULTS

Recognition Molecules of LP Bind to SARS-CoV-2 Proteins

A series of solid-phase binding ELISA were performed to identify LP recognition molecules present in NHS that bind SARS-CoV-2 proteins. MBL, FCN2 and CL-11 bind to S and N proteins, indicating possible activation of the complement system *via* the LP. Interestingly, no C1q binding with SARS-CoV-2 proteins was observed using non-immune NHS, suggesting that the classical pathway is not activated in the absence of specific antibody (**Figure 1**).

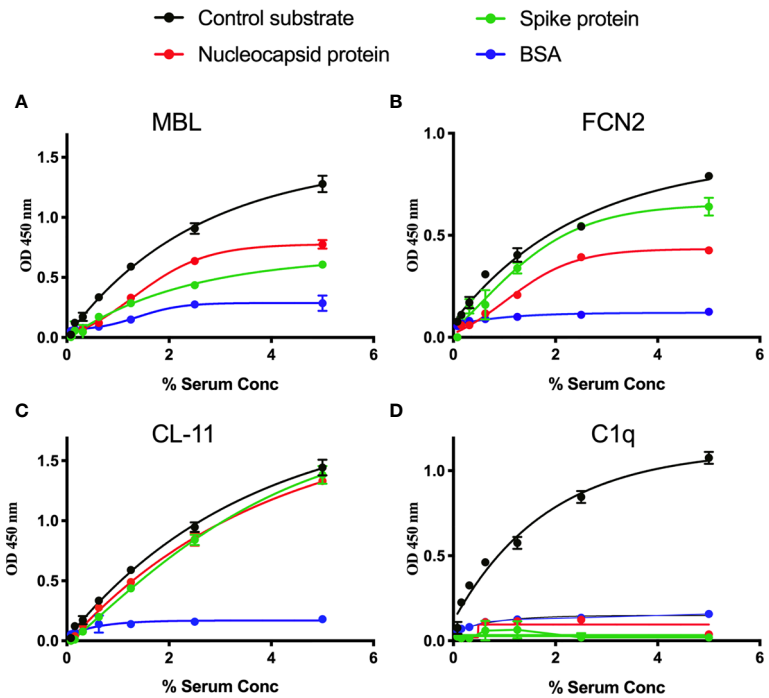


FIGURE 1 | Binding of LP recognition molecules and C1q to SARS-CoV-2 proteins. A microtitre ELISA plate was coated with either S, N or control ligands (mannan for MBL, N-acetyl BSA for FCN2, zymosan for CL-11 or immune complexes for C1q). Following incubation with blocking buffer and washing steps, serial dilutions of NHS, starting at 5% were added to detect binding of LP recognition molecules and C1q from NHS to SARS-CoV-2 proteins. Human MBL **(A)**, FCN2 **(B)**, Cl-11 **(C)** and C1q **(D)** were assayed by ELISA. A significant binding of LP recognition molecules to S and N proteins was clearly observed. No C1q binding to any of the viral proteins was detected. Results are means of duplicates ± SD.

The Lectin Pathway Drives Complement Deposition on SARS-CoV-2

We measured complement C3b and C4b deposition on SARS-CoV-2 proteins immobilised on microtiter plates. When serial dilutions of pre-pandemic NHS were incubated on the plates, there was a dose-dependent and saturable deposition of C3b and C4b, indicative of LP activation, and comparable with the control substrates. Essentially no C3b or C4b deposition was detected on wells that were just blocked with BSA ($p<0.01$, 2-way ANOVA vs. the control) (**Figures 2A, B**).

HG4 Inhibits LP Mediated C4b Deposition

The ability of the fully humanised monoclonal antibody HG4 to inhibit LP functional activity was assessed using a C4b deposition

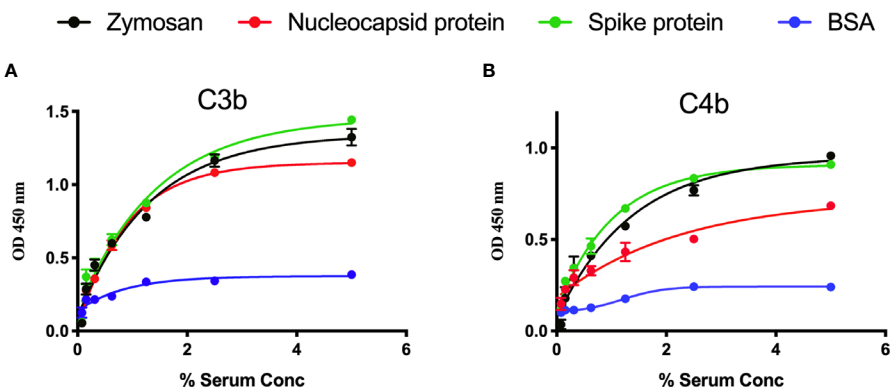


FIGURE 2 | Detection of C3b and C4b deposition on SARS-CoV-2 proteins. An ELISA plate was coated with SARS-CoV-2 proteins (S or N) or zymosan and incubated with serial dilutions of NHS (starting at 5%) for 1 h at 37°C. C3b or C4b deposition were detected using antibodies against human C3c or C4c. High levels of C3b **(A)** and C4b **(B)** deposition were observed on surface immobilised SARS-CoV-2 proteins.

inhibition assay. Our results showing that HG4 significantly inhibits LP functional activity with an IC50 around 0.74 nM (**Figure 3**).

MASP-2 Binds Directly to N Protein and Promotes MASP-2-Mediated C4 Cleavage

The ability of MASP-2 to bind directly to SARS-CoV-2 N protein was tested using ELISA. A significant binding of rMASP-2 to N protein was detected. To further confirm the functional significance of this finding, ELISA wells coated with N-protein or BSA were incubated with 1µg of rMASP-2 for 1 hour at 37°C. After washing, 2.5 µg of purified C4 in BBS was then added to the wells and incubated at 37°C. After 1 hour, the supernatant was removed from the wells and the degree of C4 cleavage was analysed using SDS-PAGE. C4 cleavage was observed when purified C4 was incubated with N protein and rMASP-2 but not with BSA and rMASP-2. This experiment clearly showed that MASP-2 binds to N protein and promotes LP-mediated C4 cleavage. Interestingly, inhibition of MASP-2 activity using HG4 completely blocks LP-mediated C4 cleavage (**Figure 4**).

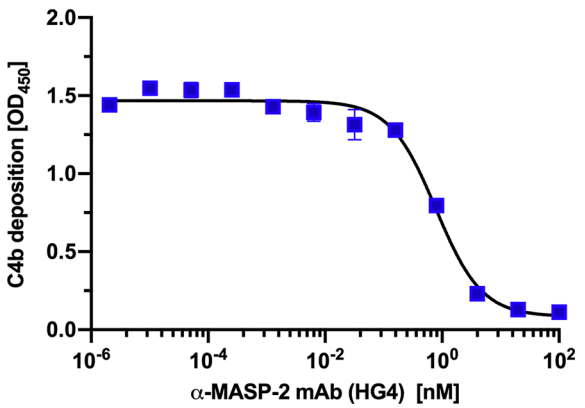


FIGURE 3 | HG4 inhibits lectin pathway mediated C4b deposition. A microtiter ELISA plate was coated with mannan and blocked with BSA. Different concentrations of HG4 antibody were mixed with 2% NHS and incubated on the plate. Bound C4b was detected using anti C4c antibodies.

Evaluation of HEK 293 T Cells Expressing SARS-CoV-2 Surface Proteins as a Model to Detect COVID-19-Related Complement Activation

To analyse complement activation on SARS-CoV-2 S protein using a model that mimics the natural surface expression in cells infected with SARS-CoV-2, we employed transiently transfected HEK 293T cells. In this model, a transient high level of expression of S protein on the surface of HEK 293T cells was achieved after transfection with the mammalian expression vector pEVAC containing the coding sequences for SARS-CoV-2 S protein. Cells expressing viral protein were incubated with NHS or pooled serum from convalescent patients, followed by detection of bound human antibodies by incubation with goat anti-human IgG Alexa fluor 647 antibodies. Fluorescence intensity was measured with The Attune NxT Flow Cytometer (Invitrogen). The level of anti-S protein antibody bound to HEK 293T cells was approximately 100 fold higher when using convalescent human serum compared to non-immune NHS (**Figure 5**). In a parallel experiment, C1q binding to HEK293 cells expressing S protein was not observed when using NHS (data not shown).

Inhibition of LP Impairs Complement C3b Deposition on SARS-CoV-2 Proteins

To evaluate complement C3b deposition on the surface of HEK 293T cells expressing S protein, cells were incubated with 2.5% NHS, and complement C3b deposition was detected using FACS analysis. A significantly higher level of complement C3b deposition was detected on cells expressing S protein compared to non-transfected cells. Inhibition of lectin pathway using HG4

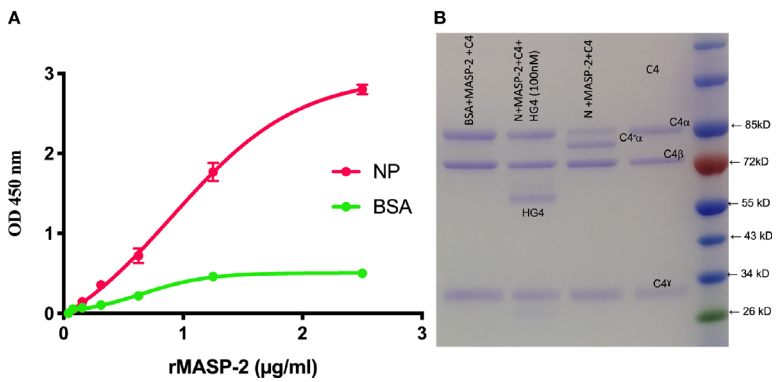


FIGURE 4 | MASP-2 binds directly to SARS-Cov-2 N-protein and mediates complement C4 activation. **(A)** Microtiter plates were coated with 2.5µg/well N-protein or BSA as a control. Residual binding sites were blocked using 1% BSA. Serial dilutions of rMASP-2 were added, and binding was detected using an anti-MASP-2 mAb. MASP-2 bound to the NP protein in a concentration-dependent and saturable manner **(A)**. **(B)** In a parallel experiment, 1µg of rMASP-2 in barbital buffered saline (BBS) was added to wells coated with NP or BSA. After 1 hr at 37°C, wells were washed and 1µg of purified human C4 was added to each well. After 1 hr incubation at 37°C, the supernatant was collected and separated on SDS-PAGE. The results showed that rMASP-2 binds directly to NP and cleaves C4. Addition of HG4 (a mAb that inhibits MASP-2) inhibited MASP-2 mediated C4 cleavage. Purified C4 was run on the gel as a control.

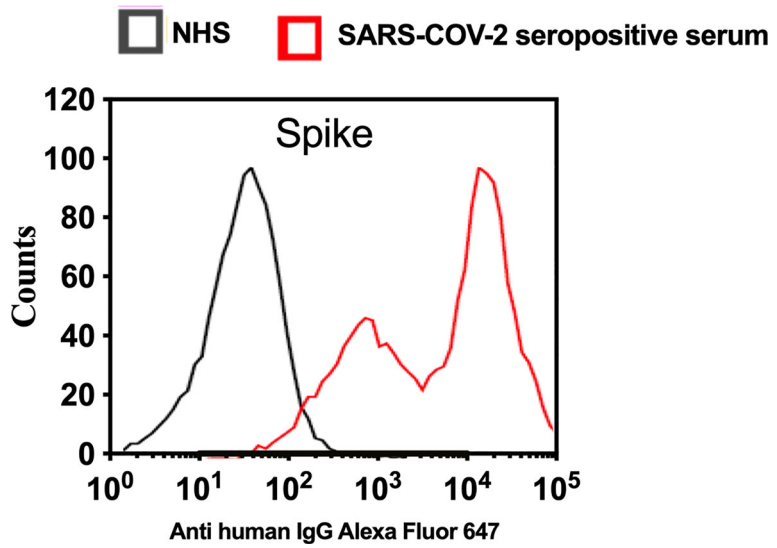


FIGURE 5 | Transfection of HEK 293T promotes high levels of expression of SARS-CoV-2 surface proteins. The expression levels of S protein on the surface of HEK 293 T cells were measured using serum from convalescent SARS-CoV-2 PCR positive patients or NHS (PCR negative) followed by anti-human IgG Alexa fluor 647 antibodies. Significant high levels of IgG binds to S were observed on the surface of HEK T293 cells.

significantly decreased complement C3b deposition from NHS (**Figure 6**).

DISCUSSION

SARS-CoV-2 is an emerging virus with a very high infectivity that causes life-threatening complications with mild to severe long-term morbidity and mortality, especially in patients with underlying medical conditions (16). The immunopathology differentiating mild from severe disease is not as yet sufficiently well understood to identify the windows of therapeutic opportunities for treatment (13, 17, 18). Excessive activation of complement, initiated in part by viral invasion of endothelial cells, causes collateral tissue injury (12). This work demonstrates that the LP recognition molecules MBL, FCN-2 and CL-11 bind to S

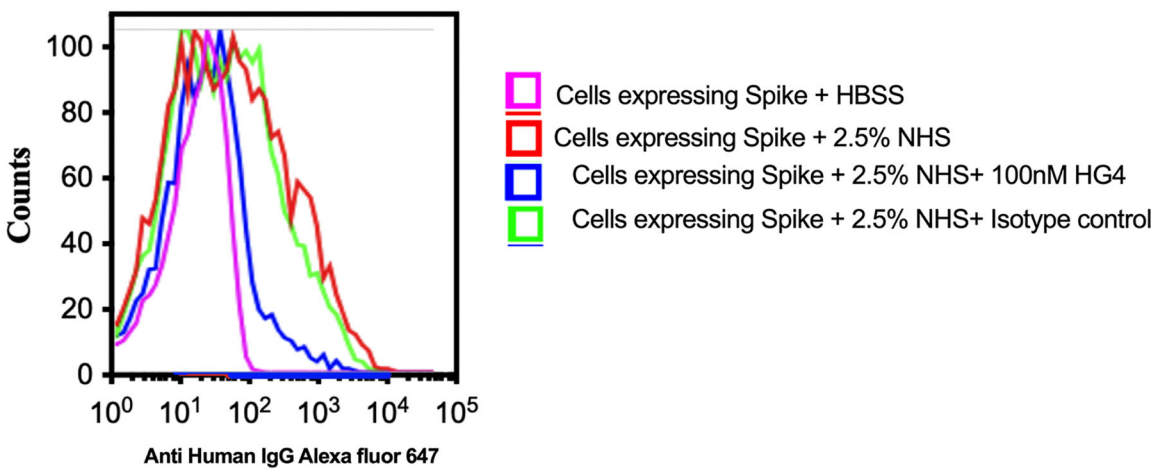


FIGURE 6 | Inhibition of LP impairs complement C3b deposition on SARS-CoV-2 surface proteins. HEK 293T cells expressing SARS-CoV-2 S protein were used. Cells were incubated with 2.5% NHS with 100nM HG4 or an isotype control antibody at 37C for 30 min. Cells were washed and C3b deposition was detected using rabbit anti human C3b followed by goat anti rabbit FITC labelled antibodies. A significant C3b deposition was detected on HEK 293T cells expressing S protein. Inhibition of MASP-2 using HG4 significantly impairs complement C3b deposition on the surface of HEK 293T cells expressing S protein.

and N proteins of SARS-CoV-2 with subsequent activation of LP-mediated C3b and C4b deposition. These findings clearly show the activation of the LP on SARS-CoV-2 surface proteins and N protein, confirming the central role of LP activation in the immunopathogenesis of COVID-19. Tissue damage consistent with complement-mediated microvascular injury has been observed in the lung and/or skin of patients with severe COVID-19, with significant deposition of the LP effector enzyme MASP-2, a hallmark of profound activation of the LP (14). Furthermore, extensive deposition of MASP-2 in the capillaries and venules of small bowel thrombotic microvascular injury in COVID-19 has also been reported, and endothelial complement staining patterns colocalized with staining of SARS-CoV-2 membrane and spike proteins (19). Our work also shows direct binding of MASP-2 to the N protein of SARS-CoV-2 with subsequent LP-mediated C4 cleavage into C4b and C4a, confirming the previous finding of Gao et al., who reported direct activation of MASP-2 on the SARS-CoV-2 N protein and showed that MASP-2-deficient mice are protected from disease (20). Since we used a truncated zymogen form of MASP-2, containing the CCP1, CCP2 and serine protease domains, our results also narrow down the N-protein binding site to these C terminal domains. Our *in vitro* study confirms that inhibition of MASP-2 blocks complement activation *via* the LP. Interestingly, Narsoplimab, a fully humanized immunoglobulin gamma 4 (IgG4) monoclonal antibody against MASP-2 that inhibits LP functional activity, has been used successfully in treatment of critically ill, mechanical ventilation-dependent COVID-19 patients. Patients who received Narsoplimab recovered and survived, demonstrating corresponding improvement/normalization of laboratory markers of inflammation (21). Many other complement inhibitors and anti-inflammatory drugs have been re-purposed and evaluated in COVID-19 clinical trials but, to date, none of those other agents have yielded a breakthrough in the treatment of severe COVID-19 (22–27). While vaccination is reducing hospitalisation, there is a fear of possible reduction in the efficacy against the new variants, which are responsible for severe cases of COVID-19 in younger age groups. Therapeutic approaches utilizing passive immunity (e.g., convalescent plasma, mono- and polyclonal antibodies), have also been disappointing, demonstrating no meaningful efficacy in severe COVID-19 and, possibly, adding to selection pressure on SARS-CoV-2. There remains a need to pursue aggressively and apply therapeutic strategies that block the COVID-19 immunopathological events and endothelial pathology, all of which appear to remain consistent across viral variants, with the objective of increasing access to any effective treatment(s) for acute as well as long-term post-COVID pathology. As an alternative to tackling the virus itself, protection from emerging new mutant viral strains could be achieved by tackling the immune physiological events that turn SARS-CoV-2 infections into generalised endothelial disease and ARDS in those susceptible to moderate-to-severe COVID-19. More research is needed to fully understand the disease processes triggered by SARS-CoV-2 at a molecular level.

The present study provides new insights into the direct triggers by SARS-CoV-2 at the protein level of LP activation in the early phase of COVID-19, and the role of the LP in types of long-haul COVID-19 are currently under 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 author.

AUTHOR CONTRIBUTIONS

YA and MF designed and performed the experiments. YA, MF, NL, JH, and WS wrote and revised the manuscript. SY, TD, SG, and GD provided essential reagents and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by National Institute for Health Research Grant G107217, awarded to WS.

REFERENCES

1. Astuti I, Ysrafil. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2): An Overview of Viral Structure and Host Response. *Diabetes Metab Syndr* (2020) 14:407–12. doi: 10.1016/j.dsx.2020.04.020
2. Elharrar X, Tr Y, Dols AM, Touchon F, Martinez S, Prud'homme E, et al. Use of Prone Positioning in Nonintubated Patients With COVID-19 and Hypoxemic Acute Respiratory Failure. *JAMA* (2020) 323:2336–8. doi: 10.1001/jama.2020.8255
3. Hirsch JS, Ng JH, Ross DW, Sharma P, Shah HH, Barnett RL, et al. Acute Kidney Injury in Patients Hospitalized With COVID-19. *Kidney Int* (2020) 98:209–18. doi: 10.1016/j.kint.2020.05.006
4. Almitairi JOM, Venkatraman Girija U, Furze CM, Simpson-Gray X, Badakshi F, Marshall JE, et al. Structure of the C1r-C1s Interaction of the C1 Complex of Complement Activation. *Proc Natl Acad Sci USA* (2018) 115:768–73. doi: 10.1073/pnas.1718709115
5. Schwaeble W, Dahl MR, Thiel S, Stover C, Jensenius JC. The Mannan-Binding Lectin-Associated Serine Proteases (MASPs) and MAp19: Four Components of the Lectin Pathway Activation Complex Encoded by Two Genes. *Immunobiology* (2002) 205:455–66. doi: 10.1078/0171-2985-00146
6. Fujita T. Evolution of the Lectin-Complement Pathway and its Role in Innate Immunity. *Nat Rev Immunol* (2002) 2:346–53. doi: 10.1038/nri800
7. Iwaki D, Fujita T. Production and Purification of Recombinants of Mouse MASP-2 and sMAP. *J Endotoxin Res* (2005) 11:47–50. doi: 10.1179/096805105225006704
8. Skjoedt MO, Hummelshoj T, Palarasah Y, Honore C, Koch C, Skjodt K, et al. A Novel Mannose-Binding Lectin/Ficolin-Associated Protein is Highly Expressed in Heart and Skeletal Muscle Tissues and Inhibits Complement Activation. *J Biol Chem* (2010) 285:8234–43. doi: 10.1074/jbc.M109.065805

9. Yaseen S, Demopulos G, Dudler T, Yabuki M, Wood CL, Cummings WJ, et al. Lectin Pathway Effector Enzyme Mannan-Binding Lectin-Associated Serine Protease-2 can Activate Native Complement C3 in Absence of C4 and/or C2. *FASEB J* (2017) 31:2210–9. doi: 10.1096/fj.201601306R
10. Lachmann PJ. The Amplification Loop of the Complement Pathways. *Adv Immunol* (2009) 104:115–49. doi: 10.1016/S0065-2776(08)04004-2
11. de Nooijer AH, Grondman I, Janssen NAF, Netea MG, Willems L, van de Veerdonk FL, et al. Complement Activation in the Disease Course of Coronavirus Disease 2019 and Its Effects on Clinical Outcomes. *J Infect Dis* (2021) 223:214–24. doi: 10.1093/infdis/jiaa646
12. Holter JC, Pischke SE, de Boer E, Lind A, Jenum S, Holten AR, et al. Systemic Complement Activation is Associated With Respiratory Failure in COVID-19 Hospitalized Patients. *Proc Natl Acad Sci USA* (2020) 117:25018–25. doi: 10.1073/pnas.2010540117
13. Gralinski LE, Sheahan TP, Morrison TE, Menachery VD, Jensen K, Leist SR, et al. Complement Activation Contributes to Severe Acute Respiratory Syndrome Coronavirus Pathogenesis. *mBio* (2018) 9:e01753–18. doi: 10.1128/mBio.01753-18
14. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement Associated Microvascular Injury and Thrombosis in the Pathogenesis of Severe COVID-19 Infection: A Report of Five Cases. *Transl Res* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
15. Ali YM, Sim RB, Schwaeble W, Shaaban MI. Enterococcus Faecalis Escapes Complement-Mediated Killing *via* Recruitment of Complement Factor H. *J Infect Dis* (2019) 220:1061–70. doi: 10.1016/j.immuni.2020.05.002
16. Vabret N, Britton GJ, Gruber C, Hegde S, Kim J, Kuksin M, et al. Immunology of COVID-19: Current State of the Science. *Immunity* (2020) 52:910–41. doi: S1074-7613(20)30183-7
17. Java A, Apicelli AJ, Liszewski MK, Coler-Reilly A, Atkinson JP, Kim AH, et al. The Complement System in COVID-19: Friend and Foe? *JCI Insight* (2020) 5:10.1172/jci.insight.140711. doi: 10.1172/jci.insight.140711
18. Risitano AM, Mastellos DC, Huber-Lang M, Yancopoulou D, Garlanda C, Ciceri F, et al. Complement as a Target in COVID-19? *Nat Rev Immunol* (2020) 20:343–4. doi: 10.1038/s41577-020-0320-7
19. Plotz B, Castillo R, Melamed J, Magro C, Rosenthal P, Belmont HM. Focal Small Bowel Thrombotic Microvascular Injury in COVID-19 Mediated by the Lectin Complement Pathway Masquerading as Lupus Enteritis. *Rheumatol (Oxford)* (2021) 60:e61–3. doi: 10.1093/rheumatology/keaa627
20. Ting G, Mingdong H, Xiaopeng Z, Hongzhen L, Lin Z, Hainan L, et al. Highly Pathogenic Coronavirus N Protein Aggravates Lung Injury by MASP-2-Mediated Complement Over-Activation. *medRxiv* [Preprint] (2020). doi: 10.1101/2020.03.29.20041962
21. Rambaldi A, Gritti G, Mico MC, Frigeni M, Borleri G, Salvi A, et al. Endothelial Injury and Thrombotic Microangiopathy in COVID-19: Treatment With the Lectin-Pathway Inhibitor Narsoplimab. *Immunobiology* (2020) 225:152001. doi: 10.1016/j.imbio.2020.152001
22. Della-Torre E, Della-Torre F, Kusanovic M, Scotti R, Ramirez GA, Dagna L, et al. Treating COVID-19 With Colchicine in Community Healthcare Setting. *Clin Immunol* (2020) 217:108490. doi: 10.1016/j.clim.2020.108490
23. Diurno F, Numis FG, Porta G, Cirillo F, Maddaluno S, Ragozzino A, et al. Eculizumab Treatment in Patients With COVID-19: Preliminary Results From Real Life ASL Napoli 2 Nord Experience. *Eur Rev Med Pharmacol Sci* (2020) 24:4040–7. doi: 10.26355/eurrev_202004_20875
24. Ho TC, Wang YH, Chen YL, Tsai WC, Lee CH, Chuang KP, et al. Chloroquine and Hydroxychloroquine: Efficacy in the Treatment of the COVID-19. *Pathogens* (2021) 10:217. doi: 10.3390/pathogens10020217
25. Jean SS, Hsueh PR. Old and Re-Purposed Drugs for the Treatment of COVID-19. *Expert Rev Anti Infect Ther* (2020) 18:843–7. doi: 10.1080/14787210.2020.1771181
26. Mastaglio S, Ruggeri A, Risitano AM, Angelillo P, Yancopoulou D, Mastellos DC, et al. The First Case of COVID-19 Treated With the Complement C3 Inhibitor AMY-101. *Clin Immunol* (2020) 215:108450. doi: 10.1016/j.clim.2020.108450
27. Mastellos DC, Pires da Silva BGP, Fonseca BAL, Fonseca NP, Auxiliadora-Martins M, Mastaglio S, et al. Complement C3 vs C5 Inhibition in Severe COVID-19: Early Clinical Findings Reveal Differential Biological Efficacy. *Clin Immunol* (2020) 220:108598. doi: 10.1016/j.clim.2020.108598

Conflict of Interest: WS, NL, and YA are consultants to Omeros Inc., which is developing inhibitors of the lectin pathway. GD, TD, SG, and SY are employed by Omeros Inc.

The remaining 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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 18 August 2021
doi: 10.3389/fimmu.2021.716361

Thromboinflammation Supports Complement Activation in Cancer Patients With COVID-19

Ellinor I. Peerschke, Alisa Valentino, Rachel J. So, Scott Shulman and Ravinder*

Department of Laboratory Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, United States

Background: COVID-19 pathology is associated with exuberant inflammation, vascular damage, and activation of coagulation. In addition, complement activation has been described and is linked to disease pathology. However, few studies have been conducted in cancer patients.

Objective: This study examined complement activation in response to COVID-19 in the setting of cancer associated thromboinflammation.

Methods: Markers of complement activation (C3a, C5a, sC5b-9) and complement inhibitors (Factor H, C1-Inhibitor) were evaluated in plasma of cancer patients with (n=43) and without (n=43) COVID-19 and stratified based on elevated plasma D-dimer levels (>1.0 μg/ml FEU). Markers of vascular endothelial cell dysfunction and platelet activation (ICAM-1, thrombomodulin, P-selectin) as well as systemic inflammation (pentraxin-3, serum amyloid A, soluble urokinase plasminogen activator receptor) were analyzed to further evaluate the inflammatory response.

Results: Increases in circulating markers of endothelial cell dysfunction, platelet activation, and systemic inflammation were noted in cancer patients with COVID-19. In contrast, complement activation increased in cancer patients with COVID-19 and elevated D-dimers. This was accompanied by decreased C1-Inhibitor levels in patients with D-dimers > 5 ug/ml FEU.

Conclusion: Complement activation in cancer patients with COVID-19 is significantly increased in the setting of thromboinflammation. These findings support a link between coagulation and complement cascades in the setting of inflammation.

Keywords: COVID-19, cancer, complement, thromboinflammation, endothelial dysfunction

OPEN ACCESS

Edited by:
Zoltán Prohászka, Semmelweis University, Hungary

Reviewed by:
Roberta Bulla, University of Trieste, Italy
Andree Kurniawan, University of Pelita Harapan, Indonesia

****Correspondence:***
Ellinor I. Peerschke
peersche@mskcc.org

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *28 May 2021*
Accepted: *02 August 2021*
Published: *18 August 2021*

Citation:
Peerschke EI, Valentino A, So RJ, Shulman S and Ravinder (2021) Thromboinflammation Supports Complement Activation in Cancer Patients With COVID-19. Front. Immunol. 12:716361. doi: 10.3389/fimmu.2021.716361

INTRODUCTION

The global pandemic of coronavirus disease (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is associated with significant morbidity and mortality (1). Many of the pathologic manifestations of COVID-19 are associated with an exuberant and dysregulated inflammatory response (2), resulting in the release of pro-inflammatory cytokines (3), development of coagulopathies (4), and vascular endothelial cell damage (5). In addition,

complement activation (6–8) and complement deposition in vital organs on autopsy specimens (9) have been described in patients with COVID-19. Indeed, the use of pharmacologic agents targeting the complement system (10), as well as coagulation and fibrinolytic cascades (11) have been proposed.

Biomarkers of inflammation represent key prognostic indicators that may inform the selection of therapeutics in patients with COVID-19. The D-dimer, which represents a fibrin degradation product, is indicative of activation of coagulation and fibrinolysis, and elevated levels are associated with poor prognosis in patients with COVID-19 (12). A recent meta-analysis further summarizes the role of C-reactive protein, lactate dehydrogenase, and D-dimers as markers of hyperinflammation, multiorgan dysfunction, and activation of coagulation, respectively, in predicting patient outcomes in the setting of COVID-19 (13). Markers of inflammation and thrombosis, however, are associated also with cancer (14). Previous reports describing systemic markers of thromboinflammation in patients with COVID-19 have not focused on cancer patients. Since complement activation culminates in the production of anaphylatoxins C3a and C5a, as well as the C5b-9 membrane attack complex, which fuel inflammation and tissue damage (15), the current study was designed to investigate the effect of COVID-19 on circulating markers of complement activation (C3a, C5a, sC5b-9), in cancer patients with and without thromboinflammation as evidenced by plasma D-dimer levels. Selected biomarkers of vascular endothelial cell dysfunction (ICAM-1, thrombomodulin) (16, 17), and endothelial cell and platelet activation (P-selectin) (18), as well as markers of systemic inflammation linked to immunomodulation (pentraxin-3, serum amyloid A, soluble urokinase plasminogen activator (suPAR)) (19–21)were examined to inform the inflammatory response. The data suggest that complement activation is significantly enhanced in cancer patients with COVID-19 in the setting of thromboinflammation.

MATERIALS AND METHODS

Patients and Blood Specimens

This retrospective study used clinical laboratory waste blood samples from hospitalized patients to examine the relationship between activation of coagulation and inflammation and the complement system in cancer patients with and without COVID-19. Specimens were selected from samples submitted for D-dimer analysis with enough residual plasma to support additional studies. Samples were selected based on D-dimer test results, across the reportable range from <0.5 μg/ml FEU to >20,000 μg/ml FEU, and COVID-19 status. Automated D-dimer analysis (STA Compact Max, Diagnostica Stago, S.A.S., Asnieres Sure Seine, France) was performed by the Main Hospital Clinical Hematology Laboratory using the STA-Liatest D-Di assay (Diagnostica Stago). This study was approved by the Institutional Review Board (FWA-00004998, Biospecimen Protocol 16-1547).

Specimens were obtained between April 19 to May 6, 2020. Samples were deidentified and a separate link to the patient medical record was maintained. The medical record was interrogated for selected clinical and laboratory information and SARS-CoV2 test results. Qualitative molecular SARS-CoV2 testing was performed by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) on nasopharyngeal swabs in the Clinical Microbiology Laboratory using several platforms and multiple targets (N_1, N_2, S, ORF_1), approved under an FDA emergency use authorization (EUA).

Due to the method of sample acquisition, specimens were obtained occasionally from the same patient on consecutive days. Duplicate samples were eliminated from the analysis so that each patient was represented once in the study. In situations where D-dimer levels increased or decreased over time, the earliest dated sample with the highest D-dimer result was included in the analysis.

Demographics of hospitalized patients testing positive (n=43) or negative (n=43) for SARS-CoV2 are summarized in **Table 1**. COVID-19 positive and negative patient cohorts were well matched for gender, age, cancer diagnosis, and D-dimer levels. Patients in both groups were diagnosied a wide range of advanced stage or metastatic solid tumors including adenocarcinoma of breast, bladder, pancreas, lung, prostate, oropharynx cancer, colon, gall bladder, appendix, rectum, and stomach, as well as a gastric neuroendocrine tumor. Hematologic malignancies included myeloma, lymphoma, acute and chronic myelogenous leukemias, as well as acute and chronic lymphocytic leukemias. Patients were under active treatment/management for primary disease, disease recurrence, or follow-up post hematopoietic stem cell transplant. Overall, 13 patients in the COVID-19 positive group were managed by Intensive Care, compared to 2 patients in the COVID-19 negative group. A slightly higher 30-day mortality was noted in the COVID-19 positive cohort. Given the small study set, evaluation of the data by disease type and/or treatment was not possible.

Sample Processing

Blood samples were anticoagulated with 0.0109 M (0.32%) trisodium citrate. Whole blood was centrifuged at 4000g for 5 min at room temperature to obtain platelet poor plasma, using the STAT SPIN-Express 4 centrifuge (Stat Spin Technologies, Westwood, MA). Residual plasma was stored at -30°C for 24 h after clinical testing had been completed. Samples were transferred subsequently to the research laboratory where they were thawed and aliquoted for additional studies. Sample aliquots were frozen at -80° C until use.

Biomarker Analysis

Selected biomarkers were evaluated using enzyme linked immunosorbent assays (ELISA), according to manufacturer instructions, as indicated below.

Biomarkers of complement activation included C3a (MicroVue Complement C3a Plus EIA, Quidel, Athens, OH), C5a (MicroVue Complement C5a EIA, Quidel, Athens, OH), and sC5b-9 (MicroVue Complement SC5b-9 Plus EIA, Quidel, Athens, OH). In addition, complement inhibitors, C1-INH (MicroVue Complement C1-Inhibitor Plus EIA, Quidel, Athens, OH) and Factor H (MicroVue Complement Factor H

TABLE 1 | Patient demographics.

Demographic	COVID (+) (n = 43)	COVID (-) (n = 43)
Male (n)	19	19
Female (n)	24	24
Age (years)		
Male	63.9	52.9
Range	17-82	5.82
Median	67	64
Female	65.9	56.08
Range	20 – 83	21-84
Median	68.5	56.5
Ethnicity (n)		
White	23	30
Black	9	7
Asian	5	1
Other/Unknown	6	5
Cancer Type (n)		
Hematologic Malignancy	21	23
Solid Tumor	17	16
Melanoma	3	0
None	2	4
Death		
All (Number, %)	15/43, 34.9%	10/43, 23.3%
Within 30 days (Number, %)	10/43, 23.3%	8/43, 18.6%
D-Dimer (μg/ml FEU)		
All	5.4 ± 6.4*	3.8 ± 5.0*
D-Dimer (> 1.0 μg/ml FEU)		
Positive (n)	35	30
Negative (n)	8	13

(*) p = 0.125.

EIA, Quidel, Athens, OH) were investigated. Since the complement system is sensitive to *in vitro* activation based on time, temperature, and anticoagulants in the absence of exogenously added inhibitors (22), baseline levels were established using blood samples from healthy male and female volunteers (n=20).

Selected biomarkers of endothelial cell dysfunction, including ICAM-1 (Quantikine ELISA, Human ICAM-1/CD54 -specific immunoassay, R&D Systems, Inc., Minneapolis, MN), and thrombomodulin (Quantikine ELISA, Human Thrombomodulin/BDCA-3 immunoassay, R&D Systems, Inc., Minneapolis, MN), as well as platelet and endothelial cell activation, P-selectin (Quantikine ELISA, Human P-Selectin/CD62P immunoassay, R&D Systems, Inc., Minneapolis, MN), were evaluated. In addition, inflammatory biomarkers such as pentraxin-3 (Human Pentraxin 3, Hycult Biotech, Uden, Netherlands), serum amyloid A (Human SAA, Hycult Biotech, Uden, Netherlands), and suPAR (suPARnostic, ViroGates, Birkerod, Denmark) were assessed.

Statistical Evaluation

Continuous variables were expressed as median, mean, and standard deviation (S.D.). Comparisons of biomarkers between patient cohorts with and without COVID-19, in the presence or absence of positive D-dimers, were performed using Wilcoxon Rank-Sum test. Spearman rank order correlation coefficients (r_s) were calculated to assess relationships between biomarkers. All statistical calculations were performed using GraphPad Prism (Version 9; San Diego, CA). A two tailed p value <0.05 and r_s of ≥ 0.30 were considered statistically significant.

RESULTS

D-Dimer Analysis

Plasma D-dimer levels in hospitalized cancer patients with and without COVID-19 (**Table 1**) were comparable (p=0.125). For purposes of this study, high D-dimer levels were defined as ≥1.0 μg/ml FEU. Using this definition, 35 of 43 patients in the COVID-19 positive cohort were classified as D-dimer positive, compared to 30 of 43 patients in the COVID-19 negative cohort (**Table 1**).

Biomarkers of Complement Activation

Levels of complement activation markers, C3a, C5a, sC5b-9, ranged widely in plasma from hospitalized cancer patients. In patients without COVID-19, levels overlapped with those seen in healthy donors (**Figure 1A**). However, significantly elevated levels were noted in hospitalized cancer patients with COVID-19. Stratification of results by plasma D-dimer levels revealed highest levels of complement activation in patients with COVID-19 and elevated plasma D-dimer levels, and lowest levels in COVID-19 negative patients with low D-dimers (**Figure 1B**). Numerical mean and median values are summarized in **Figure 1C**. Interestingly, complement activation was similar in COVID-19 positive and negative cohorts with low D-dimers. Clinical and laboratory information accompanying specimens with markedly elevated (outlier) results were examined further, but no commonalities of diagnosis, D-dimer level, or inflammatory markers emerged.

Complement Inhibitors

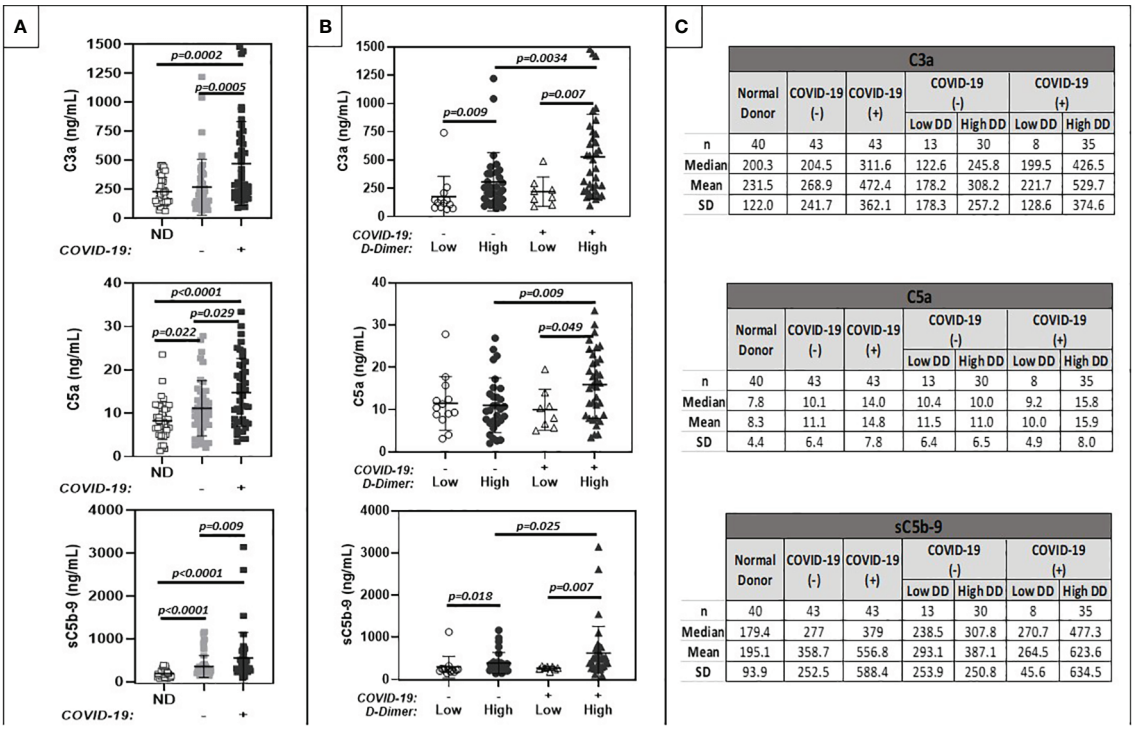
Despite evidence of systemic complement activation and activation of coagulation in cancer patient cohorts with and without COVID-19 and elevated D-dimers, C1 INH and FH levels remained within published reference ranges (Quidel MicroVue C1-Inhibitor Plus EIA. A037: 1-13; Mayo Clinic Laboratories 2020; https://www.mayocliniclabs.com/test-catalog/Clinical+and+Interpretive/64881) and were similar among all comparison groups (**Figure 2**). However, when data were stratified by D-dimer levels above and below 5 μg/ml FEU, a small (approximately 10%), but statistically significant decrease in C1 INH was noted.

Biomarkers of Endothelial Cell Dysfunction and Platelet Activation

Results are summarized in **Figure 3**. Similar levels of ICAM-1, thrombomodulin, and P-selectin levels were observed between COVID-19 positive and negative patient cohorts (Panel A). Mean levels were slightly above assay reference ranges. ICAM-1 and thrombomodulin levels trended higher in patients with positive as compared to negative D-dimers, regardless of COVID-19 positivity. Similar trends were noted for circulating P-selectin levels, but these did not reach statistical significance.

Biomarkers of Inflammation

Results are summarized in **Figure 4**. Levels of inflammatory markers were increased in cancer patients with COVID-19 compared to cancer patients without COVID-19. Values

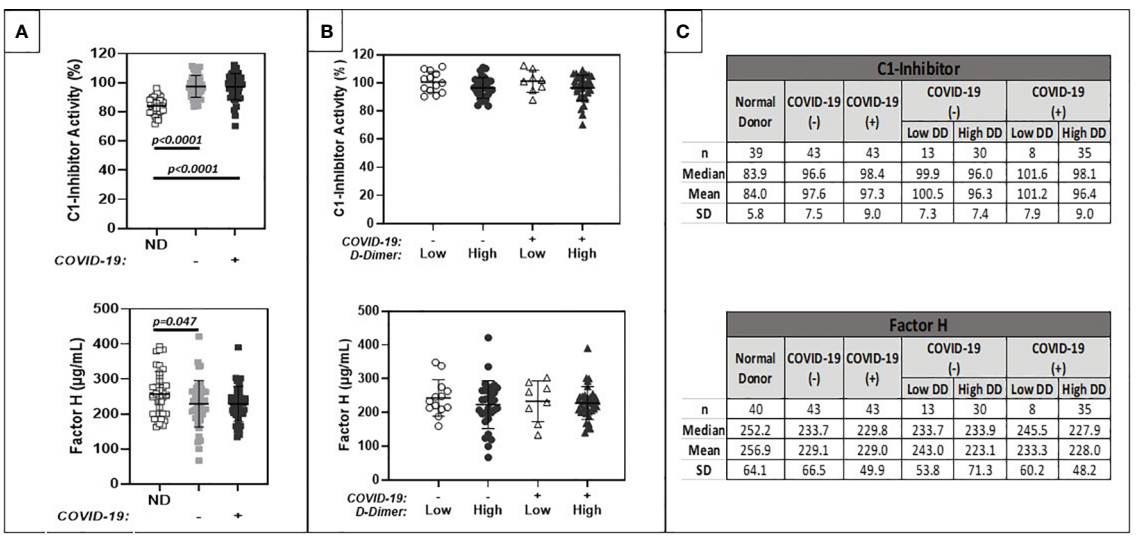


C3a	Normal Donor	COVID-19 (-)	COVID-19 (+)	COVID-19 (-)		COVID-19 (+)	
				Low DD	High DD	Low DD	High DD
n	40	43	43	13	30	8	35
Median	200.3	204.5	311.6	122.6	245.8	199.5	426.5
Mean	231.5	268.9	472.4	178.2	308.2	221.7	529.7
SD	122.0	241.7	362.1	178.3	257.2	128.6	374.6

C5a	Normal Donor	COVID-19 (-)	COVID-19 (+)	COVID-19 (-)		COVID-19 (+)	
				Low DD	High DD	Low DD	High DD
n	40	43	43	13	30	8	35
Median	7.8	10.1	14.0	10.4	10.0	9.2	15.8
Mean	8.3	11.1	14.8	11.5	11.0	10.0	15.9
SD	4.4	6.4	7.8	6.4	6.5	4.9	8.0

sC5b-9	Normal Donor	COVID-19 (-)	COVID-19 (+)	COVID-19 (-)		COVID-19 (+)	
				Low DD	High DD	Low DD	High DD
n	40	43	43	13	30	8	35
Median	179.4	277	379	238.5	307.8	270.7	477.3
Mean	195.1	358.7	556.8	293.1	387.1	264.5	623.6
SD	93.9	252.5	588.4	253.9	250.8	45.6	634.5

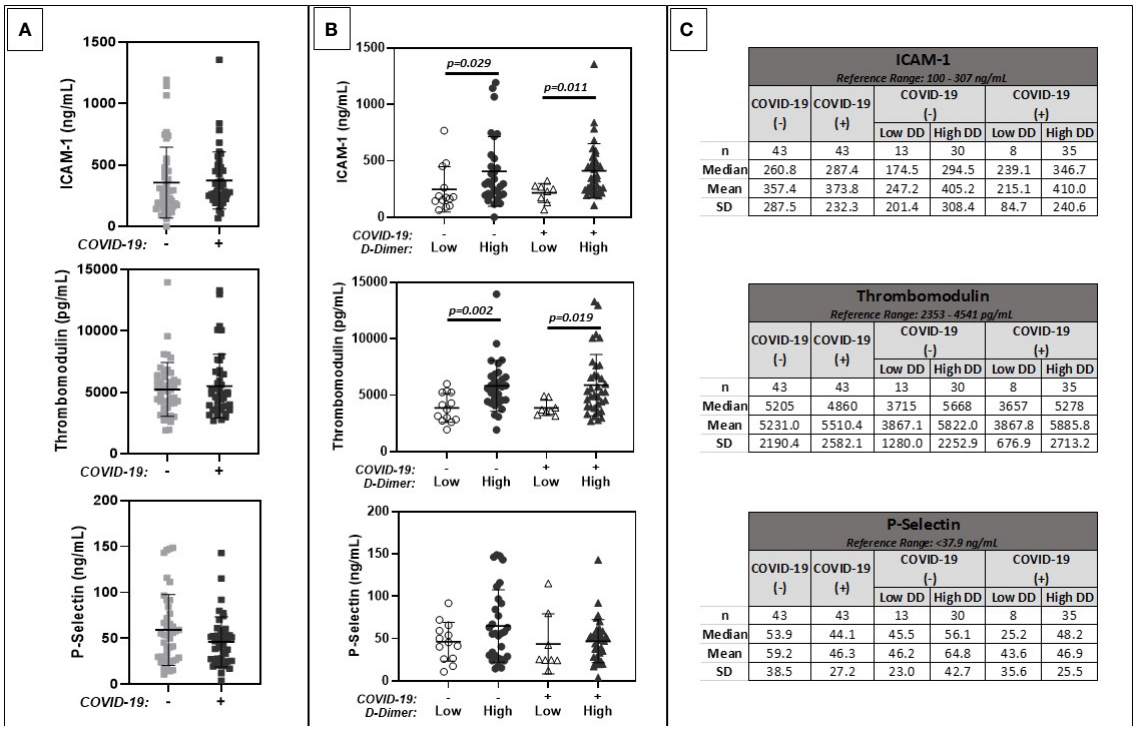
FIGURE 1 | Comparison of plasma markers of systemic complement activation (C3a, C5a, sc5b-9). **(A)** shows results for normal donors (ND) (n=20), and patients with positive (+) or negative (-) COVID-19 PCR test results. **(B)** further stratifies results based on low (<1.0 ug/ml FEU) or high (>1.0 ug/ml FEU) D-dimer concentration. Numerical results are summarized in **(C)**.



C1-Inhibitor	Normal Donor	COVID-19 (-)	COVID-19 (+)	COVID-19 (-)		COVID-19 (+)	
				Low DD	High DD	Low DD	High DD
n	39	43	43	13	30	8	35
Median	83.9	96.6	98.4	99.9	96.0	101.6	98.1
Mean	84.0	97.6	97.3	100.5	96.3	101.2	96.4
SD	5.8	7.5	9.0	7.3	7.4	7.9	9.0

Factor H	Normal Donor	COVID-19 (-)	COVID-19 (+)	COVID-19 (-)		COVID-19 (+)	
				Low DD	High DD	Low DD	High DD
n	40	43	43	13	30	8	35
Median	252.2	233.7	229.8	233.7	233.9	245.5	227.9
Mean	256.9	229.1	229.0	243.0	223.1	233.3	228.0
SD	64.1	66.5	49.9	53.8	71.3	60.2	48.2

FIGURE 2 | Comparison of plasma levels of circulating complement regulatory proteins (C1-INH, Factor H). **(A)** shows results for normal donors (ND) (n=20), and patients with positive (+) or negative (-) COVID-19 PCR test results. **(B)** further stratifies results based on low (<1.0 ug/ml FEU) or high (>1.0 ug/ml FEU) D-dimer concentration. Numerical results are summarized in **(C)**.



C

ICAM-1 Reference Range: 100 - 307 ng/mL	COVID-19 (-)	COVID-19 (+)	COVID-19 (-)		COVID-19 (+)	
			Low DD	High DD	Low DD	High DD
n	43	43	13	30	8	35
Median	260.8	287.4	174.5	294.5	239.1	346.7
Mean	357.4	373.8	247.2	405.2	215.1	410.0
SD	287.5	232.3	201.4	308.4	84.7	240.6

Thrombomodulin Reference Range: 2353 - 4541 pg/mL	COVID-19 (-)	COVID-19 (+)	COVID-19 (-)		COVID-19 (+)	
			Low DD	High DD	Low DD	High DD
n	43	43	13	30	8	35
Median	5205	4860	3715	5668	3657	5278
Mean	5231.0	5510.4	3867.1	5822.0	3867.8	5885.8
SD	2190.4	2582.1	1280.0	2252.9	676.9	2713.2

P-Selectin Reference Range: <37.9 ng/mL	COVID-19 (-)	COVID-19 (+)	COVID-19 (-)		COVID-19 (+)	
			Low DD	High DD	Low DD	High DD
n	43	43	13	30	8	35
Median	53.9	44.1	45.5	56.1	25.2	48.2
Mean	59.2	46.3	46.2	64.8	43.6	46.9
SD	38.5	27.2	23.0	42.7	35.6	25.5

FIGURE 3 | Comparison of plasma markers of endothelial cell dysfunction (ICAM 1, Thrombomodulin, P-Selectin). **(A)** shows results for patients with positive (+) or negative (-) COVID-19 PCR test results. **(B)** further stratifies results based on low (<1.0 ug/ml FEU) or high (>1.0 ug/ml FEU) D-dimer concentration. Numerical results are summarized in **(C)**.

ranged widely among patients with and without COVID-19. Levels were highest in patients with elevated D-dimers, regardless of COVID-19 status.

Biomarker Correlations

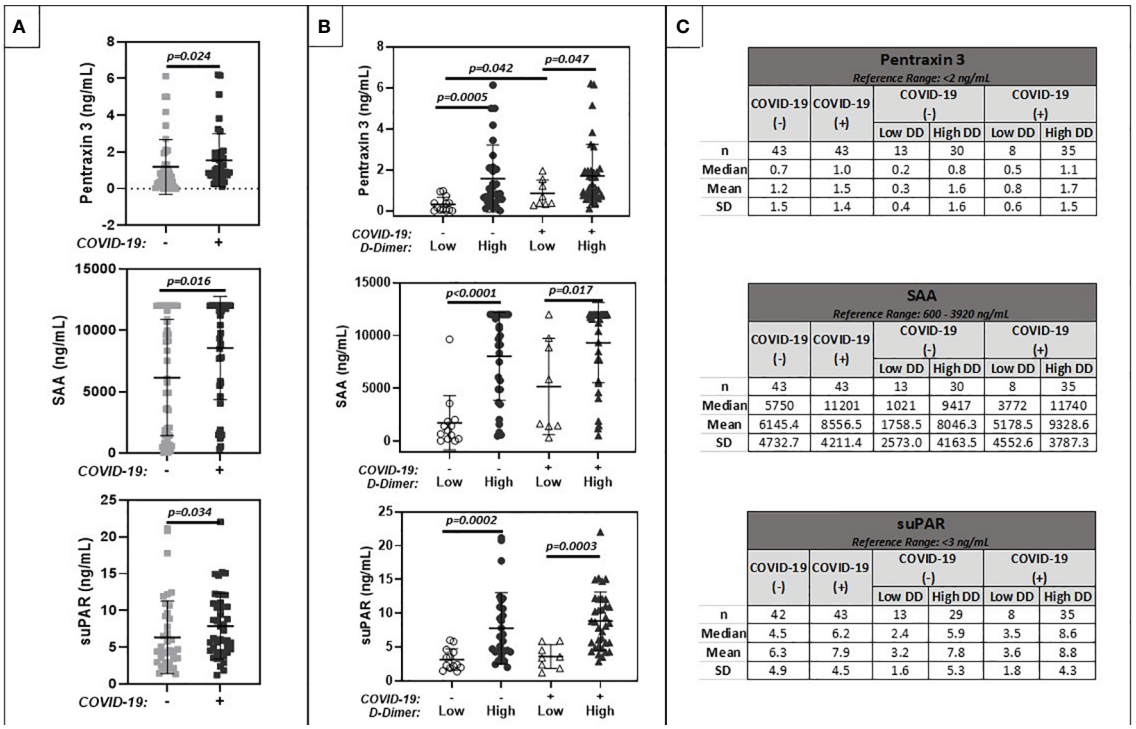
Correlations between biomarkers in cancer patients with or without COVID-19 were evaluated by determining Spearman rank order correlation coefficients (**Table 2**). Statistically significant correlations between complement activation, defined by elevated plasma C3a levels, and plasma D-dimers, thrombomodulin and suPAR were observed in both COVID-19 negative and positive patient cohorts. C1 INH levels correlated negatively with D-dimers. Interestingly, in the setting of COVID-19, marked complement activation was noted only in cancer patients with elevated D-dimers. D-dimer levels also correlated with markers of endothelial cell dysfunction and inflammation.

DISCUSSION

COVID-19 is associated with significant morbidity and mortality. Therapeutic interventions are aimed at the associated systemic inflammatory syndrome and hypercoagulability (2, 11). In addition, there is growing interest in targeting systemic complement activation (10). Systemic biomarkers may inform and optimize patient management. However, few studies have focused on cancer patients with COVID-19. Given the overlap between systemic coagulation, complement, and fibrinolytic cascades (23), this study examined selected biomarkers of complement activation, endothelial cell dysfunction and platelet activation, as well as inflammation in cancer patients in the presence or absence of COVID-19 and elevated D-dimers.

The data demonstrate that in cancer patients, D-dimer levels are associated not only with evidence of endothelial cell dysfunction and systemic inflammation but also with complement activation. In cancer patients with COVID-19, increased complement activation was noted in patients with high D-dimers (>1 μg/ml FEU). Interestingly, complement activation was similar in cancer patients with or without COVID-19 in the setting of low D-dimers. These observations suggest that complement activation during COVID-19 may be propagated by activation of coagulation and fibrinolysis, and the cross reactivity between complement and coagulation pathways (23). Consistent with this hypothesis is evidence for consumption of C1-INH, one of the major inhibitors of complement, coagulation and kinin cascades (24, 25), in cancer patients with COVID-19 and elevated D-dimers. Indeed, in the present study, C1-INH levels correlated negatively with both D-dimer levels and C3a.

Since complement activation is associated with the generation of inflammatory mediators and the potential for tissue destruction



C

Pentraxin 3
Reference Range: <2 ng/mL

	COVID-19 (-)	COVID-19 (+)	COVID-19 (-)		COVID-19 (+)	
			Low DD	High DD	Low DD	High DD
n	43	43	13	30	8	35
Median	0.7	1.0	0.2	0.8	0.5	1.1
Mean	1.2	1.5	0.3	1.6	0.8	1.7
SD	1.5	1.4	0.4	1.6	0.6	1.5

SAA
Reference Range: 600 - 3920 ng/mL

	COVID-19 (-)	COVID-19 (+)	COVID-19 (-)		COVID-19 (+)	
			Low DD	High DD	Low DD	High DD
n	43	43	13	30	8	35
Median	5750	11201	1021	9417	3772	11740
Mean	6145.4	8556.5	1758.5	8046.3	5178.5	9328.6
SD	4732.7	4211.4	2573.0	4163.5	4552.6	3787.3

suPAR
Reference Range: <3 ng/mL

	COVID-19 (-)	COVID-19 (+)	COVID-19 (-)		COVID-19 (+)	
			Low DD	High DD	Low DD	High DD
n	42	43	13	29	8	35
Median	4.5	6.2	2.4	5.9	3.5	8.6
Mean	6.3	7.9	3.2	7.8	3.6	8.8
SD	4.9	4.5	1.6	5.3	1.8	4.3

FIGURE 4 | Comparison of plasma markers of inflammation (Pentraxin 3, Serum Amyloid A, suPAR). **(A)** shows results for patients with positive (+) or negative (-) COVID-19 PCR test results. **(B)** further stratifies results based on low (<1.0 ug/ml FEU) or high (>1.0 ug/ml FEU) D-dimer concentration. Numerical results are summarized in **(C)**.

(26), complement activation may contribute to the pathology of COVID-19 and the reported increased risk of adverse outcomes in cancer patients with COVID-19 (27). Indeed, recent transcriptional profiling of nasopharyngeal swabs from patients with COVID-19 indicate that COVID-19 infection results not only in IL-6 dependent inflammatory immune responses, but also activation of complement and coagulation pathways (28), and that complement function impacts adverse COVID-19 infection outcome. Larger prospective studies are needed to further evaluate the predictive value of markers of complement activation in COVID-19.

The underlying pathophysiology of thrombosis in COVID-19 is attributed in large part to direct endothelial toxicity leading to *in situ* thrombin generation and thromboinflammation (28). Data from the present study are consistent with this concept and demonstrate that markers of endothelial cell dysfunction and inflammation were increased in the blood of cancer patients with elevated D-dimers. Since endothelial cell injury and thrombosis are hallmarks also of cancer thromboinflammation, it is not surprising that an increase in complement activation was noted in cancer patients without COVID-19 but with elevated D-dimers. Moreover, complement activation increased significantly in the setting of COVID-19 and thromboinflammation. These findings are in agreement with a recent report by Ma et al., studying non-cancer patients with respiratory failure (29), which suggests that complement activation is a general marker of critical illness, but increases the setting of COVID-19 associated respiratory failure.

These combined observations in cancer and non-cancer patients suggest a potential direct role for SARS-CoV2 mediated activation of the complement system which appear to be enhanced in the setting of thromboinflammation or critical illness. Indeed, SARS-CoV-2 activation of the lectin and alternative pathways of complement (30, 31) have been described. Moreover, the study by Ma et al. (29) demonstrates that complement activation was associated with worse outcomes in patients with COVID-19. Due to the small sample size of our study in cancer patients, we were unable to assess the impact of complement activation on disease severity and outcome.

In summary, the present study demonstrates that in cancer patients with COVID-19, D-dimer levels are associated strongly with complement activation. This study is limited by its retrospective nature and small sample size which impacts statistical power, particularly in subgroup analyses. In addition, due to retrospective sample acquisition, the available sample type and storage conditions were not optimal for analysis of complement activation products. To address this issue, complement activation assays were performed on similarly collected specimens from healthy volunteers to serve as a comparative baseline. These conditions may have influenced assay results of complement activation products, and should be considered when comparing

TABLE 2 | Correlation between activation of coagulation (D-dimers) and complement activation (C3a) and biomarkers of endothelial cell injury and inflammation in cancer patients with or without COVID-19.

A. COVID-19 Negative.

	D-dimer		C3a	
	rs	**p**	**rs**	**p**
D-dimer			0.472	0.001
C3a	0.472	0.001		
Sc5b-9	0.275	0.074	0.681	4.97E-07
C1-Inh	-0.427	0.004	-0.349	0.022
C5a	-0.013	0.935	0.129	0.408
FH	-0.100	0.523	-0.141	0.365
ICAM	0.230	0.138	0.247	0.1 10
Thombo	0.448	0.003	0.502	0.001
P-Selectin	0.224	0.149	0.331	0.030
Pentraxin	0.502	0.001	0.314	0.040
SAA	0.667	1.02E-06	0.220	0.157
suPAR	0.567	9.05E-05	0.582	5.27E-05

B. COVID-19 Posiitve.

	D-dimer		C3a	
	rs	**p**	**rs**	**p**
D-dimer			O. 424	0.005
C3a	O. 424	0.005		
Sc5b-9	0.256	0.097	0.737	1 .80E-08
C1-Inh	-0.465	0.002	-0.097	0.535
C5a	0.220	0.156	0.557	0.0001
FH	-0 .243	0.1 16	-0.121	0.438
ICAM	0.469	0.002	0.101	0.519
Thombo	0.550	0.0001	0.486	0.001
P-Selectin	0.157	0.314	0.189	0.224
Pentraxin	0.259	0.093	0.083	0.595
SAA	0.196	0.208	-0.016	0.918
suPAR	0.703	1.49E-07	0.289	0.060

data with other studies. Strengths of the current investigation include the focus on hospitalized cancer patients with COVID-19 and the evaluation of complement activation in the setting of cancer thromboinflammation. The present study suggests that D-dimer levels in cancer patients with COVID-19 are an indicator not only of systemic inflammation and activation of coagulation, but also enhanced complement activation. This link between inflammation, complement, and coagulation may further inform therapeutic intervention including the development of cocktails of anti-inflammatory agents, anticoagulants, and complement inhibitors. Interestingly, recent meta-analysis data suggest that therapy with the anti-inflammatory agent, tocilizumab, was associated not only with a reduction in inflammatory markers such as CRP and IL-6, but also D-dimers (32, 33). The effect on complement activation is not known at this time.

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

This study was approved by the Institutional Review Board/ Privacy Board at Memorial Sloan Kettering (FWA-00004998, Biospecimen Protocol 16-1547). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

EP designed the study, oversaw execution of measurements, analysis of the data, and wrote the manuscript. AV conducted measurements and analyzed the data. RS conducted measurements. SS and R provided study samples. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by in part by the NIH/NCI Cancer Center Support grant P30 CA008748.

REFERENCES

1. Martines RB, Ritter JM, Matkovic E, Gary J, Bollweg BC, Bullock H, et al. Pathology and Pathogenesis of SARS-CoV-2 Associated With Fatal Coronavirus Disease, United States. *Emerg Infect Dis* (2020) 26:2005–15. doi: 10.3201/eid2609.202095
2. Manjili RH, Zarei M, Habibi M, Manjili MH. COVID-19 as an Acute Inflammatory Disease. *J Immunol* (2020) 205:12–9. doi: 10.4049/jimmunol.2000413
3. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical Features of Patients Infected With 2019 Novel Coronavirus in Wuhan, China. *Lancet* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
4. Levi M, Thachil J, Iba T, Levy JH. Coagulation Abnormalities and Thrombosis in Patients With COVID-19. *Lancet Haematol* (2020) 7:e438–40. doi: 10.1016/S2352-3026(20)30145-9
5. Varga Z, Flammer AJ, Steiger P, Haberecker M, Andermatt R, Zinkernagel AS, et al. Endothelial Cell Infection and Endotheliitis in COVID-19. *Lancet* (2020) 395:1417–8. doi: 10.1016/S0140-6736(20)30937-5
6. de Nooijer AH, Grondman I, Janssen NAF, Netea MG, Willems L, van de Veerdonk FL, et al. Complement Activation in the Disease Course of Coronavirus Disease 2019 and Its Effects on Clinical Outcomes. *J Infect Dis* (2021) 223:214–24.
7. Holter JC, Pischke SE, de Boer E, Lind A, Jenum S, Holten AR, et al. Systemic Complement Activation Is Associated With Respiratory Failure in COVID-19 Hospitalized Patients. *Proc Natl Acad Sci USA* (2020) 117:25018–25. doi: 10.1073/pnas.2010540117
8. Sinkovits G, Mezo B, Reti M, Muller V, Ivanyi Z, Gal J, et al. Complement Overactivation and Consumption Predicts In-Hospital Mortality in SARS-CoV-2 Infection. *Front Immunol* (2021) 12:663187. doi: 10.3389/fimmu.2021.663187
9. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement Associated Microvascular Injury and Thrombosis in the Pathogenesis of Severe COVID-19 Infection: A Report of Five Cases. *Transl Res* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
10. Risitano AM, Mastellos DC, Huber-Lang M, Yancopoulou D, Garlanda C, Ciceri F, et al. Complement as a Target in COVID-19? *Nat Rev Immunol* (2020) 20:343–4. doi: 10.1038/s41577-020-0320-7
11. Bikdeli B, Madhavan MV, Gupta A, Jimenez D, Burton JR, Der Nigoghossian C, et al. Pharmacological Agents Targeting Thromboinflammation in COVID-19: Review and Implications for Future Research. *Thromb Haemost* (2020) 120:1004–24. doi: 10.1055/s-0040-1713152
12. Tang N, Li D, Wang X, Sun Z. Abnormal Coagulation Parameters are Associated With Poor Prognosis in Patients With Novel Coronavirus Pneumonia. *J Thromb Haemost* (2020) 18:844–7. doi: 10.1111/jth.14768
13. Hariyanto TI, Japar KV, Kwenandar F, Damay V, Siregar JI, Lugito NPH, et al. Inflammatory and Hematologic Markers as Predictors of Severe Outcomes in COVID-19 Infection: A Systematic Review and Meta-Analysis. *Am J Emerg Med* (2021) 41:110–9. doi: 10.1016/j.ajem.2020.12.076
14. Varki A. Trousseau's Syndrome: Multiple Definitions and Multiple Mechanisms. *Blood* (2007) 110:1723–9. doi: 10.1182/blood-2006-10-053736
15. Dunkelberger JR, Song WC. Complement and its Role in Innate and Adaptive Immune Responses. *Cell Res* (2010) 20:34–50. doi: 10.1038/cr.2009.139
16. Takahashi H, Ito S, Hanano M, Wada K, Niwano H, Seki Y, et al. Circulating Thrombomodulin as a Novel Endothelial Cell Marker: Comparison of its Behavior With Von Willebrand Factor and Tissue-Type Plasminogen Activator. *Am J Hematol* (1992) 41:32–9. doi: 10.1002/ajh.2830410107
17. Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW. ICAM-1 Regulates Neutrophil Adhesion and Transcellular Migration of TNF-Alpha-Activated Vascular Endothelium Under Flow. *Blood* (2005) 106:584–92. doi: 10.1182/blood-2004-12-4942
18. Gotsch U, Jager U, Dominis M, Vestweber D. Expression of P-Selectin on Endothelial Cells is Upregulated by LPS and TNF-Alpha In Vivo. *Cell Adhes Commun* (1994) 2:7–14. doi: 10.3109/15419069409014198
19. Ma YJ, Garred P. Pentraxins in Complement Activation and Regulation. *Front Immunol* (2018) 9:3046. doi: 10.3389/fimmu.2018.03046
20. Rovina N, Akinosoglou K, Eugen-Olsen J, Hayek S, Reiser J, Giamarellos-Bourboulis EJ. Soluble Urokinase Plasminogen Activator Receptor (suPAR) as an Early Predictor of Severe Respiratory Failure in Patients With COVID-19 Pneumonia. *Crit Care* (2020) 24:187. doi: 10.1186/s13054-020-02897-4
21. Sack GH Jr. Serum Amyloid A - A Review. *Mol Med* (2018) 24:46. doi: 10.1186/s10020-018-0047-0
22. Mollnes TE, Garred P, Bergseth G. Effect of Time, Temperature and Anticoagulants on *In Vitro* Complement Activation: Consequences for Collection and Preservation of Samples to be Examined for Complement Activation. *Clin Exp Immunol* (1988) 73:484–8.
23. Amara U, Flierl MA, Rittirsch D, Klos A, Chen H, Acker B, et al. Molecular Intercommunication Between the Complement and Coagulation Systems. *J Immunol* (2010) 185:5628–36. doi: 10.4049/jimmunol.0903678
24. Ratnoff OD, Pensky J, Ogston D, Naff GB. The Inhibition of Plasmin, Plasma Kallikrein, Plasma Permeability Factor, and the C'1r Subcomponent of the First Component of Complement by Serum C'1 Esterase Inhibitor. *J Exp Med* (1969) 129:315–31. doi: 10.1084/jem.129.2.315
25. Schreiber AD, Kaplan AP, Austen KF. Inhibition by C1INH of Hagemann Factor Fragment Activation of Coagulation, Fibrinolysis, and Kinin Generation. *J Clin Invest* (1973) 52:1402–9. doi: 10.1172/JCI107313
26. Ricklin D, Lambris JD. Complement in Immune and Inflammatory Disorders: Pathophysiological Mechanisms. *J Immunol* (2013) 190:3831–8. doi: 10.4049/jimmunol.1203487
27. Perico L, Benigni A, Casiraghi F, Ng LFP, Renia L, Remuzzi G. Immunity, Endothelial Injury and Complement-Induced Coagulopathy in COVID-19. *Nat Rev Nephrol* (2021) 17:46–64. doi: 10.1038/s41581-020-00357-4
28. Ramlall V, Thangaraj PM, Meydan C, Foox J, Butler D, May B, et al. Immune Complement and Coagulation Dysfunction in Adverse Outcomes of SARS-CoV-2 Infection. *Nat Med* (2020) 26(10):1609–15. doi: 10.1038/s41591-020-1021-2
29. Ma L, Sahu SK, Cano M, Kuppuswamy V, Bajwa J, McPhatter J, et al. Increased Complement Activation Is a Distinctive Feature of Severe SARS-CoV-2 Infection. *Sci Immunol* (2021) 6(59):eabh2259. doi: 10.1126/sciimmunol.abh2259
30. Polycarpou A, Howard M, Farrar CA, Greenlaw R, Fanelli G, Wallis R, et al. Rationale for Targeting Complement in COVID-19. *EMBO Mol Med* (2020) 12:e12642. doi: 10.15252/emmm.202012642
31. Yu J, Yuan X, Chen H, Chaturvedi S, Braunstein EM, Brodsky RA. Direct Activation of the Alternative Complement Pathway by SARS-CoV-2 Spike Proteins Is Blocked by Factor D Inhibition. *Blood* (2020) 136:2080–9. doi: 10.1182/blood.2020008248
32. Ivan Hariyanto T, Kurniawan A. Tocilizumab Administration Is Associated With the Reduction in Biomarkers of Coronavirus Disease 2019 Infection. *J Med Virol* (2021) 93:1832–6. doi: 10.1002/jmv.26698
33. Hariyanto TI, Hardyson W, Kurniawan A. Efficacy and Safety of Tocilizumab for Coronavirus Disease 2019 (Covid-19) Patients: A Systematic Review and Meta-Analysis. *Drug Res (Stuttg)* (2021) 71:265–74. doi: 10.1055/a-1336-2371

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 10 September 2021
doi: 10.3389/fimmu.2021.742446

Complement Alternative and Mannose-Binding Lectin Pathway Activation Is Associated With COVID-19 Mortality

Federica Defendi[1], Corentin Leroy[2,3], Olivier Epaulard[4,5], Giovanna Clavarino[1], Antoine Vilotitch[2], Marion Le Marechal[4], Marie-Christine Jacob[1], Tatiana Raskovalova[1], Martine Pernollet[1], Audrey Le Gouellec[5,6], Jean-Luc Bosson[5], Pascal Poignard[7,8], Matthieu Roustit[9,10], Nicole Thielens[7], Chantal Dumestre-Pérard[1,7†] and Jean-Yves Cesbron[1†]*

[1] Laboratoire d'Immunologie, Institut de Biologie et Pathologie, Centre Hospitalier Universitaire Grenoble Alpes, Grenoble, France, [2] Cellule d'Ingénierie des Données, Centre Hospitalier Universitaire Grenoble Alpes, Grenoble, France, [3] Centre d'Investigation Clinique de l'Innovation et de la Technologie (CIC-IT), Centre Hospitalier Universitaire Grenoble Alpes, Grenoble, France, [4] Service des Maladies Infectieuses et Tropicales, Centre Hospitalier Universitaire Grenoble Alpes, Grenoble, France, [5] Université Grenoble Alpes, TIMC-IMAG, Grenoble, France, [6] Laboratoire de Biochimie, Institut de Biologie et Pathologie, Centre Hospitalier Universitaire Grenoble Alpes, Grenoble, France, [7] Université Grenoble Alpes, CNRS, CEA, Institut de Biologie Structurale (IBS), Grenoble, France, [8] Laboratoire de Virologie, Institut de Biologie et Pathologie, Centre Hospitalier Universitaire Grenoble Alpes, Grenoble, France, [9] Département de Pharmacologie Clinique INSERM CIC 1406, Centre Hospitalier Universitaire Grenoble Alpes, Grenoble, France, [10] Université Grenoble Alpes, UMR 1042-HP2, INSERM, Grenoble, France

OPEN ACCESS

Edited by:
Zoltán Prohászka, Semmelweis University, Hungary

Reviewed by:
Lubka T. Roumenina, INSERM U1138 Centre de Recherche des Cordeliers (CRC), France
Marten Trendelenburg, University Hospital of Basel, Switzerland

***Correspondence:**
Federica Defendi
fdefendi@chu-grenoble.fr

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *16 July 2021*
Accepted: *23 August 2021*
Published: *10 September 2021*

Citation:
Defendi F, Leroy C, Epaulard O, Clavarino G, Vilotitch A, Le Marechal M, Jacob M-C, Raskovalova T, Pernollet M, Le Gouellec A, Bosson J-L, Poignard P, Roustit M, Thielens N, Dumestre-Pérard C and Cesbron J-Y (2021) Complement Alternative and Mannose-Binding Lectin Pathway Activation Is Associated With COVID-19 Mortality. Front. Immunol. 12:742446. doi: 10.3389/fimmu.2021.742446

Background: The SARS-CoV-2 infection triggers excessive immune response resulting in increased levels of pro-inflammatory cytokines, endothelial injury, and intravascular coagulopathy. The complement system (CS) activation participates to this hyperinflammatory response. However, it is still unclear which activation pathways (classical, alternative, or lectin pathway) pilots the effector mechanisms that contribute to critical illness. To better understand the immune correlates of disease severity, we performed an analysis of CS activation pathways and components in samples collected from COVID-19 patients hospitalized in Grenoble Alpes University Hospital between 1 and 30 April 2020 and of their relationship with the clinical outcomes.

Methods: We conducted a retrospective, single-center study cohort in 74 hospitalized patients with RT-PCR-proven COVID-19. The functional activities of classical, alternative, and mannose-binding lectin (MBL) pathways and the antigenic levels of the individual components C1q, C4, C3, C5, Factor B, and MBL were measured in patients' samples during hospital admission. Hierarchical clustering with the Ward method was performed in order to identify clusters of patients with similar characteristics of complement markers. Age was included in the model. Then, the clusters were compared with the patient clinical features: rate of intensive care unit (ICU) admission, corticoid treatment, oxygen requirement, and mortality.

Results: Four clusters were identified according to complement parameters. Among them, two clusters revealed remarkable profiles: in one cluster (n = 15), patients exhibited activation of alternative and lectin pathways and low antigenic levels of MBL, C4, C3, Factor B, and C5 compared to all the other clusters; this cluster had the higher proportion of patients who died (27%) and required oxygen support (80%) or ICU care (53%). In contrast, the second cluster (n = 19) presented inflammatory profile with high classical pathway activity and antigenic levels of complement components; a low proportion of patients required ICU care (26%) and no patient died in this group.

Conclusion: These findings argue in favor of prominent activation of the alternative and MBL complement pathways in severe COVID-19, but the spectrum of complement involvement seems to be heterogeneous requiring larger studies.

Keywords: COVID-19, complement, alternative pathway, MBL, lectin pathway

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection drives sustained inflammatory response considered to be a major cause of disease severity and death in patients with COVID-19 (1). Growing evidence suggests that the complement system (CS) activation instigates this dysregulated inflammatory reaction in COVID-19: elevated levels of the anaphylatoxin C5a have been reported to be proportional to disease severity (2); the membrane attack complex concentration has been linked with respiratory failure and systemic inflammation in infected patients (3); and deposits of mannose-binding lectin (MBL) and MBL-associated protease MASP-2 have been found in the microvasculature of critical patients with SARS CoV-2 infection (4). On the other hand, patients treated with complement blockers (anti-C5a mAb [eculizumab] or C3-inhibitor) exhibited a drop in inflammatory markers and significant clinical improvement (5–9). However, it is still not fully understood which of the three complement activation pathways (classical, alternative, or lectin pathway) drives the effector mechanisms that contribute to the tissue injury. To address these questions, we performed extensive analysis of CS activation pathways and components in samples collected from hospitalized COVID-19 patients and their relationship with the clinical outcomes.

METHODS

Study Participants

This retrospective, single center study included 74 patients with RT-PCR-proven COVID-19 admitted to Grenoble Alpes University Hospital from April 1 to 30, 2020. Samples were collected during hospitalization in infectious/pneumology/internal medicine department or intensive care unit (ICU) of our hospital. The study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice guidelines, and CNIL (Commission Nationale de l'Informatique et des Libertés) methodology reference. Patients were informed and consent was obtained, according to French law. Demographic, clinical characteristics (oxygen requirements, ICU admission, mortality, steroid treatment) and laboratory data were collected from electronic clinical records and included in an anonymized database. Patients were classified as severe on the basis of oxygen requirement (>2 L O_2/min), ICU admission, limitation of therapeutic effort (LTE), and mortality, according to (10).

Complement Testing

Peripheral blood samples were collected in citrate anticoagulated or without anticoagulant tubes for hemolytic and functional assays or antigenic level measurement, respectively. Total hemolytic assays for classical pathway (CP, TH50c) and alternative pathway (AP, TH50a) were assessed as previously published (11); 100% lysis is defined by the TH50c/TH50a of the control sample. Reference values (TH50c: 86–126%; TH50a: 84–150%) were established by testing samples from 50 blood donors.

Antigenic levels of C1q, C4, C3, C5, and Factor B proteins in the serum samples were measured using a laser nephelometer BNII (Dade Behring, GmbH, Marburg, Germany). Reference intervals (RI; C1q: 154–258 mg/L; C4: 100–380 mg/L; C3: 880–1650 mg/L; C5: 120–220 mg/L; Factor B: 216–504 mg/L) were obtained by testing samples from 50 blood donors.

Determination of MBL protein concentration and function was realized by an enzyme-linked immunosorbent assay (ELISA) as described previously (12). The characterization of MBL protein expression deficiency was established by the combination of three assays: ELISA for antigen and functional MBL and hemolytic activity of C4 (C4H) (13) normal values of C1q confirmed the absence of CP activation. Low values of antigenic (<100 μg/L) (14) and functional MBL associated with a normal value of C4H defined patients with MBL protein expression deficiency. Low levels of antigenic and functional MBL associated with decreased value of C4H identified patients

Abbreviations: AP, alternative pathway; COVID-19, coronavirus disease 19; CP, classical pathway; CS, Complement system; C4H, C4 hemolytic activity; LTE, limitation of the therapeutic effort; LP, lectin pathway; MBL, mannose-binding lectin; RI, reference interval.

with MBL pathway activation. Reference values for MBL protein concentration and function and C4H were determined from 50 blood donors (MBL antigen: 30–3000 µg/L; MBL function: 35–115%; C4H: 70–130%).

Statistical Analysis

Statistical analysis was performed using hierarchical ascendant clustering (HAC) in order to identify groups of COVID-19 patients with similar characteristics ("clusters") in terms of complement variables: TH50c, TH50a, C1q, C4, C3, C5, Factor B, and MBL antigen. Age was included in the model (**Supplementary Methods**).

The biological significance of the clusters was analyzed by comparing the values of every complement parameter between the clusters. The ANOVA F-test was performed for variables with a Gaussian distribution, and the Kruskal-Wallis test for the variables with other distribution. Mean (standard deviation [sd]) or median (interquartile range [IQR]) were presented for the parametric or non-parametric variables, respectively. For the markers with a significant difference between the clusters, specific cluster by cluster tests were performed using Student or Wilcoxon tests to identify the cluster significantly different from the others. Post-hoc analysis with the Fisher's exact test was used to test specific difference between the clusters.

RESULTS

Table 1 details the main demographic and clinical characteristics of our cohort. The median age of patients was 72 years (IQR: 62;82; range: 32–96); more than half of patients were men (58%). Of the 74 patients, 43 (58%) were severe, 8 (11%) died, 23 (31%) were admitted in the ICU, 3 (4%) were with LTE, and 23 (31%) required treatment by corticoids.

The results of complement proteins and activation pathways analysis performed in samples collected during hospitalization are summarized in **Table 2**.

Four clusters of individuals were identified in the studied cohort by HAC analysis according to the complement data (**Supplementary Figure S1**). The comparison between the clusters (cluster 2 against others and/or cluster 4 against others) is presented in **Figure 1** and **Table 3**. There was no statistically significant difference among the four clusters for sex, age, CRP, severity, ICU admission, O_2 requirement, or corticoid treatment (**Table 4**).

TABLE 1 | Patient characteristics.

Clinical and biological characteristics	
n	74
Age: median (IQR) (min-max), years	72 (62;82) (32–96)
Sex: men/women	43/31
CRP: mean (sd), mg/L	71 (66.4)
CRP: median (IQR), mg/L	59 (22-92)
Prognosis/outcome, n (%)	
Severe COVID-19[1], n (%)	43 (58)
Mortality	8 (11)
ICU admission	23 (31)
Limitation of therapeutic effort, n (%)	3 (4)
Corticosteroid treatment, n (%)	23 (21)
Oxygen support:	
>2 L O_2/min	39 (53)
≤2 L O_2/min	13 (18)

[1]*Severe COVID-19 defined as: O_2 > 2 L/min, ICU [intensive care unit] admission, LTE [limitation of the therapeutic effort], decease.*

For patients of the first cluster (n = 20; 27%), the complement profile was overall without anomalies (TH50c: 128%; TH50a: 132%; C1q: 201 mg/L; C4: 368 mg/L; C3: 1440 mg/L; C5: 216 mg/L; Factor B: 474 mg/L; MBL antigen: 900 µg/L; MBL function: 110%; C4H: 125%; **Table 3**). A large percentage of patients in this cluster was severe (n = 14; 70%) and required corticoid treatment (n = 10; 50%) (**Table 4**).

Patients of cluster 2 (n = 19; 26%) exhibited an inflammatory profile: high values of CP, LP, and C4 activities (TH50c: 145%; MBL function: 117%; C4H: 118%) and increased antigenic levels of C3 (1795 mg/L), C5 (278 mg/L), Factor B (586 mg/L), and MBL (1025 µg/L) were observed in this cluster compared to other clusters (**Table 3**). The majority of patients of the second cluster were men (men/women: 14/5). Interestingly, none of the patients died in this cluster (**Table 4**).

In cluster 3 (n = 20; 27%), most complement markers were within reference interval (TH50c: 118%; TH50a: 122%; C1q: 216 mg/L; C4: 317 mg/L; C3: 1150 mg/L; C5: 199 mg/L; Factor B: 437 mg/L; MBL antigen: 725 µg/L; C4H: 104%); only MBL function was decreased (43%) (**Table 3**). The third cluster was characterized by the lowest rate of ICU admission (20%) and by the lowest rate of patients having required corticoid treatment (10%); the rate of mortality was also low in this cluster (10%) (**Table 4**).

TABLE 2 | Complement parameters.

	RI	Mean	sd	Min	Lower quart	Median	Upper quart	Max
TH50c (%)	86–126	124	30	67	101	121	142	234
TH50a (%)	84–150	122	70	23	71	102	155	317
C1q (mg/L)	154–258	208	48	71	195	212	234	336
C4 (mg/L)	100–380	318	114	57	249	327	402	551
C3 (mg/L)	880–1650	1339	357	603	1050	1355	1550	2210
Factor B (mg/L)	216–504	466	148	182	345	465	581	828
C5 (mg/L)	120–220	225	52	142	186	214	260	367
MBL antigen (µg/L)	30–3000	1344	1586	20	270	775	1750	9000
MBL function (%)	35–115	94	79	0	22	66	178	200
C4 hemolytic activity (%)	70–130	106	51	5	76	100	132	251

RI, reference interval.

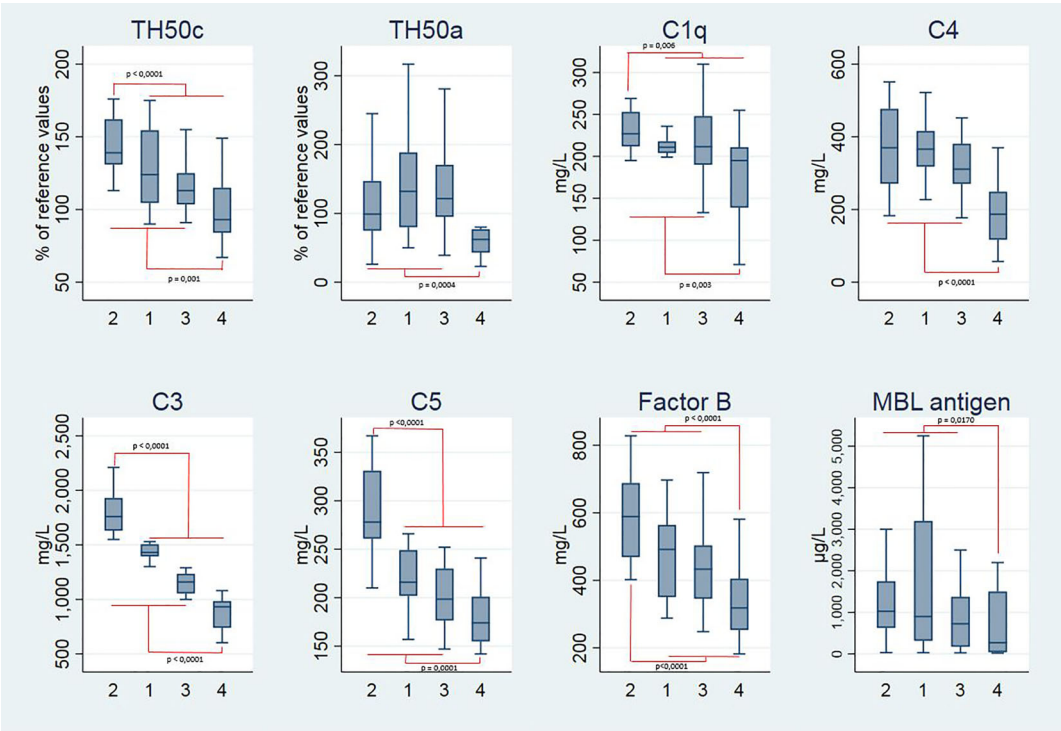


FIGURE 1 | Boxplots representing the complement parameters of the four clusters of patients. Statistical analysis by hierarchical ascendant clustering discriminates the 74 patients with COVID-19 of the cohort in four distinct clusters according to complement variables: TH50c, TH50a, C1q, C4, C3, C5, Factor B, and MBL antigen. Age was included in the model. Boxplots represent the median and 25th to 75th percentiles, the whiskers denote the maximum and minimum values, and the horizontal bars indicate the medians. Outside values were excluded.

Finally, the fourth cluster (n = 15; 20%) (**Tables 3**, **4**; **Supplementary Table S1**) was specifically characterized by significant activation of alternative (TH50a: 62%; p = 0.0004 against other clusters) and lectin (MBL antigen: 270 µg/L: p = 0.0170 against other clusters) complement pathways. Decreased MBL concentration was associated with reduced MBL function (13%) and C4H (67%) confirming the MBL pathway activation. TH50c was also decreased compared to the other clusters (101%; p = 0.001), but remained in the RI (86–126%). Interestingly, in this cluster, 13 patients (87%) exhibited AP activation and 7 patients (47%) exhibited MBL pathway activation; 6 (40%) patients presented simultaneously AP and MBL pathway activation (**Supplementary Table S1**). A large percentage of patients of the fourth cluster was severe (73%) and required ICU admission (53%) and corticoid treatment (47%) (**Table 4**). Among the 11 severe patients, 10 exhibited AP activation; 6 exhibited MBL pathway activation, and 5 presented at the same time AP and MBL pathway activation (**Supplementary Table S1**). Of note, cluster 4 was characterized by a higher mortality rate (27% versus 10%, 0% and 10% in clusters 1, 2, and 3, respectively; p = 0.048) (**Table 4**). Among the four patients who died, three presented AP activation, two exhibited MBL pathway activation, and two patients showed concurrent activation of alternative and MBL pathways (**Supplementary Table S1**). Regarding the four other patients who died, all presented a normal complement profile except patient 1 who exhibited an MBL deficiency (**Supplementary Table S2**).

DISCUSSION

SARS-CoV-2 infection triggers an innate immune response including CS activation which is a key weapon both implicated in disease resolution and organ damage depending on the time of infection (15).

Recent studies addressing the role of complement in the pathogenesis of COVID-19 have shown a relationship between respiratory failure, intravascular coagulopathy, and complement overactivation in COVID-19 patients (3, 16). There are several lines of evidence for local deposition of complement proteins and activation products in lung, skin, and other tissues showing activation of the three pathways, CP, LP, and AP (17–21). Furthermore, systemic complement activation and consumption were related to severe COVID-19 and predictive of in-hospital mortality (22). However, despite *in vitro* lines of evidence suggesting that the SARS-CoV-2 spike proteins activate the AP (23), it remains incompletely understood which complement activation pathways contribute to critical illness in COVID-19.

Concerning the possible involvement of MBL in coronavirus infection, it has been described so far for SARS-CoV in several

TABLE 3 | Clusters of COVID-19 patients according to complement parameters.

Number	RI	Cluster 1 20	Cluster 2 19	Cluster 3 20	Cluster 4 15	p-value	p-value comparison 2 by 2
TH50c % mean (sd)	86–126	128.1 (27.1)	145.2 (27.3)	117.5 (22.7)	100.6 (24.8)	<0.0001[1]	p = 0.001 cluster 4 against others; p < 0.0001 cluster 2 against others
TH50a % median (IQR)	84–150	132 (80;189)	99 (75;147)	122 (95;171)	62 (43;77)	0.003[2]	p = 0.0004 cluster 4 against others
C1q mg/L mean (sd)	154–258	201.4 (40.1)	234.3 (32.5)	215.9 (52.2)	175.2 (52.4)	0.003[1]	p = 0.003 cluster 4 against others: p = 0.006 cluster 2 against others
C4 mg/L mean (sd)	100–380	367.8 (77.8)	374.7 (112.9)	317.1 (79.5)	182.3 (85.1)	<0.0001[1]	p < 0.0001 cluster 4 against others
C3 mg/L mean (sd)	880–1650	1440 (66.7)	1795.3 (189.8)	1149.5 (99.0)	878.1 (149.9)	<0.0001[1]	p < 0.0001cluster 2 against others; cluster 4 against others
Factor B mg/L mean (sd)	216–504	473.5 (134.0)	585.6 (122.6)	436.9 (110.5)	345 (134.8)	<0.0001[1]	p < 0.0001 cluster 2 against others; cluster 4 against others
C5 mg/L median (IQR)	120–220	216 (202;249)	278 (261;331)	199 (177;230)	174 (155;201)	<0.0001[2]	p < 0.0001 cluster 2 against others; p = 0.0001 cluster 4 against others
MBL antigen µg/L median (IQR)	30–3000	900 (318;3200)	1025 (625;1750)	725 (171;1375)	270 (42;1500)	0.0374[2]	p = 0.0170 cluster 4 against others
MBL function* % median (IQR)	35–115	110 (24;187)	117 (62;200)	43 (28;181)	13 (0;168)	0.0558[2]	
C4 hemolytic activity* % mean (sd)	70–130	125 (8.1)	117.3 (50.8)	104.1 (40.8)	66.6 (49.0)	0.004[1]	p = 0.001 cluster 4 against others

Mean (sd) or median (IQR) were presented for the parametric or non-parametric variables, respectively. RI, reference interval.

**Post-hoc analysis was performed for this parameter. [1] Fisher ANOVA test. [2] Kruskal-Wallis test.*

studies. Among those, two *in vitro* studies demonstrated binding of MBL to SARS-CoV or viral particles pseudotyped with SARS-CoV spike protein and activation of the lectin complement pathway (24, 25). More recently, Ali and *al* showed binding of LP recognition molecules to S- and N- proteins of SARS-CoV-2, as also robust LP activation on the surface of HEK 293 cells expressing SARS-CoV-2 S protein (26).

One of the limitation of the study is that it reports data about the original variant of the virus, while the delta variant is now a majority among the infected patients. The crucial mutations leading to delta variant concern the S1 subunit (intimately involved in the initiation of infection) for which we have no data concerning its molecular interaction with complement components. However, the replication rate of delta strain is much higher. It would therefore be interesting to compare our results with samples collected from patients infected with the delta variant of the virus.

Using an original, unsupervised statistical approach by hierarchical clustering on complement and clinical parameters, this study reveals for the first time the association between AP and LP activation, and the mortality in COVID-19 patients (cluster 4). Our data are in line with Ma et al. showing increased AP components in COVID-19 patients with worse prognosis (27) and with Sinkovits et al. relating significant association between AP activity and COVID-19 severity (22).

Furthermore, our results provide additional evidence for an association between MBL pathway activation and mortality, supported by the data of Eriksson et al. (4). In contrast, Sinkovits et al. reported that the LP activity showed no difference between severity groups (22). Our data are consistent with recent reports describing deposits of MBL and MASP2 in affected tissues of COVID-19 patients and *in vitro* MBL pathway activation by recombinant SARS-CoV2 proteins (17, 28). Collectively, these findings are consistent with the CS implication in the pathogenesis of severe COVID-19 infection. As consequences of unrestrained complement activation, the strong pro-inflammatory C5a-C5aR axis promotes neutrophil/monocyte infiltration and the "cytokine storm" driving lung inflammation and injury, responsible for complications in hospitalized COVID-19 patients (2).

If complement activation is evident from our results and associated with the severity of the disease, the spectrum of involvement of the complement cascade in COVID-19 seems to be heterogeneous and depending on patients: notably, complement activation could be deleterious in some ones and expression of the severity of the disease in other ones. A recent review summarizes the current knowledge about modulating complement cascade as therapeutic approach in COVID-19 patients (29). In brief, despite nonconclusive studies, the available data suggested favorable outcomes in a small number of patients with severe COVID-19 treated either with C1 inhibitor, MASP-2 monoclonal antibodies, compstatin-based complement C3 inhibitor, anti-C5 drugs, or C5a-C5aR1 antagonists. Clinical trials of complement inhibitors in COVID-19 are ongoing (NCT04288713, NCT04414631, NCT04395456). However, awaiting final results from the clinical trials, the potential benefits from complement inhibition in COVID-19 remain to be elucidated.

TABLE 4 | Overall comparison of clinical and biological characteristics between the clusters.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	p-value global test
Number	**20**	**19**	**20**	**15**	
Men, n (%)	12 (60%)	14 (74%)	8 (40%)	9 (60%)	0.2109[1]
Age, (years) mean, sd	70 (15)	69 (14)	72 (14)	73 (14)	0.6265[2]
CRP, (mg/L) mean (sd) (n=73)	89.4 (84.89)	82.1 (70.77)	47.8 (44.08)	63.7 (53.86)	0.2598[2]
Severe COVID-19[3], n (%)	14 (70%)	9 (47.37%)	9 (45%)	11 (73.33%)	0.1912[1]
ICU admission, n (%)	6 (30%)	5 (26.32%)	4 (20%)	8 (53.33%)	0.199[1]
Oxygen requirement, n (%)	18 (90%)	13 (68.42%)	11 (55%)	12 (80%)	0.774[1]
Mortality, dead, n (%)	2 (10%)	0 (0%)	2 (10%)	4 (26.67%)	0.048[4]
Corticoid requirement, n (%)	10 (50%)	4 (21.05%)	2 (10%)	7 (46.67%)	0.0181[1]

Reference values: CRP < 10 mg/L.

[1]Fisher exact test. [2] Kruskal-Wallis test. [3] Severe COVID-19 defined as: O_2 > 2 L/min, ICU [intensive care unit] admission, LTE [limitation of the therapeutic effort], decease. [4] Post-hoc analysis with Fisher exact test.

Interestingly, we found high incidence of MBL expression deficiency in patients of our cohort (16%, established as described in Methods), all clusters combined. Further studies based on MBL genotyping would be of interest to support biochemical data. MBL deficiency is fairly common, affecting approximately 5–10% of individuals and usually associated with increased susceptibility to bacterial infections of the upper respiratory in young children (30).

Our results highlight the dichotomous nature of the complement MBL pathway: on one hand, MBL appears to contribute to the pathogenesis of disease because it mediates complement activation that is related to clinical deterioration of patients; on the other hand, MBL could have a protective role against SARS-CoV-2 infection by promoting phagocytosis and virus lysis or neutralization. Further *in vitro* studies using pseudoviral particles or the SARS-CoV-2 virus are necessary to check the latter hypothesis.

In summary, our study suggests that alternative and lectin pathways assessment might be useful as biomarker of disease severity. Extensive investigations of complement pathways have to be performed on a larger cohort of patients with SARS-CoV-2 infection to help rationalize therapeutic choices.

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 human participants were reviewed and approved by Commission Nationale de l'Informatique et des Libertés (CNIL). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Study conception: FD, CD-P, and J-YC. Immunological determinations: FD, CD-P, M-CJ, TR, and MP. Methodology: MR. Statistical analysis: CL, AV, and J-LB. Collection of patients' samples and clinical information: OE and MM. Funding acquisition: FD, CD-P, NT, OE, GC, AG, and PP. Writing original draft: FD and CD-P. Review manuscript: J-YC and NT. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by funding from the Université Grenoble Alpes (projects COMPLEC-COV and BIOMARCOVID).

ACKNOWLEDGMENTS

The authors are grateful for the excellent work from the staff of technicians at Complement Laboratory of Grenoble-Alpes University Hospital: Marion Allegret-Cadet, Véronique Bergerot, Nadège Fondraz, Marie-Anne Pasquier and from Pierre Audoin for help in project conception.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.742446/full#supplementary-material

REFERENCES

1. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. HLH Across Speciality Collaboration, UK. COVID-19: Consider Cytokine Storm Syndromes and Immunosuppression. *Lancet* (2020) 395:1033–4. doi: 10.1016/S0140-6736(20)30628-0
2. Carvelli J, Demaria O, Vély F, Batista L, Benmansour NC, Fares J, et al. Association of COVID-19 Inflammation With Activation of the C5a-C5aR1 Axis. *Nature* (2020) 588:146–50. doi: 10.1038/s41586-020-2600-6
3. Holter JC, Pischke SE, de Boer E, Lind A, Jenum S, Holten AR, et al. Systemic Complement Activation is Associated With Respiratory Failure in COVID-19

Hospitalized Patients. *Proc Natl Acad Sci USA* (2020) 117:25018–25. doi: 10.1073/pnas.2010540117
4. Eriksson O, Hultström M, Persson B, Lipcsey M, Ekdahl KN, Nilsson B, et al. Mannose-Binding Lectin Is Associated With Thrombosis and Coagulopathy in Critically Ill COVID-19 Patients. *Thromb Haemost* (2020) 120:1720–4. doi: 10.1055/s-0040-1715835
5. Diurno F, Numis FG, Porta G, Cirillo F, Maddaluno S, Ragozzino A, et al. Eculizumab Treatment in Patients With COVID-19: Preliminary Results From Real Life ASL Napoli 2 Nord Experience. *Eur Rev Med Pharmacol Sci* (2020) 24:4040–7. doi: 10.26355/eurrev_202004_20875
6. Laurence J, Mulvey JJ, Seshadri M, Racanelli A, Harp J, Schenck EJ, et al. Anti-Complement C5 Therapy With Eculizumab in Three Cases of Critical COVID-19. *Clin Immunol* (2020) 219:108555. doi: 10.1016/j.clim.2020.108555
7. Peffault de Latour R, Bergeron A, Lengline E, Dupont T, Marchal A, Galicier L, et al. Complement C5 Inhibition in Patients With COVID-19 - A Promising Target? *Haematologica* (2020) 105:2847–50. doi: 10.3324/haematol.2020.260117
8. Annane D, Heming N, Grimaldi-Bensouda L, Frémeaux-Bacchi V, Vigan M, Roux A-L, et al. Eculizumab as an Emergency Treatment for Adult Patients With Severe COVID-19 in the Intensive Care Unit: A Proof-of-Concept Study. *EClinicalMedicine* (2020) 28:100590. doi: 10.1016/j.eclinm.2020.100590
9. Mastaglio S, Ruggeri A, Risitano AM, Angelillo P, Yancopoulou D, Mastellos DC, et al. The First Case of COVID-19 Treated With the Complement C3 Inhibitor AMY-101. *Clin Immunol* (2020) 215:108450. doi: 10.1016/j.clim.2020.108450
10. WHO. *COVID-19 Therapeutic Trial Synopsis* World Health Organisation. (2020). Novel Coronavirus. Available at: https://www.who.int/publications/i/item/covid-19-therapeutic-trial-synopsis [Accessed June 9, 2020].
11. Dumestre-Pérard C, Lamy B, Aldebert D, Lemaire-Vieille C, Grillot R, Brion J-P, et al. Aspergillus Conidia Activate the Complement by the Mannan-Binding Lectin C2 Bypass Mechanism. *J Immunol* (2008) 181:7100–5. doi: 10.4049/jimmunol.181.10.7100
12. Dumestre-Perard C, Ponard D, Arlaud GJ, Monnier N, Sim RB, Colomb MG. Evaluation and Clinical Interest of Mannan Binding Lectin Function in Human Plasma. *Mol Immunol* (2002) 39:465–73. doi: 10.1016/s0161-5890(02)00119-0
13. Dumestre-Perard C, Ponard D, Drouet C, Leroy V, Zarski J-P, Dutertre N, et al. Complement C4 Monitoring in the Follow-Up of Chronic Hepatitis C Treatment. *Clin Exp Immunol* (2002) 127:131–6. doi: 10.1046/j.1365-2249.2002.01729.x
14. Garred P, Madsen HO, Hofmann B, Svejgaard A. Increased Frequency of Homozygosity of Abnormal Mannan-Binding-Protein Alleles in Patients With Suspected Immunodeficiency. *Lancet* (1995) 346:941–3. doi: 10.1016/S0140-6736(95)91559-1
15. Kim AHJ, Wu X, Atkinson JP. The Beneficial and Pathogenic Roles of Complement in COVID-19. *Cleve Clin J Med* (2020) 88:1–4. doi: 10.3949/ccjm.87a.ccc065
16. de Nooijer AH, Grondman I, Janssen NAF, Netea MG, Willems L, van de Veerdonk FL, et al. RCI-COVID-19 Study Group. Complement Activation in the Disease Course of Coronavirus Disease 2019 and Its Effects on Clinical Outcomes. *J Infect Dis* (2021) 223:214–24. doi: 10.1093/infdis/jiaa646
17. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement Associated Microvascular Injury and Thrombosis in the Pathogenesis of Severe COVID-19 Infection: A Report of Five Cases. *Transl Res* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
18. Prendecki M, Clarke C, Medjeral-Thomas N, McAdoo SP, Sandhu E, Peters JE, et al. Temporal Changes in Complement Activation in Haemodialysis Patients With COVID-19 as a Predictor of Disease Progression. *Clin Kidney J* (2020) 13:889–96. doi: 10.1093/ckj/sfaa192
19. Macor P, Durigutto P, Mangogna A, Bussani R, D'Errico S, Zanon M, et al. Multi-Organ Complement Deposition in COVID-19 Patients. *medRxiv* (2021) 9:1–15. doi: 10.1101/2021.01.07.21249116
20. Satyam A, Tsokos MG, Brook OR, Hecht JL, Moulton VR, Tsokos GC. Activation of Classical and Alternative Complement Pathways in the Pathogenesis of Lung Injury in COVID-19. *Clin Immunol* (2021) 226:108716. doi: 10.1016/j.clim.2021.108716
21. Pfister F, Vonbrunn E, Ries T, Jäck H-M, Überla K, Lochnit G, et al. Complement Activation in Kidneys of Patients With COVID-19. *Front Immunol* (2020) 11:594849. doi: 10.3389/fimmu.2020.594849
22. Sinkovits G, Mező B, Réti M, Müller V, Iványi Z, Gál J, et al. Complement Overactivation and Consumption Predicts In-Hospital Mortality in SARS-CoV-2 Infection. *Front Immunol* (2021) 12:663187. doi: 10.3389/fimmu.2021.663187
23. Yu J, Yuan X, Chen H, Chaturvedi S, Braunstein EM, Brodsky RA. Direct Activation of the Alternative Complement Pathway by SARS-CoV-2 Spike Proteins is Blocked by Factor D Inhibition. *Blood* (2020) 136:2080–9. doi: 10.1182/blood.2020008248
24. Zhou Y, Lu K, Pfefferle S, Bertram S, Glowacka I, Drosten C, et al. A Single Asparagine-Linked Glycosylation Site of the Severe Acute Respiratory Syndrome Coronavirus Spike Glycoprotein Facilitates Inhibition by Mannose-Binding Lectin Through Multiple Mechanisms. *J Virol* (2010) 84:8753–64. doi: 10.1128/JVI.00554-10
25. Ip WKE, Chan KH, Law HKW, Tso GHW, Kong EKP, Wong WHS, et al. Mannose-Binding Lectin in Severe Acute Respiratory Syndrome Coronavirus Infection. *J Infect Dis* (2005) 191:1697–704. doi: 10.1086/429631
26. Ali YM, Ferrari M, Lynch NJ, Yaseen S, Dudler T, Gragerov S, et al. Lectin Pathway Mediates Complement Activation by SARS-CoV-2 Proteins. *Front Immunol* (2021) 12:2645–52. doi: 10.3389/fimmu.2021.714511
27. Ma L, Sahu SK, Cano M, Kuppuswamy V, Bajwa J, McPhatter J, et al. Increased Complement Activation is a Distinctive Feature of Severe SARS-CoV-2 Infection. *Sci Immunol* (2021) 6:1–18. doi: 10.1126/sciimmunol.abh2259
28. Gao T, Hu M, Zhang X, Li H, Zhu L, Dong Q, et al. Highly Pathogenic Coronavirus N Protein Aggravates Lung Injury by MASP-2-Mediated Complement Over-Activation. *medRxiv* [preprint]. doi: 10.1101/2020.03.29.20041962v3.
29. Fodil S, Annane D. Complement Inhibition and COVID-19: The Story So Far. *Immunotargets Ther* (2021) 10:273–84. doi: 10.2147/ITT.S284830
30. Heitzeneder S, Seidel M, Förster-Waldl E, Heitger A. Mannan-Binding Lectin Deficiency — Good News, Bad News, Doesn't Matter? *Clin Immunol* (2012) 143:22–38. doi: 10.1016/j.clim.2011.11.002

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 28 October 2021
doi: 10.3389/fimmu.2021.765330

Functional Activity of the Complement System in Hospitalized COVID-19 Patients: A Prospective Cohort Study

Panteleimon Charitos[1], Ingmar A. F. M. Heijnen[2], Adrian Egli[3,4], Stefano Bassetti[1], Marten Trendelenburg[1,5,6†] and Michael Osthoff[1,5,6†]*

[1] Division of Internal Medicine, University Hospital Basel, Basel, Switzerland, [2] Division of Medical Immunology, Laboratory Medicine, University Hospital Basel, Basel, Switzerland, [3] Clinical Bacteriology and Mycology, Laboratory Medicine, University Hospital Basel, Basel, Switzerland, [4] Applied Microbiology Research, Department of Biomedicine, University of Basel, Basel, Switzerland, [5] Department of Biomedicine, University of Basel, Basel, Switzerland, [6] Department of Clinical Research, University of Basel, Basel, Switzerland

OPEN ACCESS

Edited by:
Zoltán Prohászka, Semmelweis University, Hungary

Reviewed by:
Maciej Cedzynski, Institute for Medical Biology (PAN), Poland
Brandon Henry, Cincinnati Children's Hospital Medical Center, United States

***Correspondence:**
Michael Osthoff
michael.osthoff@usb.ch

†These authors have contributed equally to this work and share last authorship

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *26 August 2021*
Accepted: *14 October 2021*
Published: *28 October 2021*

Citation:
Charitos P, Heijnen IAFM, Egli A, Bassetti S, Trendelenburg M and Osthoff M (2021) Functional Activity of the Complement System in Hospitalized COVID-19 Patients: A Prospective Cohort Study. Front. Immunol. 12:765330. doi: 10.3389/fimmu.2021.765330

Aims: Although the exact factors promoting disease progression in COVID-19 are not fully elucidated, unregulated activation of the complement system (CS) seems to play a crucial role in the pathogenesis of acute lung injury (ALI) induced by SARS-CoV-2. In particular, the lectin pathway (LP) has been implicated in previous autopsy studies. The primary purpose of our study is to investigate the role of the CS in hospitalized COVID-19 patients with varying degrees of disease severity.

Methods: In a single-center prospective observational study, 154 hospitalized patients with PCR-confirmed SARS-CoV-2 infection were included. Serum samples on admission to the COVID-19 ward were collected for analysis of CS pathway activities and concentrations of LP proteins [mannose-binding lectin (MBL) and ficolin-3 (FCN-3)] & C1 esterase inhibitor (C1IHN). The primary outcome was mechanical ventilation or in-hospital death.

Results: The patients were predominately male and had multiple comorbidities. ICU admission was required in 16% of the patients and death (3%) or mechanical ventilation occurred in 23 patients (15%). There was no significant difference in LP activity, MBL and FCN-3 concentrations according to different peak disease severities. The median alternative pathway (AP) activity was significantly lower (65%, IQR 50-94) in patients with death/invasive ventilation compared to patients without (87%, IQR 68-102, p=0.026). An optimal threshold of <65.5% for AP activity was derived from a ROC curve resulting in increased odds for death or mechanical ventilation (OR 4,93; 95% CI 1.70-14.33, p=0.003) even after adjustment for confounding factors. Classical pathway (CP) activity was slightly lower in patients with more severe disease (median 101% for death/mechanical ventilation vs 109%, p=0.014). C1INH concentration correlated positively with length of stay, inflammatory markers and disease severity on admission but not during follow-up.

Conclusion: Our results point to an overactivated AP in critically ill COVID-19 patients *in vivo* leading to complement consumption and consequently to a significantly reduced AP activity *in vitro.* The LP does not seem to play a role in the progression to severe COVID-19. Apart from its acute phase reaction the significance of C1INH in COVID-19 requires further studies.

Keywords: COVID-19, C1 esterase inhibitor, SARS-CoV-2, inflammation, complement system, mannose-binding lectin, ficolin-3

INTRODUCTION

In December 2019, a novel coronavirus was identified as the cause of a cluster of pneumonia cases in Wuhan, a city in the Hubei Province of China. It rapidly spread, resulting in an epidemic throughout China, followed by an unprecedented worldwide pandemic with more than 202 million identified cases and more than 4 million deaths (1). In February 2020, the World Health Organization designated the disease COVID-19, which stands for coronavirus disease 2019. The virus that causes COVID-19 was designated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The clinical spectrum of COVID-19 ranges from asymptomatic carriers to respiratory failure requiring respiratory support in the intensive care unit (ICU). In the latter setting, a dysregulated immune response characterized by a decrease in suppressor T cell counts, excessive release of pro-inflammatory cytokines, and activation of several inflammatory cascades including the contact activation, coagulation and complement cascade, contributes to the observed organ dysfunction (2–4).

The complement system is an integral part of the innate immune system and acts as a first line of defence by inducing an inflammatory response after opsonisation of pathogens and dying cells (5, 6). The complement cascade is initiated by at least three pathways, i.e. the classical, the lectin, and the alternative pathway. The lectin pathway (LP) of complement is activated after binding of its pattern-recognition receptors (PRR) including mannose-binding lectin (MBL) and the ficolins to carbohydrate patterns, acetyl groups or immunoglobulin M with subsequent activation of MBL-associated serine protease (MASP)-1 and -2 and assembly of the C3 convertase (7). Inter-individual serum concentrations of LP PRR vary to a considerable degree with the greatest differences observed in MBL levels (from undetectable to about 10 μg/mL) (8, 9).

The complement system and particularly the LP has been found to interact with and be involved in the clearance of a number of viruses (10–14). Importantly, its contribution to the risk and severity of SARS-CoV, that emerged in 2002 to 2003 and led to a global outbreak of SARS, has been evaluated in detail previously. Binding of MBL to SARS-CoV was demonstrated, which consequently interfered with efficient viral entry (15, 16). More importantly, several case-control studies have documented an increased susceptibility to SARS-CoV infection in patients with low MBL concentrations or certain low-producing genotypes (16–18) but not for the presence of MASP-2 polymorphisms (19). In COVID-19, convincing data has emerged showing that complement system activation mediates thrombo-inflammation and may contribute to respiratory failure and mortality (20, 21). Regarding the lectin pathway, MASP-2 was found to interact with SARS-CoV-2 leading to uncontrolled activation of the complement cascade (22). In line, pulmonary vascular MASP-2 deposits were demonstrated in an autopsy study of critically ill COVID-19 patients, and MBL concentrations were higher in patients with thromboembolic events (21, 23). However, data on the role of the LP are controversial. In a recent study, *MBL2* variants associated with lower MBL levels were more frequently encountered in COVID-19 compared to control patients and were associated with a more severe disease suggesting a protective role of MBL (24).

Ficolin-3 (FCN3) is the most abundant of the three ficolins in serum, and the only one that is expressed in the lungs even exceeding its expression in the liver (25). Although data on SARS-CoV-2 are lacking, interactions with seasonal influenza viruses have been described (26, 27). In a small study, increased FCN3 concentrations were observed in severe COVID-19 in Asian patients with significant renal disease (28). Lastly, C1 esterase inhibitor (C1INH) is a serine-protease inhibitor of manifold targets including the classical pathway (CP) and LP of the complement system (29). In line with its role as an acute phase protein, elevated serum concentrations have been documented in severe COVID-19 (30, 31), whereas its expression in bronchoalveolar lavage samples was significantly decreased (32). Interestingly, C1INH was able to block MASP-2 mediated overactivation of the complement system and lung injury induced by several pathogenic coronaviruses (22).

With regards to COVID-19, it remains to be elucidated, which complement pathway crucially contributes to complement activation and its clinical consequences. Previous studies have pointed to the LP and alternative pathway (AP) (31, 33–35).

Given the paucity of data regarding the lectin pathway of complement in SARS-CoV-2 infection, we aimed to investigate serum concentrations of two important PRR of the LP and of its predominant inhibitor (i.e. C1INH) as well as to clarify the role of complement pathway activities in a well-characterized cohort of COVID-19 patients with respect to severity and outcome.

MATERIALS AND METHODS

Ethics Statement

The study protocol was approved by the Ethics Committee of Northwest and Central Switzerland (EKNZ 2020-00769) with a

waiver for informed consent. The study was performed in accordance with the latest version of the declaration of Helsinki and the guidelines for good clinical practice.

Patient Inclusion and Sample Collection

This prospective observational cohort study was performed at a single tertiary care center in Switzerland. Consecutive patients with a SARS-CoV-2 infection confirmed by polymerase chain reaction (PCR) from a nasopharyngeal swab and admitted to the hospital between March and May 2020 were included. Serum samples were collected on admission to the COVID-19 ward during routine blood sampling, transferred to the laboratory immediately after sampling, centrifuged, aliquoted and stored at −80°C until measurement of the complement pathway activities and concentrations of MBL, FCN-3 and C1INH. According to the institutional protocol at that time most patients received lopinavir/ritonavir and hydroxychloroquine treatment, but not corticosteroids. In addition, patients who deteriorated with a C-reactive protein (CRP) above 70 mg/L and/or progressive lung involvement on repeat chest computed tomography (CT) scan were treated with tocilizumab (8 mg/kg body weight up to 800 mg with a repeat dose after 24 to 48 hours if required) on a compassionate use basis.

Patients were excluded if they had refused the general research consent of our institution, if a serum sample was not available on admission or if patients were transferred to another institution within 72 hours (lost to follow-up).

Laboratory Assessment

Complement pathway activities (WIESLAB® Complement system Screen kit, Svar Life Science AB, Sweden) and C1INH antigen concentration (Siemens, Marburg, Germany) were determined on semi-automated platforms in the Clinical Laboratory of the University Hospital according to the manufacturer's instruction and standard operating procedures. Low CP and AP activities are usually caused by *in-vivo* activated pathways with consumption of complement components, whereas low LP activity is usually due to low MBL concentrations.

For FCN-3 a research-use-only enzyme-linked immunosorbent assay (ELISA) kit was used according to the manufacturer's instruction (Hycult Biotech, Uden, the Netherland). MBL concentration was quantified using an in-house ELISA with mannan coating as previously described (36). A biotinylated mouse anti-human MBL antibody (HYB131-01B, Bioporto Diagnostics, Denmark) was used for detection and a pooled human serum with known MBL concentration (BioPorto Diagnostics, Denmar) was used to generate a standard curve.

Primary and Secondary Outcomes and Data Extraction

The primary outcome of this study was a composite clinical outcome combining in-hospital all-cause mortality and need of intubation and mechanical ventilation, corresponding to a score of 6-8 on the WHO ordinal scale for clinical improvement (37). Secondary outcomes were length of stay (LOS) until discharge to home or a non-acute care (rehabilitation) center or death occurred and correlation with inflammatory markers.

Clinical data and laboratory results were collected from the electronic health records and recorded in an electronic database (Research Electronic Data Capture). Patients were followed up for the primary and secondary clinical outcome until death or discharge occurred.

Semi-automatic quantification of affected lung tissue on a CT scan was performed using software for lung density analysis in Chest CT [CT Pulmo 3D included in Syngo.Via VB30A, Siemens Healthineers, Forchheim, Germany; method similar as described (38)]. After semi-automatic segmentation of the lungs a threshold analysis of Hounsfield units (HU) was pursued, where pulmonary involvement was defined as the percentage of lung parenchyma with a CT-density between -600 and 0 HU.

Statistical Analysis

Statistical analysis was performed using SPSS software, version 25.0 (IBM, USA), and GraphPad Prism 7 software was used for visualization (GraphPadSoftwares Inc., La Jolla, Ca, USA). Most of the variables showed skewed distributions and for this reason data are represented as medians with interquartile range (IQR), if not mentioned otherwise. Data from normally distributed variables are presented as means with standard deviation (SD). Nominal data are presented as frequencies (%). Differences between patient groups were assessed using the Mann-Whitney U test for continuous variables, and the chi-square or Fisher's exact test for nominal variables. Correlations between complement factors and the secondary clinical outcome (LOS) or inflammatory markers were assessed using Spearman rank correlation tests. Multivariable logistic regression models were performed to analyze associations between complement parameters and the composite outcome of mechanical ventilation and/or in-hospital death. Results are presented as odds ratios (OR) with corresponding 95% confidence intervals (CI). Variable selection was based on biologic plausibility and/or demonstrated associations. Differences between groups or correlations were considered statistically significant at the level of $p < 0.05$ (2-tailed).

RESULTS

Patient Characteristics

Overall, 154 of 189 patients with PCR-confirmed SARS-CoV-2 infection and hospitalized during the first wave were included in this study. Patients were predominately male (94/154, 61%) with a median age of 62 years (IQR 49-73). Frequent comorbidities included arterial hypertension, obesity (body mass index (BMI) ≥30 kg/m^2], and cardiovascular disease (**Table 1**). The median duration of symptoms before admission was 7 days [IQR 3-11). 57 patients (37%) had a SOFA score of at least 2 points on admission, indicating severe disease. Median peak viral load in nasopharyngeal swab samples and median peak affected lung volume on computed tomography scan of the chest were 118'900 copies/ml (IQR 9'700-1'705'825) and 14% (IQR 6.6-25) of the total lung volume, respectively. Further demographic and baseline characteristics are reported in **Table 1**.

TABLE 1 | Demographic and baseline characteristics, therapeutic management and outcomes of the entire patient cohort and according to the composite outcome of mechanical ventilation and/or in-hospital death.

Variables	Total n=154	Patients without the composite outcome n=131	Patients with the composite outcome n=23	p-values
Demographics				
Male sex, n (%)	94 (61)	77 (59)	17 (74)	0.170
Age on admission in years, mean (SD)	61 (16)	60 (16)	65 (14)	0.205
Body mass index (BMI) in kg/m^{2,} median (IQR)	27 (24-32)	27 (24-31)	30 (26-34)	0.093
Comorbidities				
Arterial hypertension, n (%)	81 (53)	64 (49)	17 (74)	**0.026**
Obesity (BMI>=30 kg/m^2), n (%)	52 (34)	40 (31)	12 (52)	**0.043**
Diabetes mellitus, n (%)	32 (21)	26 (20)	6 (26)	0.496
Chronic lung disease, n (%)	31 (20)	24 (18)	7 (30)	0.181
Cardiovascular disease, n (%)	46 (30)	36 (27)	10 (43)	0.122
Chronic renal failure, n (%)	25 (16)	19 (15)	6 (26)	0.170
Solid or haematological cancer, n (%)	21 (14)	17 (13)	4 (17)	
Immunosuppression, n (%)	21 (14)	17 (13)	4 (17)	0.569
Charlson comorbidity index, median (IQR)	3 (1-5)	2 (1-5)	4 (2-7)	0.109
Clinical characteristics				
Symptom duration before admission in days, median (IQR)	7 (3-11)	7 (3-11)	8 (6-12)	0.449
SOFA score on admission, median (IQR)	1 (0-2)	1 (0-2)	2 (1-3)	**0.000**
NEWS2 score on admission, median (IQR)	3 (2-5)	3 (2-5)	4.5 (2.5-7.5)	**0.028**
Oxygen saturation on admission, median (IQR)	95 (93-97)	96 (94-97)	93 (90-96)	**0.001**
Presenting symptoms				
Cough, n (%)	101 (66)	86 (66)	15 (65)	0.968
Fever, n (%)	91 (59)	74 (56)	17 (74)	0.117
Shortness of breath, n (%)	54 (35)	45 (34)	9 (39)	0.658
Routine laboratory findings (with normal ranges) on admission, median (IQR)				
Leukocytes x10^9/l (3.5-10.0)	6.0 (4.2-8.2)	6.0 (4.1-8.2)	5.9 (4.8-8.1)	0.94
Lymphocytes x10^9/l (0.9-3.3)	1.0 (0.6-1.3)	1.0 (0.7-1.4)	0.7 (0.5-1.1)	**0.014**
Platelets x10^9/l (150-450)	206 (146-242)	209 (150-275)	171 (131-220)	**0.032**
C-reactive protein in mg/l (<10.0)	39.5 (16.4-76.2)	38 (14.5-72.1)	72.2 (33.5-112.6)	**0.016**
Interleukin-6 in ng/l (<7.0)	80.8 (36.1-107)	57.4 (24.8-87.3)	105.0 (56.9-210.5)	**0.003**
Ferritin in μg/l (10-200)	586 (288-1234)	555 (279-1202)	865 (406-2243)	0.146
D-dimer in μg/l (0.19-0.50)	0.72 (0.42-1.58)	0.73 (0.40-1.68)	0.66 (0.47-1.27)	1.0
LDH in IU/l (135-225)	269 (222-360)	265 (218-352)	340 (232-455)	0.085
Peak viral load in nasopharyngeal swab in copies/ml	118`900 (9`700-1`705`825)	78`900 (7`900-1`425`600)	692`400 (115`100-3`095`900)	**0.02**
Peak affected lung volume as percentage (%) on computed tomography scan of the chest, median (IQR)	14.0 (6.6-25.0)	12.0 (6.0-20.1)	29.0 (24.3-36.3)	**<0.001**
Treatment				
Lopinavir/ritonavir, n (%)	100 (65)	81 (62)	19 (83)	0.054
Hydroxychloroquine, n (%)	125 (81)	103 (79)	22 (96)	0.054
Remdesivir, n (%)	7 (5)	0 (0)	7 (30)	**<0.001**
Tocilizumab, n (%)	42 (27)	25 (19)	17 (74)	**<0.001**
Antibiotics, n (%)	67 (44)	46 (35)	21 (91)	**<0.001**
Outcomes				
In-hospital mortality, n (%)	4 (3)	0 (0)	4 (17)	**<0.001**
ICU admission, n (%)	25 (16)	6 (5)	19 (83)	**<0.001**
[with median (IQR) LOS in ICU in days]	[8 (3-13)]	[1 (1-3)]	[10 (6-13)]	
LOS in days, median (IQR)	8 (5-11)	6 (4-10)	20 (12-28)	**<0.001**

BMI, body mass index; ICU, intensive-care unit; IQR, interquartile range; LDH, lactate dehydrogenase; LOS, length of stay; NEWS2, National Early Warning Score 2; SOFA, sepsis-related organ failure assessment score; SD, standard deviation.
Statistically significant results ($p < 0.05$) are marked in boldface font.

In total, 25 patients (16%) required transfer to the intensive care unit (ICU) during the disease course, 10 (8%) of whom were transferred less than 24 hours after their initial admission to the COVID-19 ward. Death (4/154, 3%) and/or mechanical ventilation occurred in 23 patients (15%). These patients suffered more frequently from arterial hypertension or obesity, presented with more advanced disease, and were more likely to receive antibiotic, antiviral and anti-inflammatory therapies (**Table 1**).

Association With Disease Severity

Neither FCN-3, MBL, C1INH serum concentrations nor LP activity on admission were associated with the occurrence of the composite outcome of mechanical ventilation or in-hospital

death (**Table 2**). Furthermore, moderate and severe MBL deficiencies, defined as serum concentration below 500 ng/ml and below 100 ng/ml, respectively, were not associated with the clinical outcome. In contrast, CP activity was slightly lower and AP activity significantly lower in patients who required mechanical ventilation and/or died [CP activity, median (IQR) 101% (91-110) vs. 109% (97-119), p=0.014; AP activity, median (IQR) 65% (50-94) vs. 87% (68-102), p=0.026] (**Figure 1**) suggesting complement consumption due to *in-vivo* activation *via* the AP and to a lesser degree *via* the CP of complement. Due to the larger median difference, we explored receiver-operator characteristics analysis of the AP activity to evaluate if a dichotomous cut-off might differentiate between patients with and without the composite outcome (**Figure S1**). An optimal cut-off of 65.5% for the AP activity was derived from this analysis (AUC 0.656, p=0.026) and was tested in the multivariable analysis. Patients with an AP activity of less than 65.5% on admission had higher odds for in-hospital death or mechanical ventilation (OR 4.93 (IQR 1.70-14.33), p = 0.003) after adjustment for comorbidities and disease severity on admission (**Table 3**).

Regarding the secondary outcomes, only C1INH and the AP activity showed a weak correlation with disease severity on admission as assessed by the SOFA score. Only C1INH correlated weakly with length of stay (r = 0.266, p = 0.002; **Table 4**). In line, an AP activity below 65.5% was more frequently encountered in patients with a SOFA score of at least 2 on admission (38.6% vs. 20.6%, p=0.016).

Correlation With Inflammation

Admission CP, LP activity and MBL concentrations correlated only weakly with CRP and ferritin sampled at the same time point, but not with D-Dimer or lymphocyte count (**Table 4**). In line, MBL deficiency < 500 ng/ml was only associated with a lower ferritin concentration on admission [median 433 µg/l (IQR 238-738) vs. 844 µg/l (IQR 335-1392) in patients without MBL deficiency < 500 ng/ml, p=0.008]. For FCN-3, correlations with inflammatory markers were essentially absent, whereas C1INH – the natural inhibitor of the LP and CP – correlated positively with several inflammatory markers on admission, in particular CRP (r = 0.455, p < 0.001) and ferritin (r = 0.477, p < 0.001 (**Table 4**). In addition, C1INH correlated positively with peak CRP (r = 0.469, p<0.001), peak ferritin and lactate dehydrogenase concentrations (r = 0.546, p<0.001 and r = 0.545, p<0.001, respectively), and peak affected lung volume on CT scan of the chest (r = 0.381, p<0.001). Interestingly, a higher C1INH concentration on admission was associated with the subsequent administration of tocilizumab [median 0.51 (IQR 0.46-0.57) g/L vs. 0.45 (IQR 0.39-0.53) g/L in patients not receiving tocilizumab, p=0.003].

DISCUSSION

The complement system has been implicated in the pathogenesis and severity of COVID-19 in several studies (20, 21, 35). The present study is the largest study to assess serum MBL, ficolin-3 and C1INH concentrations as well as CP, AP and LP activity in a well-characterized cohort of COVID-19 patients. In addition, it extends previous data on the role of the AP in COVID-19 by presenting data on the association of AP activity measured on admission with a composite outcome of mechanical ventilation and/or in-hospital death.

Neither serum concentrations of MBL and FCN-3 nor LP activity on admission were associated with mechanical ventilation and/or in-hospital death, and only a very weak correlation with markers of inflammation was observed. This is surprising given the proposed involvement of MBL and the LP in the pathogenesis of SARS-CoV-2 infection. MBL and other pattern-recognition receptors of the LP bind directly to SARS-CoV-2 nucleocapsid and spike proteins, and LP activation was demonstrated upon binding (39). In addition, complement mediated injury has been documented in the lungs and skin of patients with severe COVID-19 with significant deposition of MASP-2, MBL and even ficolin-3 (21, 40), and MASP-2 deficient mice were protected from severe disease (22). Lastly, Shen B et al. documented a significant upregulation of mannose in sera of severe COVID-19 patients, which may lead to complement activation upon binding of MBL to mannose (41). Our results are in line with observations from smaller cohorts that have not found a an association of MBL or FCN-3 serum concentrations (20, 23, 28) or LP activity (34) with outcome or severity in

TABLE 2 | Complement parameters in the entire cohort and according to the composite outcome of mechanical ventilation and/or in-hospital death.

Complement parameter Median (IQR) or n (%)	Total n=154	Patients without the composite outcome n=131	Patients with the composite outcome n=23	p-values
Lectin pathway activity, %	69 (5-126)	77 (5-126)	57 (5-112)	0.392
Classical pathway activity, %	108 (95-119)	109 (97-119)	101 (91-110)	**0.014**
Alternative pathway activity, %	86 (65-100)	87 (68-102)	65 (50-94)	**0.026**
MBL in ng/ml	1`913 (261-4419)	1`858 (305-4`320)	2`280 (36-5`177)	0.763
MBL < 500 ng/ml	44 (28.6)	37 (28.2)	7 (30.4)	0.805
MBL < 100 ng/ml	32 (20.8)	25 (19.1)	7 (30.4)	0.260
FCN-3 in ng/ml	40`728 (28`036-50`971)	41`063 (30`121-51`080)	34`681 (23`725-48`444)	0.341
C1INH in g/l	0.47 (0.40-0.54)	0.46 (0.40-0.54)	0.50 (0.41-0.57)	0.444

C1INH, C1 esterase inhibitor; FCN-3, ficolin-3; MBL, mannose-binding lectin.
Statistically significant results (p < 0.05) are marked in boldface font.

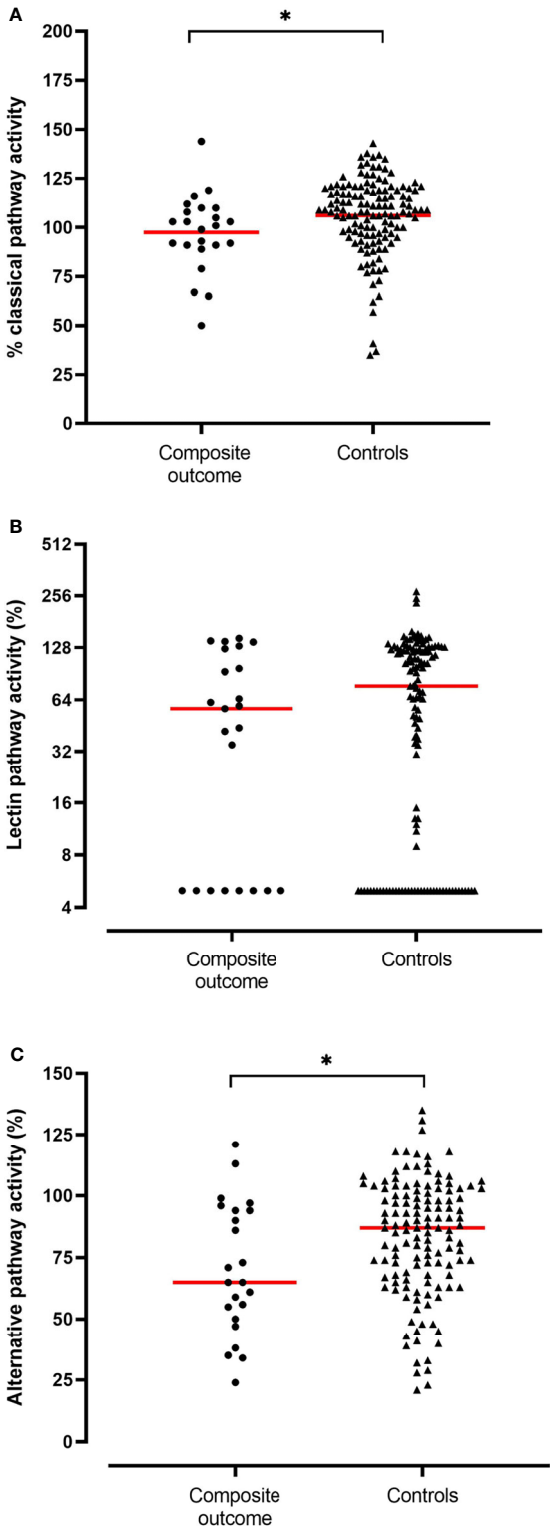


FIGURE 1 | Activity levels (in %) of the classical **(A)**, lectin **(B)** and alternative pathway **(C)** in patients who required mechanical ventilation or died during the hospitalization (n = 23, composite outcome) compared to patients who survived without requiring mechanical ventilation (n = 131, controls). Medians are depicted (red line). Significant differences ($p<0.05$) are marked with an asterisk.

TABLE 3 | Predictors of mechanical ventilation or in-hospital death in the multivariable analysis.

Variable	Multivariable OR (95% CI)	P-value
Alternative pathway activity <65.5%	4.93 (1.70-14.33)	**0.003**
Obesity	2.96 (1.02-8.58)	**0.046**
Arterial Hypertension	2.15 (0.65-7.10)	0.211
SOFA score on admission (per 1 point increase)	1.59 (1.02-2.16)	**0.038**
CRP on admission (per 1 mg/L increase)	1.01 (0.99-1.01)	0.167

CI, confidence interval; CRP, C-reactive protein; OR, odds ratio; SOFA, sepsis-related organ failure assessment score.
Statistically significant results (p < 0.05) are marked in boldface font.

COVID-19. In contrast, MBL (elevated proteomic signature) was found to be associated with 28-day mortality in a proteomic analysis (31). However, this study only included sera from 62 ICU patients, and the predictive performance of the MBL proteomic signature could not be tested in the much larger validation cohort (31). Of note, MBL genotypes associated with lower protein concentrations (i.e. the opposite as observed in the proteomic study) were more frequently encountered in patients with severe disease and need for ICU support in a study from Turkey (24). Lastly, a small study of 65 ICU patients reported no association of MBL with survival or need of mechanical ventilation similar to our cohort but higher MBL levels in patients with thromboembolic events, which correlated with D-dimer levels (23). Due to a very low number of thromboembolic events we were not able to confirm or refute this finding in the present study, but MBL concentrations or activity did not correlate with D-dimer levels in our cohort, even when limiting the analysis to ICU patients (data not shown). In summary, current human studies measuring LP proteins or activity have not been able to support the current concept of a core role of the LP in the overactivation of the complement system and the pathogenesis of COVID-19.

In contrast to the LP, low AP activity measured on admission to the COVID-19 and suggesting *in-vivo* overactivation *via* the AP emerged as a significant predictor of mechanical ventilation or in-hospital death in the present analysis. Patients with AP activity below the cut-off derived from the ROC analysis were almost five times more likely to develop acute lung injury requiring mechanical ventilation and/or to die compared to patients with AP activity above this cut-off. In addition, a lower AP activity also correlated with a higher disease severity on admission as assessed by the SOFA score. Our study cohort is the largest to date assessing AP activity and provides independent evidence regarding the predictive value of AP activity when assessed early during admission for COVID-19. Sinkovits G et al. have previously assessed AP activity in a cohort of 102 COVID-19 patients, too (34). However, AP activity was not uniformly measured on admission (but up to 63 days later; one third of patients were already in the ICU during sampling). They report decreased AP activity in critically ill patients at the time of sampling compared to non-ICU patients and outpatients. In addition, AP activity was significantly lower in deceased patients compared to ICU or non-ICU patients. Our results extend their findings by demonstrating that a decreased AP activity on admission is associated with a poor outcome in COVID-19. AP activity was also measured in a subgroup of patients (n=38) in the study by Ma L et al. but was found not to be significantly different when comparing ICU vs. non-ICU patients, a result that is probably influenced by the small number of patients analyzed (33). The measurement of AP

TABLE 4 | Correlation of complement pathway activities and protein concentrations and C1INH with length of stay, disease severity on admission and inflammatory markers on admission.

Complement variables r (p-value)*	LOS,	SOFA score	CRP	Ferritin	IL-6	D-Dimer	Lymphocyte count	LDH	Peak SARS-CoV-2 viral load in nasopharyngeal swab
Lectin pathway activity	0.008 (0.926)	-0.013 (0.870)	**0.172 (0.041)**	**0.269 (0.006)**	-0.184 (0.237)	0.041 (0.697)	-0.001 (0.988)	0.060 (0.461)	-0.099 (0.314)
Classical pathway activity	-0.039 (0.629)	-0.016 (0.847)	**0.172 (0.040)**	**0.229 (0.020)**	**-0.316 (0.039)**	0.017 (0.874)	0.044 (0.607)	0.106 (0.194)	-0.033 (0.736)
Alternative pathway activity	-0.104 (0.200)	**-0.163 (0.044)**	-0.048 (0.570)	-0.007 (0.945)	**-0.338 (0.027)**	**-0.207 (0.046)**	**0.277 (0.001)**	-0.153 (0.061)	-0.070 (0.478)
MBL	0.053 (0.524)	0.041 (0.624)	**0.234 (0.006)**	**0.364 (0.000)**	-0.079 (0.628)	0.091 (0.392)	-0.035 (0.691)	0.134 (0.107)	-0.097 (0.330)
FCN-3	-0.103 (0.212)	-0.123 (0.135)	**0.170 (0.046)**	0.103 (0.307)	-0.103 (0.517)	-0.200 (0.054)	-0.083 (0.336)	0.152 (0.066)	-0.076 (0.441)
C1INH	**0.266 (0.002)**	**0.224 (0.009)**	**0.455 (0.000)**	**0.477 (0.000)**	0.019 (0.914)	0.103 (0.358)	**-0.240 (0.008)**	**0.491 (0.000)**	0.048 (0.647)

**Spearman correlation coefficients and p-values are presented.*
C1INH, C1 esterase inhibitor; CRP, C-reactive protein; FCN-3, ficolin-3; IL-6, interleukin-6; LOS, length of stay; MBL, mannose-binding lectin; r, Spearman correlation coefficient; p, p-value; SARS-CoV-2, severe acute respiratory syndrome coronavirus type 2; SOFA, sepsis-related organ failure assessment score.
Statistically significant results (p < 0.05) are marked in boldface font.

activity provides a global assessment and characterization of several proteins that regulate AP activity and determine total AP function. As such, our analyses suggest that the occurrence of an overactivated AP and consumption of its associated complement proteins already on admission to the COVID-19 ward may be associated with a worse prognosis. In line, several studies have documented higher concentrations of complement activation products upstream of the terminal complement pathway (such as C3a) being associated with ICU admission, death and thromboembolic events (23, 33, 34). Both, Ma L et al. and Sinkovits G et al. reported that markers of AP activation and consumption may identify SARS-CoV-2 infected patients with a poor prognosis (33, 34). For example, the ratios of C3a/C3 and iC3b/C3 were associated with mortality and ICU admission, respectively (33, 34), and factor D strongly correlated with markers of endothelial cell activation and coagulation (33). Interestingly, SARS-CoV-2 spike protein has been found to activate the alternative pathway directly (42), which was blocked by factor D inhibition. Apart from the AP, the CP seems to be overactivated as a result of SARS-CoV-2 infection in our cohort. These data support a role of the C3 and the AP axis in complement activation in severe COVID-19 either as a consequence of direct activation by SARS-CoV-2 or as an amplifier secondary to CP (and to a lesser degree LP) activation.

We observed markedly elevated C1INH concentrations already on admission (median 0.47 g/L; upper limit of normal of 0.39 g/l at our institution). This should be viewed as biological feedback mechanisms in response to activation of several plasmatic cascades including the complement system in an attempt to inhibit or control inappropriate activation (43), which has also been documented in sepsis patients (44). C1INH was the most significantly upregulated protein in COVID-19 patients compared to controls with COVID-19 like symptoms in a preprint study (45). Similarly, a significant increase in C1INH protein signature was documented in severe compared to non-severe COVID-19 patients (41). In line, C1INH levels correlated positively not only with inflammatory markers on admission but also with their peak values in the present study. Moreover, C1INH levels on admission provided information on peak lung involvement as assessed on CT scans of the chest and on subsequent escalation of treatment (tocilizumab in our center). However, C1INH was not associated with the composite outcome in agreement with a previous smaller study (46). Given that C1INH is only a very weak regulator of the alternative pathway the lack of its association may support the observed significant association of AP activity with the outcome in our study. As even elevated C1INH concentrations may not be sufficient to inhibit or modulate all potential downstream effectors within the complement and contact activation cascade (47) and due to the detection of an increased amount of modified (cleaved) inactive C1INH in patients with severe sepsis (48) and a decreased expression of C1INH in the lungs of COVID-19 patients (32), results from trials evaluating additional C1INH supplementation treatment (49, 50) will be informative if C1INH may influence COVID-19 severity and outcome independent of AP activation (e.g. by inactivating the contact activation system or MASP-2).

Our study did not include a control population of SARS-CoV-2 negative patients with similar disease severity (e.g. influenza). This is an important limitation, as AP activation and consumption and its association with severe SARS-CoV-2 infection and detrimental outcomes may not be specific for COVID-19. In fact, Bain W et al. demonstrated that decreased AP activity was associated with an increased 30-day and one-year mortality in critically ill patients with acute lung injury as a consequence of infectious and non-infectious etiologies (51). Interestingly, a "hyperinflammatory" subphenotype was more frequently observed in patients with decreased AP activity as were bloodstream infections. This may point towards a detrimental effect of an overactive AP leading to a diminished AP function, in particular if sustained over time. In line, Bibert S et al. observed a similar pattern of increased expression of complement component-encoding genes in COVID-19 and influenza compared to healthy controls (52) with the exception of C3 that was over-expressed in early COVID-19 compared to influenza patients.

Our study has several additional limitations including the lack of data on other important proteins of the LP (other ficolins, collectin liver 1, MASP-2 and MASP-3) and AP and the single-center design. Importantly, none of our patients was treated with corticosteroids, which were not yet standard of care at the time of inclusion of patients. On the other hand, a significant number of patients was already treated with tocilizumab, a therapy that has shown to improve outcome in moderate to severe COVID-19 (53, 54). Consequently, outcome results generated in this study may even be valid in the current area of COVID-19 treatment. In-hospital mortality was lower than reported for our center during the first wave [e.g. 3% vs. 9.5% for in-hospital mortality (55)] and much lower compared to reported rates in the literature, which may have influenced our results. In particular, a serum sample may have not been available on admission of patients that required immediate transfer to the ICU from the emergency department (and subsequently died). Adjustment for confounders in the multivariable analysis of the composite outcome was limited. The significance of our results is limited by the small sample size of the analyzed cohort and the multiple comparisons investigated. Significant differences as described might be a chance result in the setting of multiple statistical analyses. Vice versa, it is possible that small differences in the composite outcome according to lectin pathway protein or C1INH concentrations may only be detectable in a larger patient cohort. Lastly, protein concentrations were only determined on admission but not during the disease course or at admission to the ICU.

CONCLUSION

Our results point to an overactivated AP in critically ill COVID-19 patients *in-vivo* leading to complement consumption and consequently to a significantly reduced AP activity *in-vitro*. The

LP does not seem to play a role in the progression to severe COVID-19. Apart from its acute phase reaction the significance of C1INH in COVID-19 requires further studies.

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 human participants were reviewed and approved by Ethics Committee of Northwest and Central Switzerland. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MT and MO designed the study. PC, IH, AE, SB, MT, and MO performed the study, collected, analyzed and interpreted the data. PC and MO drafted the manuscript. All authors critically revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Funded by personal and departmental funds MO.

ACKNOWLEDGMENTS

We are devoted to the nurses and physicians who cared for the COVID-19 patients and to the lab technicians for their excellent work during these stressful times.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.765330/full#supplementary-material.

Supplementary Figure 1 | Receiver-operator characteristics (ROC) analysis of alternative pathway activity according to the composite outcome of mechanical ventilation and/or in-hospital death.

REFERENCES

1. John Hopkins University & Medicine. *Coronarvirus Resource Center. Coronarvirus COVID-19 Global Cases by the CSSE [09 August 2021]*. Available at: https://coronavirus.jhu.edu/map.html.
2. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, et al. Dysregulation of Immune Response in Patients With COVID-19 in Wuhan, China. *Clin Infect Dis* (2020) 15:762–8. doi: 10.1093/cid/ciaa248
3. Ramlall V, Thangaraj PM, Meydan C, Foox J, Butler D, Kim J, et al. Immune Complement and Coagulation Dysfunction in Adverse Outcomes of SARS-CoV-2 Infection. *Nat Med* (2020) 26(10):1609–15. doi: 10.1038/s41591-020-1021-2
4. Englert H, Rangaswamy C, Deppermann C, Sperhake JP, Krisp C, Schreier D, et al. Defective NET Clearance Contributes to Sustained FXII Activation in COVID-19-Associated Pulmonary Thrombo-Inflammation. *EBioMedicine* (2021) 67:103382. doi: 10.1016/j.ebiom.2021.103382
5. Walport MJ. Complement. Second of Two Parts. *N Engl J Med* (2001) 344 (15):1140–4. doi: 10.1056/NEJM200104123441506
6. Walport MJ. Complement. First of Two Parts. *N Engl J Med* (2001) 344 (14):1058–66. doi: 10.1056/NEJM200104053441406
7. Kjaer TR, Thiel S, Andersen GR. Toward a Structure-Based Comprehension of the Lectin Pathway of Complement. *Mol Immunol* (2013) 56(4):413–22. doi: 10.1016/j.molimm.2013.05.007
8. Garred P, Honore C, Ma YJ, Rorvig S, Cowland J, Borregaard N, et al. The Genetics of Ficolins. *J Innate Immun* (2010) 2(1):3–16. doi: 10.1159/000242419
9. Garred P, Larsen F, Seyfarth J, Fujita R, Madsen HO. Mannose-Binding Lectin and its Genetic Variants. *Genes Immun* (2006) 7(2):85–94. doi: 10.1038/sj.gene.6364283
10. Thielens NM, Tacnet-Delorme P, Arlaud GJ. Interaction of C1q and Mannan-Binding Lectin With Viruses. *Immunobiology* (2002) 205(4-5):563–74. doi: 10.1078/0171-2985-00155
11. Kase T, Suzuki Y, Kawai T, Sakamoto T, Ohtani K, Eda S, et al. Human Mannan-Binding Lectin Inhibits the Infection of Influenza A Virus Without Complement. *Immunology* (1999) 97(3):385–92. doi: 10.1046/j.1365-2567.1999.00781.x
12. Bibert S, Piret J, Quinodoz M, Collinet E, Zoete V, Michielin O, et al. Herpes Simplex Encephalitis in Adult Patients With MASP-2 Deficiency. *PLoS Pathog* (2019) 15(12):e1008168. doi: 10.1371/journal.ppat.1008168
13. Schiela B, Bernklau S, Malekshahi Z, Deutschmann D, Koske I, Banki Z, et al. Active Human Complement Reduces the Zika Virus Load via Formation of the Membrane-Attack Complex. *Front Immunol* (2018) 9:2177. doi: 10.3389/fimmu.2018.02177
14. Bermejo-Jambrina M, Eder J, Helgers LC, Hertoghs N, Nijmeijer BM, Stunnenberg M, et al. C-Type Lectin Receptors in Antiviral Immunity and Viral Escape. *Front Immunol* (2018) 9:590. doi: 10.3389/fimmu.2018.00590
15. Zhou Y, Lu K, Pfefferle S, Bertram S, Glowacka I, Drosten C, et al. A Single Asparagine-Linked Glycosylation Site of the Severe Acute Respiratory Syndrome Coronavirus Spike Glycoprotein Facilitates Inhibition by Mannose-Binding Lectin Through Multiple Mechanisms. *J Virol* (2010) 84 (17):8753–64. doi: 10.1128/JVI.00554-10
16. Ip WK, Chan KH, Law HK, Tso GH, Kong EK, Wong WH, et al. Mannose-Binding Lectin in Severe Acute Respiratory Syndrome Coronavirus Infection. *J Infect Dis* (2005) 191(10):1697–704. doi: 10.1086/429631
17. Zhang H, Zhou G, Zhi L, Yang H, Zhai Y, Dong X, et al. Association Between Mannose-Binding Lectin Gene Polymorphisms and Susceptibility to Severe Acute Respiratory Syndrome Coronavirus Infection. *J Infect Dis* (2005) 192 (8):1355–61. doi: 10.1086/491479
18. Tu X, Chong WP, Zhai Y, Zhang H, Zhang F, Wang S, et al. Functional Polymorphisms of the CCL2 and MBL Genes Cumulatively Increase Susceptibility to Severe Acute Respiratory Syndrome Coronavirus Infection. *J Infect* (2015) 71(1):101–9. doi: 10.1016/j.jinf.2015.03.006
19. Wang Y, Yan J, Shi Y, Li P, Liu C, Ma Q, et al. Lack of Association Between Polymorphisms of MASP2 and Susceptibility to SARS Coronavirus Infection. *BMC Infect Dis* (2009) 9:51. doi: 10.1186/1471-2334-9-51
20. Holter JC, Pischke SE, de Boer E, Lind A, Jenum S, Holten AR, et al. Systemic Complement Activation is Associated With Respiratory Failure in COVID-19 Hospitalized Patients. *Proc Natl Acad Sci USA* (2020) 117(40):25018–25. doi: 10.1073/pnas.2010540117
21. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement Associated Microvascular Injury and Thrombosis in the

Pathogenesis of Severe COVID-19 Infection: A Report of Five Cases. *Transl Res* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
22. Gao T, Hu M, Zhang X. Highly Pathogenic Coronavirus N Protein Aggravates Lung Injury by MASP-2-Mediated Complement Over-Activation. *medRxiv* (2020) 2020.03.29.20041962. doi: 10.1101/2020.03.29.20041962
23. Eriksson O, Hultstrom M, Persson B, Lipcsey M, Ekdahl KN, Nilsson B, et al. Mannose-Binding Lectin is Associated With Thrombosis and Coagulopathy in Critically Ill COVID-19 Patients. *Thromb Haemost* (2020) 120(12):1720–4. doi: 10.1055/s-0040-1715835
24. Medetalibeyoglu A, Bahat G, Senkal N, Kose M, Avci K, Sayin GY, et al. Mannose Binding Lectin Gene 2 (Rs1800450) Missense Variant may Contribute to Development and Severity of COVID-19 Infection. *Infect Genet Evol* (2021) 89:104717. doi: 10.1016/j.meegid.2021.104717
25. Hummelshoj T, Fog LM, Madsen HO, Sim RB, Garred P. Comparative Study of the Human Ficolins Reveals Unique Features of Ficolin-3 (Hakata Antigen). *Mol Immunol* (2008) 45(6):1623–32. doi: 10.1016/j.molimm.2007.10.006
26. White MR, Tripathi S, Verma A, Kingma P, Takahashi K, Jensenius J, et al. Collectins, H-Ficolin and LL-37 Reduce Influence Viral Replication in Human Monocytes and Modulate Virus-Induced Cytokine Production. *Innate Immun* (2017) 23(1):77–88. doi: 10.1177/1753425916678470
27. Verma A, White M, Vathipadiekal V, Tripathi S, Mbianda J, Ieong M, et al. Human H-Ficolin Inhibits Replication of Seasonal and Pandemic Influenza A Viruses. *J Immunol* (2012) 189(5):2478–87. doi: 10.4049/jimmunol.1103786
28. Medjeral-Thomas NR, Troldborg A, Hansen AG, Gisby J, Clarke CL, Prendecki M, et al. Plasma Lectin Pathway Complement Proteins in Patients With COVID-19 and Renal Disease. *Front Immunol* (2021) 12:671052. doi: 10.3389/fimmu.2021.671052
29. Beinrohr L, Dobo J, Zavodszky P, Gal P. C1, MBL-MASPs and C1-Inhibitor: Novel Approaches for Targeting Complement-Mediated Inflammation. *Trends Mol Med* (2008) 14(12):511–21. doi: 10.1016/j.molmed.2008.09.009
30. Urwyler P, Moser S, Charitos P, Heijnen I, Rudin M, Sommer G, et al. Treatment of COVID-19 With Conestat Alfa, a Regulator of the Complement, Contact Activation and Kallikrein-Kinin System. *Front Immunol* (2020) 11:2072. doi: 10.3389/fimmu.2020.02072
31. Gutmann C, Takov K, Burnap SA, Singh B, Ali H, Theofilatos K, et al. SARS-CoV-2 RNAemia and Proteomic Trajectories Inform Prognostication in COVID-19 Patients Admitted to Intensive Care. *Nat Commun* (2021) 12 (1):3406. doi: 10.1038/s41467-021-23494-1
32. Mast AE, Wolberg AS, Gailani D, Garvin MR, Alvarez C, Miller JI, et al. SARS-CoV-2 Suppresses Anticoagulant and Fibrinolytic Gene Expression in the Lung. *Elife* (2021) 10:e64330. doi: 10.7554/eLife.64330
33. Ma L, Sahu SK, Cano M, Kuppuswamy V, Bajwa J, McPhatter JN, et al. Increased Complement Activation is a Distinctive Feature of Severe SARS-CoV-2 Infection. *Sci Immunol* (2021) 6(59):eabh2259. doi: 10.1126/sciimmunol.abh2259
34. Sinkovits G, Mezo B, Reti M, Muller V, Ivanyi Z, Gal J, et al. Complement Overactivation and Consumption Predicts In-Hospital Mortality in SARS-CoV-2 Infection. *Front Immunol* (2021) 12:663187. doi: 10.3389/fimmu.2021.663187
35. de Nooijer AH, Grondman I, Janssen NAF, Netea MG, Willems L, van de Veerdonk FL, et al. Complement Activation in the Disease Course of Coronavirus Disease 2019 and Its Effects on Clinical Outcomes. *J Infect Dis* (2021) 223(2):214–24. doi: 10.1093/infdis/jiaa646
36. Vogt S, Trendelenburg M, Tamm M, Stolz D, Hostettler KE, Osthoff M. Local and Systemic Concentrations of Pattern Recognition Receptors of the Lectin Pathway of Complement in a Cohort of Patients With Interstitial Lung Diseases. *Front Immunol* (2020) 11:562564. doi: 10.3389/fimmu.2020.562564
37. World Health Organization. WHO R&D Blueprint Novel Coronavirus COVID-19 Therapeutic Trial Synopsis. *R–D Blueprint* (2020), 1–12.
38. Colombi D, Bodini FC, Petrini M, Maffi G, Morelli N, Milanese G, et al. Well-Aerated Lung on Admitting Chest CT to Predict Adverse Outcome in COVID-19 Pneumonia. *Radiology* (2020) 296(2):E86–96. doi: 10.1148/radiol.2020201433
39. Ali YM, Ferrari M, Lynch NJ, Yaseen S, Dudler T, Gragerov S, et al. Lectin Pathway Mediates Complement Activation by SARS-CoV-2 Proteins. *Front Immunol* (2021) 12:714511. doi: 10.3389/fimmu.2021.714511
40. Malaquias MAS, Gadotti AC, Motta-Junior JDS, Martins APC, Azevedo MLV, Benevides APK, et al. The Role of the Lectin Pathway of the Complement System in SARS-CoV-2 Lung Injury. *Transl Res* (2021) 231:55–63. doi: 10.1016/j.trsl.2020.11.008
41. Shen B, Yi X, Sun Y, Bi X, Du J, Zhang C, et al. Proteomic and Metabolomic Characterization of COVID-19 Patient Sera. *Cell* (2020) 182(1):59–72 e15. doi: 10.1016/j.cell.2020.05.032
42. Yu J, Yuan X, Chen H, Chaturvedi S, Braunstein EM, Brodsky RA. Direct Activation of the Alternative Complement Pathway by SARS-CoV-2 Spike Proteins is Blocked by Factor D Inhibition. *Blood* (2020) 136(18):2080–9. doi: 10.1182/blood.2020008248
43. Panagiotou A, Trendelenburg M, Osthoff M. The Lectin Pathway of Complement in Myocardial Ischemia/Reperfusion Injury-Review of Its Significance and the Potential Impact of Therapeutic Interference by C1 Esterase Inhibitor. *Front Immunol* (2018) 9:1151. doi: 10.3389/fimmu.2018.01151
44. Igonin AA, Protsenko DN, Galstyan GM, Vlasenko AV, Khachatryan NN, Nekhaev IV, et al. C1-Esterase Inhibitor Infusion Increases Survival Rates for Patients With Sepsis*. *Crit Care Med* (2012) 40(3):770–7. doi: 10.1097/CCM.0b013e318236edb8
45. Geyer PE, Arend FM, Doll S, Louiset M-L, Winter SV, Müller-Reif JB, et al. High-Resolution Longitudinal Serum Proteome Trajectories in COVID-19 Reveal Patients-Specific Seroconversion. *medRxiv* (2021) 2021.02.22.21252236. doi: 10.1101/2020.03.29.20041962
46. Henry BM, Szergyuk I, de Oliveira MHS, Lippi G, Benoit JL, Vikse J, et al. Complement Levels at Admission as a Reflection of Coronavirus Disease 2019 (COVID-19) Severity State. *J Med Virol* (2021) 93(9):5515–22. doi: 10.1002/jmv.27077
47. Peoples N, Strang C. Complement Activation in the Central Nervous System: A Biophysical Model for Immune Dysregulation in the Disease State. *Front Mol Neurosci* (2021) 14:620090. doi: 10.3389/fnmol.2021.620090
48. Nuijens JH, Eerenberg-Belmer AJ, Huijbregts CC, Schreuder WO, Felt-Bersma RJ, Abbink JJ, et al. Proteolytic Inactivation of Plasma C1-Inhibitor in Sepsis. *J Clin Invest* (1989) 84(2):443–50. doi: 10.1172/JCI114185
49. Urwyler P, Charitos P, Moser S, Heijnen I, Trendelenburg M, Thoma R, et al. Recombinant Human C1 Esterase Inhibitor (Conestat Alfa) in the Prevention of Severe SARS-CoV-2 Infection in Hospitalized Patients With COVID-19: A Structured Summary of a Study Protocol for a Randomized, Parallel-Group, Open-Label, Multi-Center Pilot Trial (PROTECT-COVID-19). *Trials* (2021) 22(1):1. doi: 10.1186/s13063-020-04976-x
50. Mansour E, Bueno FF, de Lima-Junior JC, Palma A, Monfort-Pires M, Bombassaro B, et al. Evaluation of the Efficacy and Safety of Icatibant and C1 Esterase/Kallikrein Inhibitor in Severe COVID-19: Study Protocol for a Three-Armed Randomized Controlled Trial. *Trials* (2021) 22(1):71. doi: 10.1186/s13063-021-05027-9
51. Bain W, Li H, van der Geest R, Moore SR, Olonisakin TF, Ahn B, et al. Increased Alternative Complement Pathway Function and Improved Survival During Critical Illness. *Am J Respir Crit Care Med* (2020) 202(2):230–40. doi: 10.1164/rccm.201910-2083OC
52. Bibert S, Guex N, Lourenco J, Brahier T, Papadimitriou-Olivgeris M, Damonti L, et al. Transcriptomic Signature Differences Between SARS-CoV-2 and Influenza Virus Infected Patients. *Front Immunol* (2021) 12:666163. doi: 10.3389/fimmu.2021.666163
53. Investigators R-C, Gordon AC, Mouncey PR, Al-Beidh F, Rowan KM, Nichol AD, et al. Interleukin-6 Receptor Antagonists in Critically Ill Patients With Covid-19. *N Engl J Med* (2021) 384(16):1491–502. doi: 10.1056/NEJMoa2100433
54. Group RC. Tocilizumab in Patients Admitted to Hospital With COVID-19 (RECOVERY): A Randomised, Controlled, Open-Label, Platform Trial. *Lancet* (2021) 397(10285):1637–45. doi: 10.1016/S0140-6736(21)00676-0
55. Diebold M, Martinez AE, Adam KM, Bassetti S, Osthoff M, Kassi E, et al. Temporal Trends of COVID-19 Related in-Hospital Mortality and Demographics in Switzerland - a Retrospective Single Centre Cohort Study. *Swiss Med Wkly* (2021) 151:w20572. doi: 10.4414/smw.2021.20572

Conflict of Interest: MT reports receiving a grant from the Swiss National Science Foundation, and having research collaborations with Roche, Novartis, and Idorsia (all outside of the submitted work). MO reports receiving grants from the Swiss

National Science foundation outside of the submitted work, and consulting fees from Pharming Technologies B.V. during the conduct of the study and grants from Pharming Biotechnologies B.V. outside the submitted work. AE reports receiving grants from the Swiss National Science foundation outside of the submitted work. No conflict of interest for this work.

The remaining 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Immunology

ORIGINAL RESEARCH
published: 02 November 2021
doi: 10.3389/fimmu.2021.767981

SARS-CoV-2 Antibodies Mediate Complement and Cellular Driven Inflammation

Ida Jarlhelt[1], Sif Kaas Nielsen[1], Camilla Xenia Holtermann Jahn[1], Cecilie Bo Hansen[1], Laura Pérez-Alós[1], Anne Rosbjerg[1,2], Rafael Bayarri-Olmos[1,2†], Mikkel-Ole Skjoedt[1,3†] and Peter Garred[1†]*

[1] Laboratory of Molecular Medicine, Department of Clinical Immunology, Section 7631, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark, [2] Recombinant Protein and Antibody Laboratory, Department of Clinical Immunology, Section 7631, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark, [3] Institute of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark

OPEN ACCESS

Edited by:
Nicolas Stephane Merle, National Heart, Lung, and Blood Institute (NHLBI), United States

Reviewed by:
Christine Gaboriaud, UMR5075 Institut de Biologie Structurale (IBS), France
Valarmathy Murugaiah, The Ohio State University, United States

***Correspondence:**
Peter Garred
Peter.Garred@regionh.dk

[†]These authors have contributed equally to this work and share senior authorship

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: 31 August 2021
Accepted: 18 October 2021
Published: 02 November 2021

Citation:
Jarlhelt I, Nielsen SK, Jahn CXH, Hansen CB, Pérez-Alós L, Rosbjerg A, Bayarri-Olmos R, Skjoedt M-O and Garred P (2021) SARS-CoV-2 Antibodies Mediate Complement and Cellular Driven Inflammation. Front. Immunol. 12:767981. doi: 10.3389/fimmu.2021.767981

The ongoing pandemic of coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to constitute a serious public health threat worldwide. Protective antibody-mediated viral neutralization in response to SARS-CoV-2 infection has been firmly characterized. Where the effects of the antibody response are generally considered to be beneficial, an important biological question regarding potential negative outcomes of a SARS-CoV-2 antibody response has yet to be answered. We determined the distribution of IgG subclasses and complement activation levels in plasma from convalescent individuals using in-house developed ELISAs. The IgG response towards SARS-CoV-2 receptor-binding domain (RBD) after natural infection appeared to be mainly driven by IgG1 and IgG3 subclasses, which are the main ligands for C1q mediated classical complement pathway activation. The deposition of the complement components C4b, C3bc, and TCC as a consequence of SARS-CoV-2 specific antibodies were depending primarily on the SARS-CoV-2 RBD and significantly correlated with both IgG levels and disease severity, indicating that individuals with high levels of IgG and/or severe disease, might have a more prominent complement activation during viral infection. Finally, freshly isolated monocytes and a monocyte cell line (THP-1) were used to address the cellular mediated inflammatory response as a consequence of Fc-gamma receptor engagement by SARS-CoV-2 specific antibodies. Monocytic Fc gamma receptor charging resulted in a significant rise in the secretion of the pro-inflammatory cytokine TNF-α. Our results indicate that SARS-CoV-2 antibodies might drive significant inflammatory responses through the classical complement pathway and *via* cellular immune-complex activation that could have negative consequences during COVID-19 disease. We found that increased classical complement activation was highly associated to COVID-19 disease severity. The combination of antibody-mediated complement activation and subsequent cellular priming could constitute a significant risk of exacerbating COVID-19 severity.

Keywords: SARS-CoV-2, antibody response, complement, monocytes, spike, receptor binding domain

INTRODUCTION

Coronavirus disease 2019 (COVID-19) constitutes a global pandemic, caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). COVID-19 related morbidity and mortality have been attributed to an exaggerated immune response and the ambiguous role of the complement system and its contribution to illness severity is being increasingly recognized (1). The complement system constitutes an essential part of innate immunity and the proteolytic cascade is initiated by three distinct pathways; the classical-, the lectin- and the alternative pathway. Each pathway differs on the mode of initiation, but all converge at the level of complement factor C3 to generate central and terminal effector molecules (2). The classical pathway can be initiated by the binding of C1q directly to the Fc portion of antibodies bound to antigens. The lectin pathway is initiated by binding of the so-called collectins and ficolins to carbohydrates directly on pathogens. Finally, the alternative pathway functions as an amplification loop for the classical and lectin pathway or might to some degree be initiated spontaneously when activated by C3 hydrolysis (3). Proper activation of the complement system will result in the elimination of the pathogen through effector functions including opsonization, pro-inflammatory secreted cleavage products, and microbial lysis by the terminal complement complex (TCC) (4). While complement may effectively contribute to the control of viral infection, activation of the complement pathways may also contribute to several pathologies observed in severe COVID-19 patients, due to its potent proinflammatory effects (5).

Several studies have suggested that the complement system is implicated in the pathogenesis of COVID-19 (6–8). It is generally accepted that the complement system plays a central role in acute respiratory distress syndrome (ARDS), which is also typically seen in severely COVID-19 disease (9). In a previous study regarding SARS-CoV-1, which is closely related to SARS-CoV-2, it was found that the activation of complement component C3 can aggravate the disease in SARS-CoV-associated ARDS (10). Moreover, *in vitro* studies have suggested that the spike protein of SARS-CoV-2 activates the alternative pathway (11) and that the nucleocapsid (N) protein from SARS-CoV-1, MERS-CoV and SARS-CoV-2 binds to MASP-2, which is the central serine protease in the lectin pathway (12). This binding results in increased lectin pathway-mediated complement activation and exacerbates inflammatory lung damage. Blockade of the N-protein:MASP-2 interaction or complement inhibition reduces hyperactivation of the complement system and lung damage. In addition, patients with age-related macular degeneration (AMD), in which exaggerated complement activation plays a central role in the etiology, have been shown to have a significantly increased risk of adverse clinical outcomes following SARS-CoV-2 infection (13). Conversely, a group of patients with genetic backgrounds of complement deficiency had no need for mechanical ventilation and they overcame their illness with less complications (13). Together, these data suggest that an exaggerated complement activation level predispose individuals to adverse outcomes associated with SARS-CoV-2 infection. Additionally, several studies have demonstrated an accumulation of activated complement proteins in damaged tissues and organs (14, 15) as well as elevated plasma levels of C5a and soluble TCC in infected patients (7, 16, 17). It has been suggested that a blockade of C5a may be crucial for inhibition of the cytokine storm and, therefore, would be a potential therapeutic target for acute lung damage caused by pathogenic viral infections, such as SARS-CoV-1 and -2 (18). At the time being, several approaches are conceivable to control complement activation as a therapeutic principle in COVID-19 (19).

More data are emerging showing the involvement of especially the lectin and alternative pathway of complement in COVID-19 pathology and the beneficial antibody-mediated viral neutralization in response to SARS-CoV-2 infection has been firmly characterized. We have approached some of the potential adverse outcomes of a SARS-CoV-2 antibody response and the linkage to an exacerbated immune response through the classical pathway. We hypothesized that the developed SARS-CoV-2 antibodies might contribute to enhanced complement and cellular-driven inflammation, exacerbating COVID-19 disease.

MATERIALS AND METHODS

Buffers

The following buffers were used: PBS (10.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl), PBS-Tween (PBS-T) [PBS, 0.05% Tween-20 (8221840050, Merck)], PBS-T-EDTA [PBS, 0.05% Tween-20, 5 mM EDTA (EDS-500G, Merck) and Barbital-Tween (Barbital-T) [4 mM $C_8H_{11}N_2NaO_3$, 145 mM NaCl, 2.6 mM $CaCl_2$, 2.1 mM $MgCl_2$, 0.05% Tween-20].

Plasma From Convalescent and Healthy Individuals

A total of 180 recovered individuals previously tested RT-PCR-positive for SARS-CoV-2 were included in the study. The Department of Emergency Medicine at Herlev University Hospital in Denmark recruited the participants and EDTA plasma samples were stored in aliquots and kept frozen at −80°C until used. The RT-PCR positive participants are comprised of males and females aged from 18–86 and the course of disease ranged from mild to severe based on an electronic self-report questionnaire. Mild disease was defined as having few symptoms and generally feeling well, moderate disease as being bedridden at home, and severe disease as the need for hospitalization. Based on previous measurements of IgG levels (20), individuals could further be divided into groups based on IgG levels as followed: high IgG, intermediate IgG and low IgG. A total of 60 EDTA plasma samples collected from healthy blood donors before 2020, having no SARS-CoV-2 antibodies, were used as negative controls.

Measurements of IgG Subclasses by Sandwich ELISA

Nunc™ MaxiSorp Flat-Bottom 96-Well plates (442404, Thermo Fisher Scientific) were coated with 1 μg/ml RBD in PBS overnight (ON) at 4°C. The RBD antigen was produced as

previously described (20). Plates were blocked with PBS-T for 1 h. Samples were diluted 1:50 in PBS-T-EDTA, applied to the plates in a 3-fold dilution and incubated for 1 h at room temperature (RT). HRP-conjugated monoclonal antibodies against IgG1 (A10648, Thermo Fisher Scientific). IgG2 (ab99779, abcam), IgG3 (05-3620, Thermo Fisher Scientific) and IgG4 (A10654, Thermo Fisher Scientific) were applied in a concentration of 1 µg/ml in PBS-T and incubated for 1 h RT. TMB ONE (4380A, KemEnTec Diagnostics) was used as a substrate and allowed to react for 10 minutes. The reaction was stopped with 0.3 M H_2SO_4 and the optical density (OD) of the samples was measured at 450-630 nm using A Synergy HT absorbance reader (BioTek Instruments). Plates were washed three times with PBS-T between the steps mentioned above. The data is presented in signal to noise (S/N) ratios between the sample OD value and a negative quality control.

An ELISA control experiment of the detection of each IgG subtypes were performed in which normal human immunoglobulin (ZLB Behring) or Bovine Serum Albumin (BSA) (Sigma-Aldrich) were coated in a 2-fold dilution, starting in 10 µg/ml incubating ON at 4°C. Each of the 4 subclass antibodies was used for detection in a concentration of 1 µg/ml. TMB ONE and 0.3 M H_2SO_4 were used for revealing as described above.

Measurements of Complement Deposition by Sandwich ELISA

Nunc™ MaxiSorp Flat-Bottom 96-Well plates (442404, Thermo Fisher Scientific) were coated with 3 µg/ml RBD in PBS ON at 4°C. Plates were blocked with PBS-T for 1 h. Samples were diluted 1:60 (for high IgG samples) and 1:20 (for intermediate, low and negative IgG samples) in PBS-T-EDTA, applied to the plates in a 3-fold dilution and incubated for 1 h at RT. A normal human serum pool was applied in a 1:50 dilution and incubated for 45 min at 37°C. Biotinylated monoclonal antibodies against C4b (Hyb 162-02, Bioporto Diagnostics), C3bc [in-house produced, clone BH6 (21)] and TCC [in-house produced, clone aE11 (22)] were applied in a concentration of 2 µg/ml in PBS-T and incubated for 1 h RT. Streptavidin-HRP conjugate (Sigma-Aldrich) was added to the wells for 1 h in a 1:2500 dilution in PBS-T. TMB ONE (4380A, KemEnTec Diagnostics) was used as a substrate and allowed to react for 8 minutes. The reaction was stopped with 0.3 M H_2SO_4 and the OD of the samples was measured at 450-630 nm using a Synergy HT absorbance reader (BioTek Instruments). Plates were washed three times with PBS-T between the steps mentioned above.

Depletion of Antibodies Against RBD

Nunc™ MaxiSorp Flat-Bottom 96-Well plates (442404, Thermo Fisher Scientific) were used to deplete antibodies against RBD by coating with 10 µg/ml RBD or BSA in PBS ON at 4°C. Plates were blocked with PBS-T for 1 h. EDTA plasma from three convalescent individuals with high levels of IgG and healthy controls were each transferred to a RBD and BSA coated well in a 1:50 dilution and incubated for 30 min at RT shaking. The samples were transferred to the next RBD or BSA coated well and once again incubated for 30 min at RT shaking. This was repeated through 12 wells along with the ELISA plate and the sample was left in the final well ON at 4°C. To ensure that each sample was depleted for antibodies against RBD, the samples was analyzed in a new plate coated with either RBD or spike protein (1 µg/ml), and HRP-conjugated polyclonal rabbit antibodies against human IgM (P0215), IgA (P0216), or IgG (P0214) (all from Agilent Technologies) in a concentration of 1 µg/ml was used to detect the remaining antibodies bound to each of the antigens. The depleted samples were afterwards subjected to the complement deposition assay as described above and a neutralization assay as described elsewhere (23). The principle of the neutralization assay is to address the neutralizing capacity of the developed antibodies based on the interaction between recombinant human ACE-2 ectodomain and the SARS-CoV-2 RBD. Briefly, Nunc MaxiSorp microtiter plates (442404, Thermo Fisher Scientific) were coated with 1 µg/ml of ACE-2 overnight at 4°C in PBS. The following day, 2% EDTA plasma/mAbs were incubated for 1 h in low-binding round-bottom plates (Thermo Fisher Scientific) with a solution of biotinylated RBD (4 ng/ml) with HS-strep-HRP (1:16,000 dilution) in PBS-T. Afterwards, the mAbs:RBD:HS-Strep-HRP solution was transferred to ACE-2 plates and incubated for 15 min. The plates were developed with TMB One (4380A, KemEnTec Diagnostics) for 20 min and stopped with 0.3 M H_2SO_4. The absorbance was read at 450-630 nm. Between steps, the plates were washed twice with PBS-T.

Cytokine Production in Human Monocytes

A human THP-1 monocytic cell line (88081201, Sigma-Aldrich) and human monocytes isolated from a healthy blood donor were used to address the cellular mediated inflammatory response due to Fc-gamma receptor (FcγR) engagement by SARS-CoV-2 specific antibodies. MagniSort™ Human CD14 Positive Selection Kit (8802-6834-74, Invitrogen) was used for the isolation of human monocytes according to the manufacturer's instructions. TPP® tissue culture 6-well plates (Z707767, Sigma-Aldrich) were coated with 10 µg/ml RBD or LPS and incubated ON at 4°C. Heat-inactivated sera from a pool of individuals previously infected with SARS-CoV-2 were applied in a 1:50 dilution and incubated for 2h at RT. Plates were washed with sterile PBS and THP-1 cells or freshly isolated monocytes were applied in a total concentration of $1x10^6$ cells for each condition/in each well. Non-treated cells and cells incubated with fluid-phase LPS were included as controls. The cells were incubated for 24 hours in a humidified atmosphere at 37°C and 5% CO_2. Cytokine release within the resulting supernatants was analyzed by the Luminex® 100/200™ System (Invitrogen), using a custom-made Bio-Plex Pro™ Human Cytokine Panel (#12011278, Biorad). The following cytokines were measured: IL-6, IL-1β and TNF-α. Analyses were performed according to the manufacturer's instructions (Instruction Manual #10000092045). All work was performed under sterile conditions.

Statistics

Statistical analyses were performed using GraphPad Prism version 9.0.0 (GraphPad Software, CA, USA) and R (version

3.6.1 for Windows, R Foundation for Statistical Computing, Vienna, Austria). Estimation of levels of deposited C4, C3 and TCC were interpolated by regression analysis using a four-parameter logistic curve fitting, and results were given in arbitrary units/ml (AU/ml). In a 1:50 dilution, the calibrator was defined to contain 200 AU/ml. Non-parametric data were log-transformed before performing statistical analysis. Statistical differences between disease severity and IgG levels groups were analyzed using Kruskal–Wallis test with a Tukey's multiple comparison test. Multiple regression models were used to assess the relationship between complement activation and IgG subclass levels and the independent variables of disease severity, age and IgG levels groups. Spearman rank correlation tests were used to determine the correlation between antibody levels and complement activation products. An ordinary one-way ANOVA with Holm-Šídák's multiple comparisons test was used to determine the statistical differences between groups in the neutralization- and deposition assay post depletion of RBD antibodies, as well for the differences between cytokine release of monocytes under different conditions. Significance levels are as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$; $p < 0.05$ was considered statistically significant.

RESULTS

Levels of IgG1, IgG2, IgG3 and IgG4 in Convalescent Plasma

The levels of IgG in the convalescent samples were previously determined in a study, where we showed that the IgG levels were significantly correlated to COVID-19 disease severity (20). In order to look further into the IgG response during COVID-19, we determined the distribution of IgG subclasses IgG1, -2, -3 and -4, in a cohort of previously infected individuals. The quantitative relative levels of the four subclass antibodies were measured in EDTA plasma from 240 individuals in total, with 60 individuals in each of four groups divided according to total IgG levels (i.e. high IgG, intermediate IgG, low IgG and no IgG/healthy controls. IgG1 was by far the most abundant subclass antibody, followed by prominent detection of IgG3 - both with significantly different levels between each of the four IgG groups. However, low levels of IgG2 and IgG4 were also detected (**Figure 1**). All subclass antibodies correlated significantly with the levels of IgG. Results from a control experiment validating that each commercial antibody correctly binds one IgG subtype, verified in an ELISA, are presented in **Supplementary Figure 1**.

Complement Activation Mediated by SARS-CoV-2 Antibodies

To evaluate the role of the complement system during COVID-19 infection, we investigated if the developed antibodies against SARS-CoV-2 antigens would activate the classical pathway *in vitro*. The activation of complement on SARS-CoV-2 antigen/antibody complexes was measured in three setups detecting deposition of complement components C4, C3 and TCC and the data show that individuals with high levels of IgG will have equivalent high complement deposition (**Figure 2A**). A strong positive correlation ($r > 0.8$) were seen for IgG and the three complement components (**Figure 2B**). Deposition levels of complement components C4, C3 and TCC also correlated internally (**Supplementary Figure 2**). The deposition of complement did additionally correlate with disease severity, implying that plasma from individuals with either high levels of IgG and/or a more severe course of the disease, in general, would have increased complement activation through deposition of C4, C4 and TCC (**Figure 3**).

Depletion of Antibodies Against RBD in Convalescent Plasma

To assess the importance of the RBD domain for complement activation, plasma from three individuals with high levels of IgG against SARS-CoV-2, along with three negative controls, were subjected to solid phase depletion of antibodies against RBD (**Figure 4**). The depleted and non-depleted samples were afterwards subjected to immobilized RBD (**Figure 4A**) or full-length spike (**Figure 4B**) to verify that the antibodies were in fact depleted. The resulting data show that all IgM, IgA and IgG antibodies against RBD are removed, while antibodies with epitopes on the remaining parts of spike are still present in the plasma samples. Neutralization capacity for the remaining antibodies (**Figure 4C**) and deposition of complement on spike (**Figure 4D**) was decreased significantly post RBD depletion, indicating that Ig directed towards this domain will play an essential role in complement activation.

Activation of Human Monocytes by SARS-CoV-2 Immune Complexes

To address the axis connecting humoral and cellular inflammation, we primed THP-1 monocytes and freshly isolated human monocytes with SARS-CoV-2 antigen/antibody immune complexes, the antigen being RBD. The resulting Fc gamma charging with anti RBD antibodies resulted in a significant rise in pro-inflammatory cytokine, TNF-α, in the freshly isolated human monocytes ($p = 0.0194$) (**Figure 5**). LPS was included as a positive control, both immobilized in the solid phase and directly added to the cells during incubation (fluid-phase), and the latter appeared to result in more activation of the monocytes. The same tendency was seen for the THP-1 monocytes, but the differences were not significant due to higher background activation of the cells ($p = 0.1874$). Priming with immune complexes did not give rise to increased production of cytokines IL-6 and IL-1β in either freshly isolated monocytes or THP-1 monocytes (**Supplementary Figure 3**).

DISCUSSION

A hallmark of severe COVID-19 disease is excessive inflammation associated with enhanced morbidity and mortality. In addition, accumulating evidence suggests that overactivation of the complement system plays a critical role in the pathophysiology and inflammatory response during severe

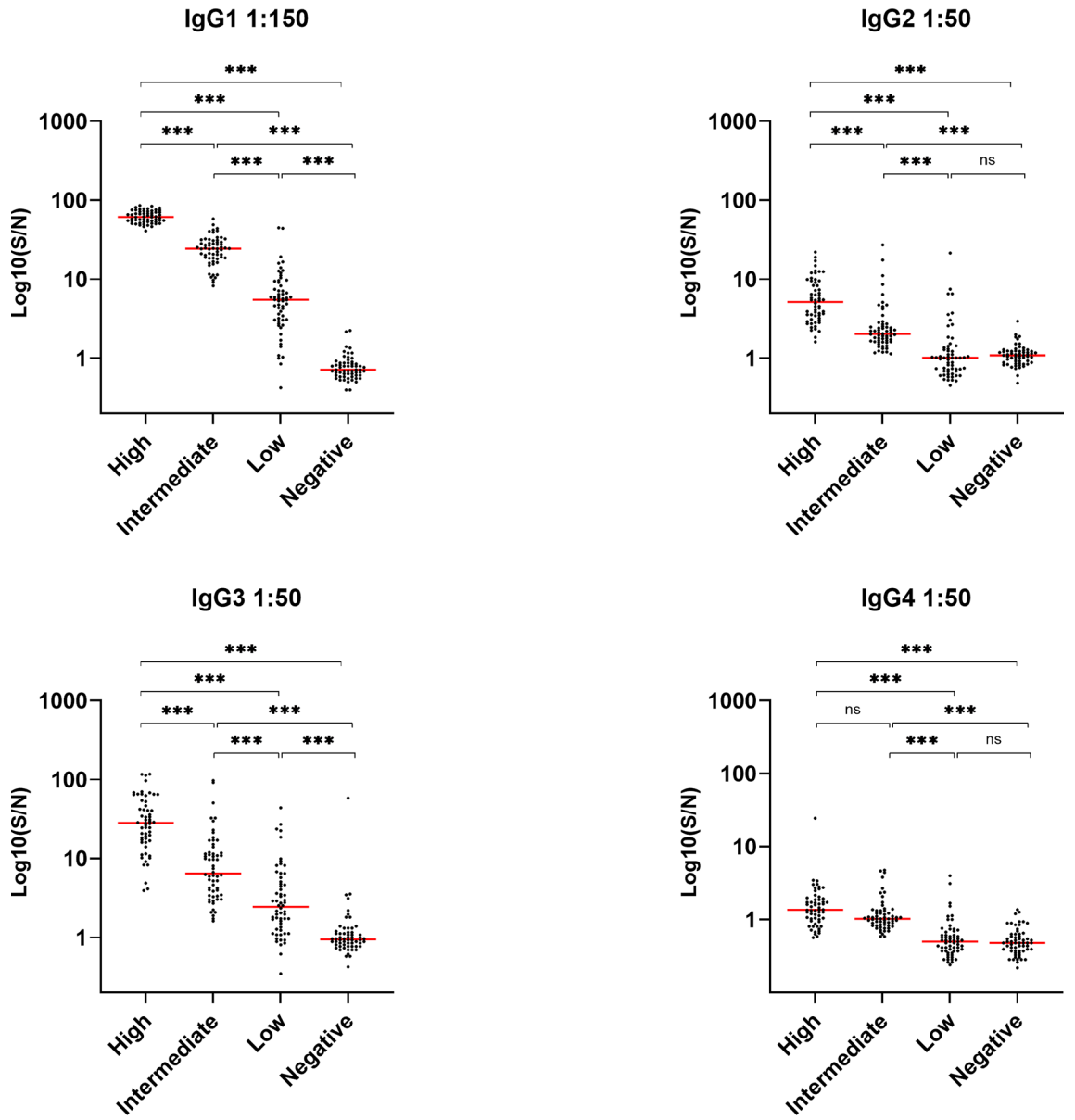


FIGURE 1 | Detection of IgG1, IgG2, IgG3 and IgG4 in recovered SARS-CoV-2 individuals. Groups divided according to levels of total IgG; high, intermediate, low and negative healthy controls, n = 240. Levels were assessed by coating plates with 1 µg/nm RBD and detecting with HRP-conjugated antibodies against IgG1, 2, 3 and 4 (1 µg/ml). Samples were measured in a 1:50 dilution, except for IgG1 which were measured in a 1:150 dilution. Dynamic range represented in signal-to-noise ratios (S/N). A p value < 0.05 was considered significant. ns, not significant, ***p < 0.001 using Tukey all-pair comparisons.

COVID-19 disease (1). We hypothesized that anti-SARS-CoV-2 antibodies may contribute to exacerbated immune responses through classical complement pathway activation. Due to the polyclonal nature of antibodies, each subclass displays distinct functions and features and the distribution of the developed antibodies therefore becomes important to control viral infection, such as COVID-19. In line with the findings in recent reports (24–27) our results indicate that SARS-CoV-2 specific IgG1 and IgG3 were the dominant subclasses of IgG; while IgG2 and IgG4 were barely detected. IgG1 and IgG3 are known to efficiently trigger the classical complement pathway while IgG2 and IgG4 have reduced binding of C1q (28). Viral infections in general lead to IgG antibodies of the IgG1 and IgG3 subclasses, with IgG3 antibodies appearing first in the course of the infection (29). In a similar manner, there appears to be a preferential generation of IgG3 during COVID-19 infection. Our findings agree with another study where a substantial difference in the spike-specific IgG subclass composition was observed. A larger proportion of S1 and RBD-specific IgG3 was associated with COVID-19 severity (30). The study shows that spike-specific

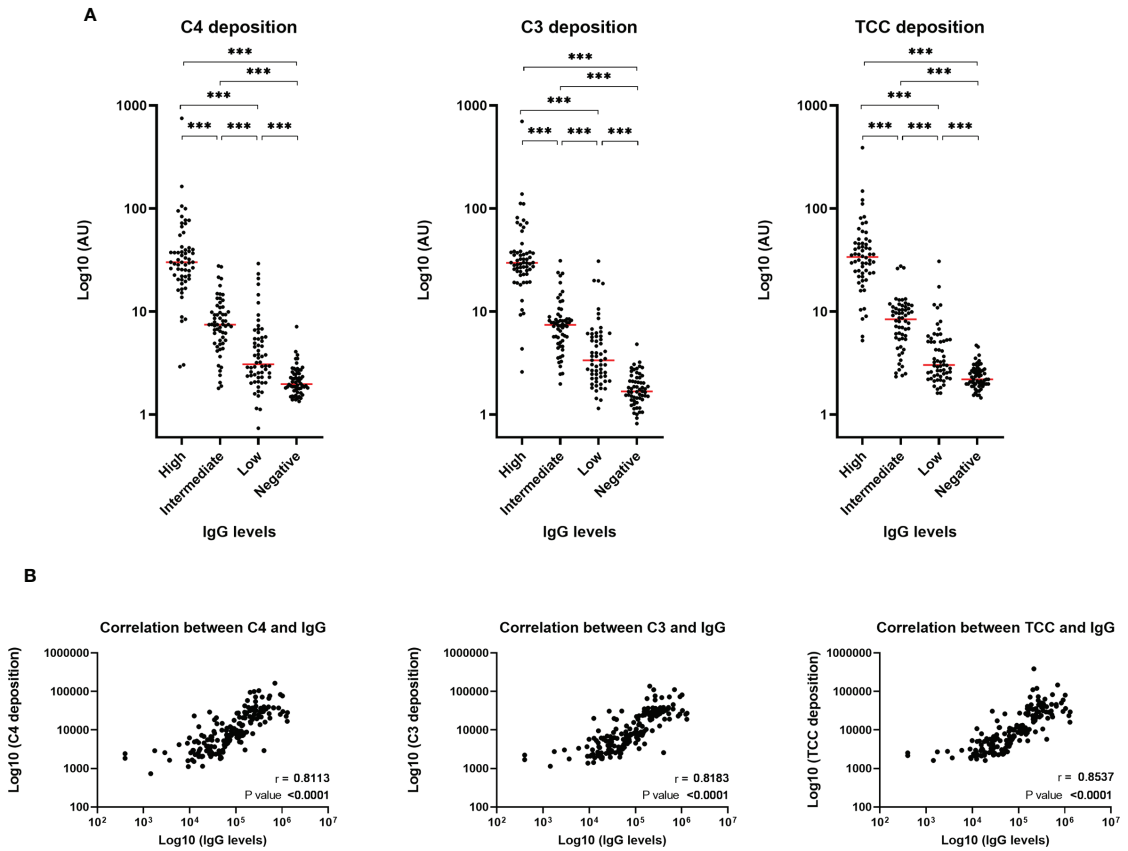


FIGURE 2 | Complement deposition in recovered SARS-CoV-2 individuals. **(A)** Measurements of complement components C4, C3 and TCC in convalescent EDTA plasma. Groups divided according to levels of total IgG; high, intermediate, low and negative healthy controls, n = 240. Levels were assessed by coating plates with 3 µg/nm RBD, followed by incubation with EDTA plasma diluted 1:60 (for high IgG samples) and 1:20 (for intermediate, low and negative IgG samples). A normal human serum pool was applied in a 1:50 dilution. Biotinylated monoclonal antibodies against C4b, C3bc and TCC (2 µg/ml) was used for detection. Dynamic range represented in arbitrary units (AU). A p value < 0.05 was considered significant. ***p < 0.001 using Tukey all-pair comparisons. **(B)** The correlation between deposition of either C4, C3 or TCC and IgG levels using Spearman rank correlation analysis.

IgG1, and not IgG3, was most closely correlated with *in vitro* viral neutralization, leading the authors to conclude that excess IgG3 may play an inflammatory role in the pathogenesis of COVID-19 in some individuals. This somehow unbalanced IgG response, enriched in IgG3, may promote immunopathology and excess inflammation rather than tissue repair, exacerbating the symptoms of COVID-19 patients. Furthermore, our study showed that complement activation through deposition of C4, C3 and TCC appears to be mediated by the developed antibodies against SARS-CoV-2. More specifically, this seems to occur through the antibodies binding to epitopes on RBD, since the complement deposition was almost completely absent post depletion of RBD antibodies in convalescent plasma samples using spike protein as a target for complement activation. The exact residues of RBD domain that are involved in triggering the complement activation through interaction with the produced antibodies are unknown. However, in a recent publication epitope binning experiments were performed and revealed several epitope hotspots within the RBD (23). Whether these are equally important for complement activation needs to be further studied. A meta-analysis has suggested that RBD specific antibodies are the most dominant in COVID-19 patients (27). In addition, recent studies have reported that deceased COVID-19 patients have more protein N-specific antibody responses while the convalescent response is mainly driven by spike specific antibodies, suggesting the antigen-specific antibodies influence the immunity effectiveness and disease development (31). However, in terms of inflammatory contribution have different modes of action from the developed antibodies been reported. A recent publication indicates that human N protein targeting antibodies might inhibit excessive complement activation (32). The authors reported a COVID-19 patient-derived human monoclonal antibody targeting the N protein, which upon binding to the SARS-CoV-2 antigen in an *ex vivo* assay, were able to avoid hyperactivation of complement in patients recovering quickly.

Our findings indicate that the levels of complement activation products are positively correlated with both IgG levels and disease severity. Although C3 and TCC measurements reached slightly different significance levels between severity groups, the

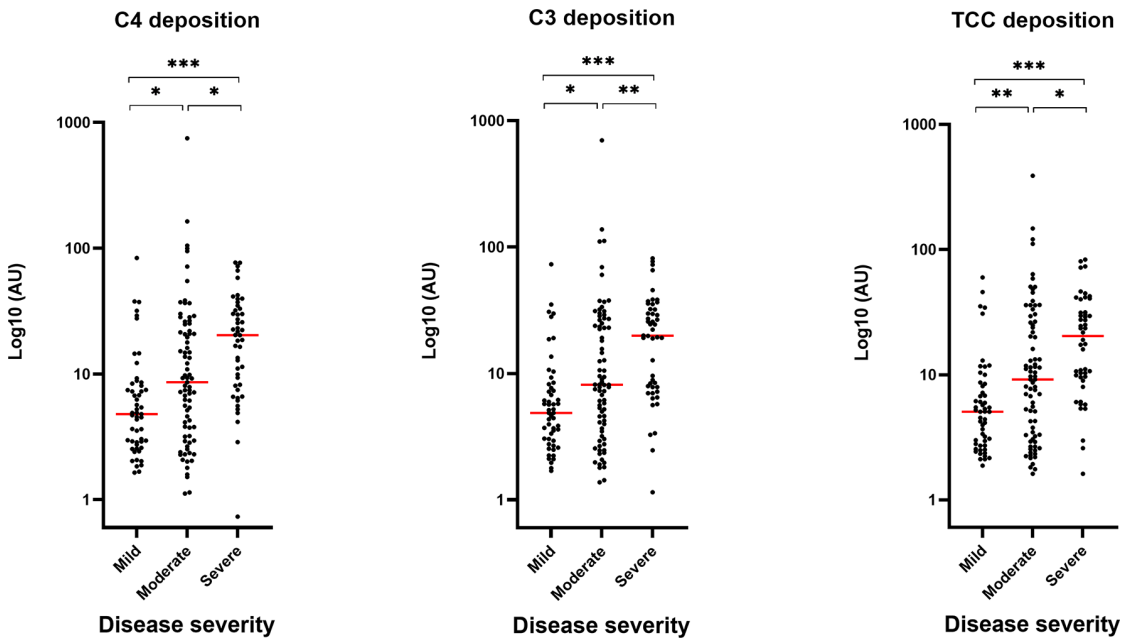


FIGURE 3 | Correlation between complement deposition, IgG levels and severity. Groups divided according to disease severity. Dynamic range represented in arbitrary units (AU). A p value < 0.05 was considered significant. *p < 0.05, **p < 0.01, ***p < 0.001 using Tukey all-pair comparisons, n = 180.

same tendency is seen for all three complement components. Of notice we see the most pronounced spreading/distribution in the moderate group. The findings agree with a recent study conducting a systematic review and meta-analysis, investigating possible differences in the serum concentrations of the complement components, C4 and C3, in COVID-19 patients with different severity and survival status (33). The conclusion was that both C4 and C3 levels were significantly lower in patients with high disease severity or non-survivor status than patients with low severity or survivor status, indicating that C4 and C3 are more readily consumed in patients with a severe or fatal course of the disease. Another study found that the complement system was one of the intracellular pathways most highly induced by SARS-CoV-2 infection in lung epithelial cells (34). The genes whose transcription was most highly induced by SARS-CoV-2 was encoding C1 proteases C1R and C1S, CFB, and complement C3. The authors conclude that the induction of complement expression and C3 protein activation in airway epithelial cells is a SARS-CoV-2–driven event and not a bystander event triggered by exacerbated inflammation. Interestingly, this may be a mechanism common to pandemic coronaviruses, because mouse models of the related SARS-CoV-1 infection, have indicated that C3, C1R, and CFB are all part of a pathogenic gene signature correlating with lethality (35). One could speculate whether increased complement activation is a broad indicator of critical disease, or whether increased complement activation contributes to severe illness. In a recent publication they investigated complement activation in blood from patients with COVID-19 compared with two non-COVID cohorts: patients hospitalized with influenza and patients admitted to the intensive care unit with acute respiratory failure requiring invasive mechanical ventilation (36). They demonstrated that circulating markers of complement activation were elevated in patients with COVID-19 compared with those with influenza and to patients with non–COVID-19 respiratory failure. Moreover, the results indicated that enhanced activation of the alternative pathway was most prevalent in patients with severe COVID-19 and was associated with markers of endothelial injury and hypercoagulability; all together identifying complement activation as a distinctive and possibly pathogenic feature of COVID-19. In addition, it might be expected that increased antibody production and complement activation are a result of high viral load. However, a study demonstrated that total levels of IgG were not correlated with viral load in COVID-19 patients, while a weak negative correlation was observed between IgG subclasses and viral load (27). These findings are consistent with two prior studies, which also reported a missing correlation between persistent SARS-CoV-2 RNA and neutralizing antibody titers (37, 38). Considering the negative correlation between viral load and antibody levels, the role of IgG subclasses, especially IgG1 and IgG3, could be very relevant parameters during COVID-19 infection. Taken together, the findings give an essential insight into the immunological response during a COVID-19 infection and further research is needed into the potentially harmful effects of SARS-CoV-2 antibodies and their interaction with the complement system during infection.

Monocytes and monocyte-derived macrophages are professional phagocytes, utilizing Fc, complement, lectin- and scavenger receptors to facilitate endocytosis. They are also highly active biosynthetic and secretory cells, contributing to inflammation, immunity and antiviral responses (39). It has

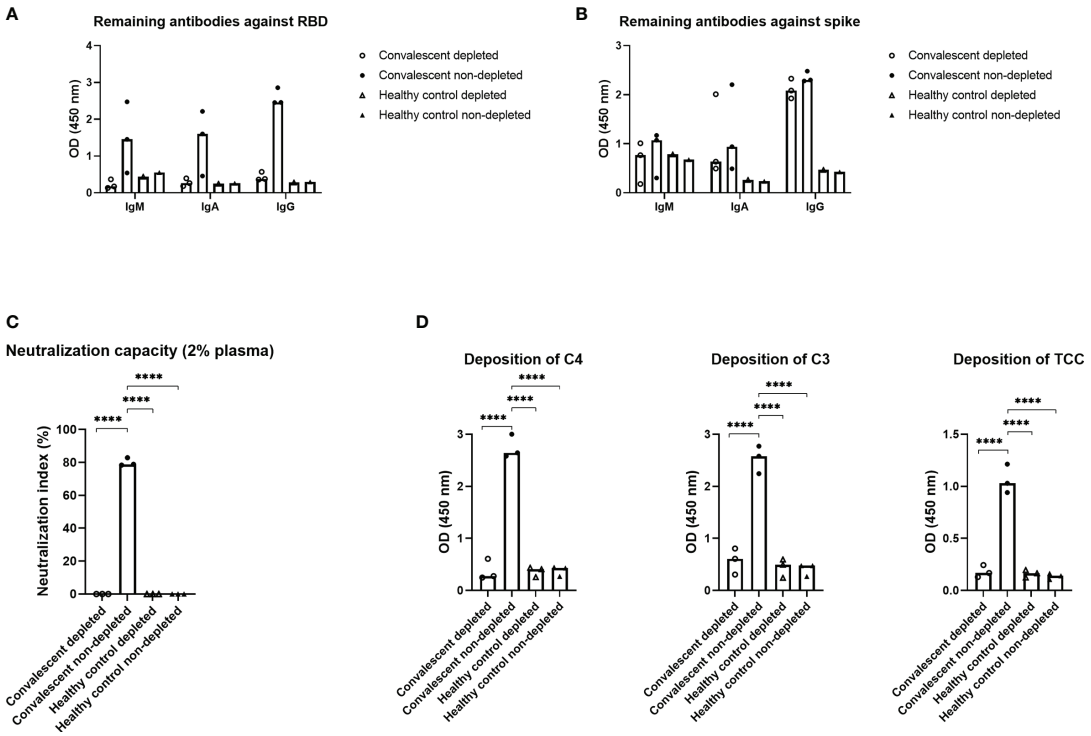


FIGURE 4 | Effect of depletion of antibodies against RBD. Plasma from three recovered convalescent individuals and healthy control individuals was depleted for anti-RBD antibodies and subjected to the following assays; **(A)** an RBD specific sandwich ELISA, **(B)** a spike specific sandwich ELISA, **(C)** a neutralizing assay and **(D)** a complement deposition assay, with spike as the antigen. The RBD and spike specific assays utilized plates coated with RBD or spike (1 µg/ml) and HRP-conjugated polyclonal rabbit antibodies against human IgM, IgA, or IgG (1 µg/ml) for detection. A p value < 0.05 was considered significant. ****p < 0.0001 using an ordinary one-way-ANOVA with Holm-Šídák's multiple comparisons test. Data are represented as mean values of two independent measurements.

been demonstrated that myeloid cell recruitment to the lungs contributes to the hyperinflammatory state and coagulation during ARDS (40). COVID-19 infection is known to be accompanied by an aggressive inflammatory response with the release of a large amount of pro-inflammatory cytokines, also referred to as the "cytokine storm" (41, 42). In order to address the cellular mediated inflammatory response as a consequence of FcγR engagement by SARS-CoV-2 specific antibodies, we evaluated cytokine secretion of IL-6, IL-1β and TNF-α by THP-1 cells and freshly isolated monocytes after incubation with RBD immune complexes. Much of the research focus has been on the role of antibodies and FcγRs during viral infections in relation to virus neutralization and antibody-dependent enhancement of infection, while data on FcγR-mediated cytokine responses in the context of viral infections is limited and somehow conflicting (43). However, it has been suggested that anti-spike antiserum can induce hyperinflammation *via* macrophage FcγR, depending on IgG glycosylation and involving Syk, which is a tyrosine kinase involved in intracellular signaling (44). Our investigations indicate that FcγR stimulation of the monocytes by SARS-CoV-2 antibodies result in a significant increase in the production of TNF-α, but not IL-6 and IL-1β. It is nevertheless important to highlight that an induced cytokine profile depends on the collaboration between FcγRs and other danger sensing receptors. For example, cross-activation of FcγRIIa and TLRs is known to strongly amplify the production of several pro-inflammatory cytokines (45, 46). In our analysis, we measure the effect of FcγR activation alone. In addition, the freshly isolated monocytes seemed to be slightly activated (although not significantly) on immobilized RBD, without SARS-CoV-2 antibodies present – this is most likely explained by the fact that human monocytes have been suggested to have small amounts of ACE-2 on the surface (47). Finally, THP-1 cells are, despite of many similarities to human monocytes, known to have distinct cytokine profiles compared to human monocytes. The lack of IL-6 or IL-1β secretion from the THP-1 cells in our experiment, even after incubation with LPS, is in agreement with previous literature (48). Taken together, it could be speculated whether an exacerbated immune response and antibody production toward SARS-CoV-2 may supplement to the mechanisms causing hyperactivation of macrophages and monocytes, being a contributing factor to the deadly cytokine storm which is a hallmark of COVID-19 disease. In relation, exaggerated type I IFN responses have additionally been reported to be involved in hyperinflammation and contribute to the severe progression of COVID-19 (49).

In conclusion, our findings support the notion that antibodies against SARS-CoV-2 could represent a two-edged sword. It is known that particularly antibody-dependent cellular cytotoxicity and complement-dependent cellular cytotoxicity can drive

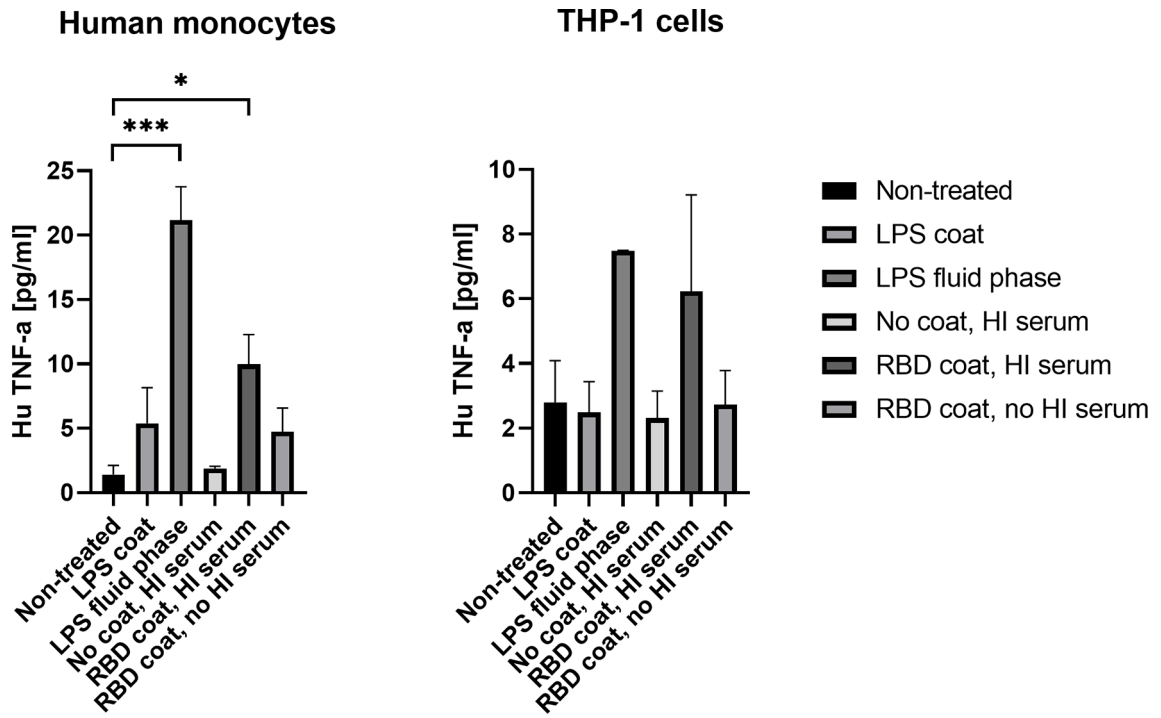


FIGURE 5 | Production of cytokine TNF-α in isolated human monocytes and THP-1 cells after stimulation with SARS-CoV-2 immune complexes. A p value < 0.05 was considered significant. *p < 0.05, ***p < 0.001 using an ordinary one-way-ANOVA with Holm-Šídák's multiple comparisons test.. Data are represented as mean values of three independent measurements.

harmful and systemic proinflammatory responses that can have severe pathophysiological consequences. Our investigations indicate that SARS-CoV-2 antibodies might drive significant inflammatory responses through the classical complement pathway and cellular immune-complex activation that could have negative consequences during COVID-19 disease. However, it is important to note that the data presented does not give direct evidence of classical pathway activation, since a contribution from the lectin pathway cannot be excluded. The combination of antibody-mediated complement activation and subsequent cellular priming could constitute a significant risk of exacerbating COVID-19 severity.

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 human participants were reviewed and approved by Regional Ethical Committee of the Capital Region of Denmark (H-20028627). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IJ, SN, and CJ performed laboratory determinations and analyzed the data. RB-O, M-OS, and PG designed the study. IJ, SN, CJ, CH, LP-A, AR, RB-O, M-OS, and PG wrote the manuscript. All authors critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was financially supported by the Carlsberg Foundation (CF20-0045) and the Novo Nordisk Foundation (205A0063505).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.767981/full#supplementary-material

Supplementary Figure 1 | An ELISA control experiment of the detection of IgG1, -2, -3 and -4.

Supplementary Figure 2 | Correlation between deposition of C4, C3 and TCC.

Supplementary Figure 3 | Production of cytokines IL-6 and IL-1β in human monocytes and THP-1 cells after stimulation with SARS-CoV-2 immune complexes.

REFERENCES

1. Java A, Apicelli AJ, Liszewski MK, Coler-Reilly A, Atkinson JP, Kim AH, et al. The Complement System in COVID-19: Friend and Foe? *JCI Insight* (2020) 5 (15):e140711. doi: 10.1172/jci.insight.140711
2. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: A Key System for Immune Surveillance and Homeostasis. *Nat Immunol* (2010) 11(9):785–97. doi: 10.1038/ni.1923
3. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement System Part I – Molecular Mechanisms of Activation and Regulation. *Front Immunol* (2015) 6:262. doi: 10.3389/fimmu.2015.00262
4. Garred P, Genster N, Pilely K, Bayarri-Olmos R, Rosbjerg A, Ma YJ, et al. A Journey Through the Lectin Pathway of Complement - MBL and Beyond. *Immunol Rev* (2016) 274(1):74–97. doi: 10.1111/imr.12468
5. Jodele S, Köhl J. Tackling COVID-19 Infection Through Complement-Targeted Immunotherapy. *Br J Pharmacol* (2020) 178(14):2832–48. doi: 10.1111/bph.15187
6. Noris M, Benigni A, Remuzzi G. The Case of Complement Activation in COVID-19 Multiorgan Impact. *Kidney Int* (2020) 53(1):1–9. doi: 10.1016/j.kint.2020.05.013
7. Holter JC, Pischke SE, de Boer E, Lind A, Jenum S, Holten AR, et al. Systemic Complement Activation Is Associated With Respiratory Failure in COVID-19 Hospitalized Patients. *Proc Natl Acad Sci USA* (2020) 117(40):25018–25. doi: 10.1073/pnas.2010540117
8. Skendros P, Mitsios A, Chrysanthopoulou A, Mastellos DC, Metallidis S, Rafailidis P, et al. Complement and Tissue Factor-Enriched Neutrophil Extracellular Traps Are Key Drivers in COVID-19 Immunothrombosis. *medRxiv* (2020) 130(11):6151–7. doi: 10.1172/JCI141374
9. Risitano AM, Mastellos DC, Huber-Lang M, Yancopoulou D, Garlanda C, Ciceri F, et al. Complement as a Target in COVID-19? *Nat Rev Immunol* (2020) 20:343–4. doi: 10.1038/s41577-020-0320-7
10. Gralinski LE, Sheahan TP, Morrison TE, Menachery VD, Jensen K, Leist SR, et al. Complement Activation Contributes to Severe Acute Respiratory Syndrome Coronavirus Pathogenesis. *MBio* (2018) 9(5):1–15. doi: 10.1128/mBio.01753-18
11. Yu J, Yuan X, Chen H, Chaturvedi S, Braunstein EM, Brodsky RA. Direct Activation of the Alternative Complement Pathway by SARS-CoV-2 Spike Proteins Is Blocked by Factor D Inhibition. *Blood* (2020) 136(18):2080–9. doi: 10.1182/blood.2020008248
12. Gao T, Hu M, Zhang X, Li H, Zhu L, Liu H, et al. Highly Pathogenic Coronavirus N Protein Aggravates Lung Injury by MASP-2-Mediated Complement Over-Activation. *medRxiv* (2020). doi: 10.1101/2020.03.29.20041962
13. Ramlall V, Thangaraj PM, Meydan C, Foox J, Butler D, Kim J, et al. Immune Complement and Coagulation Dysfunction in Adverse Outcomes of SARS-CoV-2 Infection. *Nat Med* (2020) 26:1609–15. doi: 10.1038/s41591-020-1021-2
14. Macor P, Durigutto P, Mangogna A, Bussani R, D'Errico S, Zanon M, et al. Multi-Organ Complement Deposition in COVID-19 Patients. *medRxiv* (2021). doi: 10.1101/2021.01.07.21249116
15. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement Associated Microvascular Injury and Thrombosis in the Pathogenesis of Severe COVID-19 Infection: A Report of Five Cases. *Transl Res* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
16. Carvelli J, Demaria O, Vély F, Batista L, Benmansour NC, Fares J, et al. Association of COVID-19 Inflammation With Activation of the C5a–C5aR1 Axis. *Nature* (2020) 588:146–50. doi: 10.1038/s41586-020-2600-6
17. Cugno M, Meroni PL, Gualtierotti R, Griffini S, Grovetti E, Torri A, et al. Complement Activation in Patients With COVID-19: A Novel Therapeutic Target. *J Allergy Clin Immunol* (2020) 146:215–7. doi: 10.1016/j.jaci.2020.05.006
18. Wang R, Xiao H, Guo R, Li Y, Shen B. The Role of C5a in Acute Lung Injury Induced by Highly Pathogenic Viral Infections. *Emerg Microbes Infect* (2015) 4(5):1–7. doi: 10.1038/emi.2015.28
19. Bosmann M. Complement Control for COVID-19. *Sci Immunol* (2021) 6(59): eabj1014. doi: 10.1126/sciimmunol.abj1014
20. Hansen CB, Jarlhelt I, Pérez-Alós L, Hummelshøj Landsy L, Loftager M, Rosbjerg A, et al. SARS-CoV-2 Antibody Responses Are Correlated to Disease Severity in COVID-19 Convalescent Individuals. *J Immunol* (2021) 206 (1):109–17. doi: 10.4049/jimmunol.2000898
21. Garred P, Mollnes TE, Lea T, Fischer E. Characterization of a Monoclonal Antibody MoAb Bh6 Reacting With a Neoepitope of Human C3 Expressed on C3b, Ic3b, and C3c. *Scand J Immunol* (1988) 27(3):319–27. doi: 10.1111/j.1365-3083.1988.tb02353.x
22. Mollnes TE, Lea T, Harboe M, Tschopp J. Monoclonal Antibodies Recognizing a Neoantigen of Poly(C9) Detect the Human Terminal Complement Complex in Tissue and Plasma. *Scand J Immunol* (1985) 22 (2):183–95. doi: 10.1111/j.1365-3083.1985.tb01870.x
23. Bayarri-Olmos R, Idorn M, Rosbjerg A, Pérez-Alós L, Hansen CB, Johnsen LB, et al. SARS-CoV-2 Neutralizing Antibody Responses Towards Full-Length Spike Protein and the Receptor-Binding Domain. *J Immunol* (2021) 207(3):878–87. doi: 10.4049/jimmunol.2100272
24. Chen Y, Tong X, Li Y, Gu B, Yan J, Liu Y, et al. A Comprehensive, Longitudinal Analysis of Humoral Responses Specific to Four Recombinant Antigens of SARS-CoV-2 in Severe and Non-Severe COVID-19 Patients. *PLoS Pathog* (2020) 16(9):1–16. doi: 10.1371/journal.ppat.1008796
25. Suthar MS, Zimmerman MG, Kauffman RC, Mantus G, Linderman SL, Hudson WH, et al. Rapid Generation of Neutralizing Antibody Responses in COVID-19 Patients. *Cell Rep Med* (2020) 1(3):100040. doi: 10.1016/j.xcrm.2020.100040
26. Hu YL, Luo FL, Fu JL, Chen TL, Wu SM, Zhou YD, et al. Early Increased Ficolin-2 Concentrations Are Associated With Severity of Liver Inflammation and Efficacy of Anti-Viral Therapy in Chronic Hepatitis C Patients. *Scand J Immunol* (2013) 77(2):144–50. doi: 10.1111/sji.12014
27. Luo H, Jia T, Chen J, Zeng S, Qiu Z, Wu S, et al. The Characterization of Disease Severity Associated IgG Subclasses Response in COVID-19 Patients. *Front Immunol* (2021) 12:632814. doi: 10.3389/fimmu.2021.632814
28. Vidarsson G, Dekkers G, Rispens T. IgG Subclasses and Allotypes: From Structure to Effector Functions. *Front Immunol* (2014) 5(OCT):1–17. doi: 10.3389/fimmu.2014.00520
29. Ferrante A, Beard LJ, Feldman RG. IgG Subclass Distribution of Antibodies to Bacterial and Viral Antigens. *Pediatr Infect Dis J* (1990) 9(8):516–24. doi: 10.1097/00006454-199008001-00004
30. Yates JL, Ehrbar DJ, Hunt DT, Girardin RC, Dupuis AP, Payne AF, et al. Serological Analysis Reveals an Imbalanced IgG Subclass Composition Associated With COVID-19 Disease Severity. *Cell Rep Med* (2021) 2 (7):100329. doi: 10.1101/2020.10.07.20208603
31. Atyeo C, Fischinger S, Zohar T, Slein MD, Burke J, Loos C, et al. Distinct Early Serological Signatures Track With SARS-CoV-2 Survival. *Immunity* (2020) 53 (3):524–32.e4. doi: 10.1016/j.immuni.2020.07.020
32. Kang S, Yang M, He S, Wang Y, Chen X, Chen YQ, et al. A SARS-CoV-2 Antibody Curbs Viral Nucleocapsid Protein-Induced Complement Hyperactivation. *Nat Commun* (2021) 12(1):1–11. doi: 10.1038/s41467-021-23036-9
33. Zinellu A, Mangoni AA. Serum Complement C3 and C4 and COVID-19 Severity and Mortality: A Systematic Review and Meta-Analysis With Meta-Regression. *Front Immunol* (2021) 12:1–13. doi: 10.3389/fimmu.2021.696085
34. Yan B, Freiwald T, Chauss D, Wang L, West E, Mirabelli C, et al. SARS-CoV-2 Drives JAK1/2-Dependent Local Complement Hyperactivation. *Sci Immunol* (2021) 6(58):eabg0833. doi: 10.1126/sciimmunol.abg0833
35. Rockx B, Baas T, Zornetzer GA, Haagmans B, Sheahan T, Frieman M, et al. Early Upregulation of Acute Respiratory Distress Syndrome-Associated Cytokines Promotes Lethal Disease in an Aged-Mouse Model of Severe Acute Respiratory Syndrome Coronavirus Infection. *J Virol* (2009) 83 (14):7062–74. doi: 10.1128/JVI.00127-09
36. Ma L, Sahu SK, Cano M, Kuppuswamy V, Bajwa J, McPhatter J, et al. Increased Complement Activation Is a Distinctive Feature of Severe SARS-CoV-2 Infection. *Sci Immunol* (2021) 6(59):eabh2259. doi: 10.1126/sciimmunol.abh2259
37. Zhang X, Lu S, Li H, Wang Y, Lu Z, Liu Z, et al. Viral and Antibody Kinetics of COVID-19 Patients With Different Disease Severities in Acute and Convalescent Phases: A 6-Month Follow-Up Study. *Virol Sin* (2020) 35 (6):820–9. doi: 10.1007/s12250-020-00329-9
38. Wang Y, Zhang L, Sang L, Ye F, Ruan S, Zhong B, et al. Kinetics of Viral Load and Antibody Response in Relation to COVID-19 Severity. *J Clin Invest* (2020) 130(10):5235–44. doi: 10.1172/JCI138759
39. Gordon S, Plüddemann A. Tissue Macrophages: Heterogeneity and Functions. *BMC Biol* (2017) 15(1):1–18. doi: 10.1186/s12915-017-0392-4

40. Wang J, Jiang M, Chen X, Montaner LJ. Cytokine Storm and Leukocyte Changes in Mild *Versus* Severe SARS-CoV-2 Infection: Review of 3939 COVID-19 Patients in China and Emerging Pathogenesis and Therapy Concepts. *J Leukoc Biol* (2020) 108(1):17–41. doi: 10.1002/JLB.3COVR0520-272R
41. Coperchini F, Chiovato L, Croce L, Magri F, Rotondi M. The Cytokine Storm in COVID-19: An Overview of the Involvement of the Chemokine/Chemokine-Receptor System. *Cytokine Growth Factor Rev* (2020) 53 (May):25–32. doi: 10.1016/j.cytogfr.2020.05.003
42. Song P, Li W, Xie J, Hou Y, You C. Cytokine Storm Induced by SARS-CoV-2. *Clin Chim Acta* (2020) 509(January):280–7. doi: 10.1016/j.cca.2020.06.017
43. Vogelpoel LTC, Baeten DLP, de Jong EC, den Dunnen J. Control of Cytokine Production by Human Fc Gamma Receptors: Implications for Pathogen Defense and Autoimmunity. *Front Immunol* (2015) 6:79. doi: 10.3389/fimmu.2015.00079
44. Hoepel W, Chen HJ, Geyer CE, Allahverdiyeva S, Manz XD, de Taeye SW, et al. High Titers and Low Fucosylation of Early Human Anti-SARS-CoV-2 IgG Promote Inflammation by Alveolar Macrophages. *Sci Transl Med* (2021) 13(596):eabf8654. doi: 10.1126/scitranslmed.abf8654
45. Den Dunnen J, Vogelpoel LTC, Wypych T, Muller FJM, De Boer L, Kuijpers TW, et al. IgG Opsonization of Bacteria Promotes Th17 Responses *via* Synergy Between TLRs and Fcγriia in Human Dendritic Cells. *Blood* (2012) 120(1):112–21. doi: 10.1182/blood-2011-12-399931
46. Vogelpoel LTC, Hansen IS, Rispens T, Muller FJM, Van Capel TMM, Turina MC, et al. Fc Gamma Receptor-TLR Cross-Talk Elicits Pro-Inflammatory Cytokine Production by Human M2 Macrophages. *Nat Commun* (2014) 5:5444. doi: 10.1038/ncomms6444
47. Rutkowska-Zapała M, Suski M, Szatanek R, Lenart M, Weglarczyk K, Olszanecki R, et al. Human Monocyte Subsets Exhibit Divergent Angiotensin I-Converting Activity. *Clin Exp Immunol* (2015) 181(1):126–32. doi: 10.1111/cei.12612
48. Schildberger A, Rossmanith E, Eichhorn T, Strassl K, Weber V. Monocytes, Peripheral Blood Mononuclear Cells, and THP-1 Cells Exhibit Different Cytokine Expression Patterns Following Stimulation With Lipopolysaccharide. *Mediators Inflamm* (2013) 2013:697972. doi: 10.1155/2013/697972
49. Kim YM, Shin EC. Type I and III Interferon Responses in SARS-CoV-2 Infection. *Exp Mol Med* (2021) 53(5):750–60. doi: 10.1038/s12276-021-00592-0

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Immunology

ORIGINAL RESEARCH
published: 05 November 2021
doi: 10.3389/fimmu.2021.767347

SARS-CoV-2 Exacerbates COVID-19 Pathology Through Activation of the Complement and Kinin Systems

*Anne G. Savitt[1,2], Samantha Manimala[2], Tiara White[1,2], Marina Fandaros[3], Wei Yin[3], Huiquan Duan[4], Xin Xu[4], Brian V. Geisbrecht[4], David A. Rubenstein[3], Allen P. Kaplan[5], Ellinor I. Peerschke[6] and Berhane Ghebrehiwet[1]**

[1] Department of Microbiology & Immunology, Renaissance School of Medicine of Stony Brook University, Stony Brook, NY, United States, [2] Department of Medicine, Renaissance School of Medicine of Stony Brook University, Stony Brook, NY, United States, [3] Department of Biomedical Engineering, Stony Brook University, Stony Brook, NY, United States, [4] Department of Biochemistry and Molecular Biophysics, Kansas State University, Manhattan, KS, United States, [5] Pulmonary and Critical Care Division, The Medical University of South Carolina, Charleston, SC, United States, [6] The Department of Laboratory Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, United States

OPEN ACCESS

Edited by:
Zoltán Prohászka,
Semmelweis University, Hungary

Reviewed by:
Christian Drouet,
INSERM U1016 Institut Cochin (INSERM), France
Péter Gál,
Hungarian Academy of Sciences (MTA), Hungary

***Correspondence:**
Berhane Ghebrehiwet
berhane.ghebrehiwet@stonybrook.edu

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: 30 August 2021
Accepted: 19 October 2021
Published: 05 November 2021

Citation:
Savitt AG, Manimala S, White T, Fandaros M, Yin W, Duan H, Xu X, Geisbrecht BV, Rubenstein DA, Kaplan AP, Peerschke EI and Ghebrehiwet B (2021) SARS-CoV-2 Exacerbates COVID-19 Pathology Through Activation of the Complement and Kinin Systems. Front. Immunol. 12:767347. doi: 10.3389/fimmu.2021.767347

Infection with SARS-CoV-2 triggers the simultaneous activation of innate inflammatory pathways including the complement system and the kallikrein-kinin system (KKS) generating in the process potent vasoactive peptides that contribute to severe acute respiratory syndrome (SARS) and multi-organ failure. The genome of SARS-CoV-2 encodes four major structural proteins – the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and the envelope (E) protein. However, the role of these proteins in either binding to or activation of the complement system and/or the KKS is still incompletely understood. In these studies, we used: solid phase ELISA, hemolytic assay and surface plasmon resonance (SPR) techniques to examine if recombinant proteins corresponding to S1, N, M and E: (a) bind to C1q, gC1qR, FXII and high molecular weight kininogen (HK), and (b) activate complement and/or the KKS. Our data show that the viral proteins: (a) bind C1q and activate the classical pathway of complement, (b) bind FXII and HK, and activate the KKS in normal human plasma to generate bradykinin and (c) bind to gC1qR, the receptor for the globular heads of C1q (gC1q) which in turn could serve as a platform for the activation of both the complement system and KKS. Collectively, our data indicate that the SARS-CoV-2 viral particle can independently activate major innate inflammatory pathways for maximal damage and efficiency. Therefore, if efficient therapeutic modalities for the treatment of COVID-19 are to be designed, a strategy that includes blockade of the four major structural proteins may provide the best option.

Keywords: Sars-CoV-2, complement, bradykinin, COVID-19, kinin-kallikrein system, post COVID "long-haulers"

INTRODUCTION

Coronaviruses (CoVs) are a group of related viruses which cause mild to severe diseases in both humans and animals. However, 3 of the last 7 pathogenic coronaviruses reported have caused much more severe and often fatal respiratory infections in humans and have been responsible for deadly pneumonia outbreaks in the 21st century (1–9). Coronaviruses cause a lethal disease called severe acute respiratory syndrome (SARS), in which the subsequent edema in the lungs prevents oxygen uptake, resulting in deadly hypoxia (4, 5). Since the first major outbreak of SARS in 2002, there have been two major coronavirus pandemics: MERS-CoV (Middle Eastern Respiratory Syndrome Coronavirus) in 2012, which affected 27 countries in the Middle East, Africa and South Asia, and the present COVID-19 pandemic, which is caused by SARS-CoV-2 (1–9). Therefore, as the virus adjusts and adapts to its environment, it will certainly mutate through either immunologic shift or immunologic drift, releasing respectively new strains or variants that cause novel pandemics in the future (1–9). In fact, the new variants of SARS-CoV-2 that appeared very recently in the UK and S. Africa and now also showing up in the US and other parts of the world are almost certainly the beginning of what is to come. Therefore, complete understanding of the molecular structures and the mutations that trigger and/or exacerbate the diseases caused by SARS-CoV-2 may help us identify novel pharmacological targets for the development of therapies that challenge present as well as future pandemics.

The genome of the SARS-CoV-2 encodes four major structural proteins: the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and the envelope (E) protein, all of which are required to produce a structurally complete viral particle (1, 2). Among these structural proteins however, the S protein takes center stage in SARS-CoV-2 infection as it is singularly responsible for viral attachment, fusion, and entry into target cells. Infection with SARS-CoV-2 is initiated when the spike (S) protein interacts with its cognate host cell surface receptor (5, 10). SARS-CoV-2 infects human lung alveolar type II epithelial cells by attaching *via* its S protein to angiotensin converting enzyme 2 (ACE-2) expressed at the cell surface (9). Viral entry is facilitated when the type II transmembrane serine protease (TMPRSS2) cleaves the viral S protein into S1 and S2, with the latter causing membrane fusion (7–9). This is followed by simultaneous activation of powerful, cross-reactive inflammatory pathways in plasma resulting in rapid production of vasoactive peptides, which in turn recruit leukocytes which secrete inflammatory cytokines that contribute to the vascular leakage and edema culminating in severe acute respiratory syndrome. Foremost among these cross-reactive innate pathways are the complement system, the coagulation system, and the kallikrein-kinin system (KKS), each of which is able to generate activation byproducts that collectively contribute to the 'cytokine storm', multi-organ inflammation, bilateral pneumonia, and progression to the acute respiratory distress syndrome (ARDS) requiring ventilatory support (5, 9–13). The question is: which of the SARS-CoV-2-associated molecular structures are responsible for the activation of the innate immune pathways?

The present studies were undertaken to examine in detail if any of the highly conserved SARS-CoV-2 proteins–which are encoded by all the coronaviruses–can activate the complement and/or the kinin system. Furthermore, since gC1qR (14, 15), the receptor for globular heads of C1q as well as for high molecular weight kininogen (HK), is often overexpressed and released by infected cells, we hypothesized that, if any of the SARS-CoV-2 structural proteins bind gC1qR, then a virus decorated with gC1qR, or released viral proteins bound to gC1qR, may provide a suitable complex for the assembly and activation of the complement system, the KKS and–directly or indirectly–the coagulation system (9, 11–13). Therefore, we also examined if any of the SARS-CoV-2 structural proteins directly interact with gC1qR in a manner that activates the complement system and/or the KKS.

MATERIALS AND METHODS

Chemicals and General Reagents

Recombinant proteins corresponding to the structural proteins of SARS-CoV-2 (S1, N and a fusion protein of M-E), were purchased from ViroGen Corporation (Brighton, MA), and the M and E proteins were purchased from MyBioSource, Inc. (San Diego, CA), as was biotinylated anti complement C4d fragment. Bradykinin (BK) ELISA kit was purchased from Enzo Life Sciences Inc. (East Farmingdale, NY), and normal human plasma from random donors was purchased from Oklahoma Blood Institute (Oklahoma City, OK).

Expression of Recombinant gC1qR

The strategies for expression and purification of mature gC1qR and deletion mutants have been described previously (14–19).

Solid-Phase Microplate Binding Assay

The interaction of viral proteins with gC1qR was assessed by a standard ELISA. Briefly, microtiter plate wells were coated in duplicates (60 min, at 37°C or overnight, 4°C) with 100 µl of either, concentrations of viral proteins (S1, N, M, E or a fusion M-E) ranging from 2-10 µg/ml, or heat inactivated BSA, in carbonate buffer, pH 9.6 (15 mM Na_2CO_3 and 35 mM $NaHCO_3$). The unbound protein was removed from each well; the wells washed 2x with TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween-20), and the unreacted sites blocked by incubation (30 min, room temp) with 300 µl of 1% heat inactivated BSA (1hr, 37°C). After washing (2x with TBST), the microtiter plate bound proteins were incubated (1hr, room temp.) with concentrations of biotinylated gC1qR, ranging from 0 to 5 µg/ml. This was followed by sequential reaction (1 hr each, room temp) with alkaline-phosphatase conjugated streptavidin and pNPP, and the color developed at the end of the incubation read spectrophotometrically at 405nm.

Similarly, the binding of FXII or HK to microtiter plate coated S1, N, M-E was performed using the same strategy and the

bound FXII or HK was detected using specific antibodies. In all cases, each experiment was repeated at least three times in duplicates (n=3).

Surface Plasmon Resonance

Biosensor surfaces were prepared by immobilizing either wild-type gC1qR (i.e., gC1qR-WT) or a gC1qR deletion mutant that removed the flexible, negatively charged loops (i.e., gC1qR-ΔΔ). Then, a two-fold dilution series of recombinant forms of various SARS-CoV-2 structural proteins was injected over each surface. The reference-corrected sensorgrams were then fit to kinetic models to obtain the apparent equilibrium dissociation constant for each interaction pair. Of note, the gC1qR-ΔΔ used in these studies (not published) was generated–as an alternate to gC1qR-WT for crystallographic experiments–by removing the highly charged D and E rich loops. Interestingly, despite the missing charged loops, we found that the gC1qR-ΔΔ not only crystallizes readily, but also forms a trimer both in solution and crystal, demonstrating that the protein is indeed intact. More importantly, HK binds to both gC1qR and gC1qR-ΔΔ equally well (unpublished data).

Hemolytic Assays for Complement Activation

To assess whether the viral antigens that bound to C1q also activate complement in serum, we used a standard hemolytic assay for complement activity using AggIgG as a positive control. To make AggIgG, 10 mg/ml of IgG was incubated at 62°C for 20 min and then the large precipitates formed were removed by centrifugation at 1000xg for 30 min. Each AggIgG was then tested for complement activation before use (20). Briefly, 10 µl of normal human serum (NHS, Complement Technology, Inc., Tyler, TX) in 100 µl GVB^{++} (gelatin containing veronal buffer) was first incubated (1hr, 37°C) with or without various concentrations of recombinant viral proteins S1, N, M, E, or the fusion of M-E. As a positive control for complement activation, NHS was incubated with 10 µl of aggregated IgG (AggIgG) in 100 µl of GVB^{++}. After incubation, 50 µl of sensitized sheep erythrocytes (EAs) ($2x10^8$/ml sheep red blood cells sensitized with anti-sheep IgG) were added to each tube, the volume brought up to 500 µl with GVB^{++} and further incubated (1hr, 37°C). The tubes were then centrifuged and the degree of hemolysis in the supernatant in each tube determined spectrophotometrically at 412 nm. The total, releasable hemoglobin (100%), was achieved by lysis of 50 µl EAs with 450 µl of H_2O, against which the hemoglobin released in each experimental tube was compared.

To assess the deposition of complement C1q and C4d fragment on viral proteins under the conditions of the hemolytic assay, viral proteins were coated in wells of microtiter plates (10 µg/mL) and blocked with 1% heat inactivated BSA as described under Solid Phase Microtiter Plate Assay, above. Wells were then incubated with 50 µL of a 1:10 dilution of NHS in GVB^{++} and incubated at 37°C for 2 hrs. After washing, the wells were then probed with either biotinylated anti C4d or goat anti C1q in GVB^{++}, followed by streptavidin AP or rabbit anti goat AP. Following washing with GVB^{++}, wells were incubated with pNPP and color development monitored at 405 nm.

Activation of the KKS by Viral Proteins

To test if the binding of the viral proteins resulted in KKS activation, we used a commercial BK ELISA kit (Enzo Life Sciences Inc., East Farmingdale, NY) and followed the manufacturer's recommendations. Briefly, 1 ml of normal human plasma (from random donors, purchased from Oklahoma Blood Institute, Oklahoma City, OK) diluted 1:16 in assay buffer (with 50 µM $ZnCl_2$) was mixed with various concentrations (1, 2, 5 or 10 µg/mL) of S, M, and N proteins and incubated at 37°C for 1 hour. The plasma samples were then diluted and added to rabbit-IgG antibody-coated wells, along with biotin-conjugated bradykinin and a rabbit polyclonal antibody to bradykinin. Through a competitive binding process, biotin-conjugated bradykinin was captured on the bottom of the wells, which was detected using horseradish peroxidase-conjugated streptavidin. Color development was read at 405nm in a Spectramax i3x microplate reader (Molecular Devices).

Statistical Analysis

Student t-tests were performed using statistical software (Excel; Microsoft, Redmond, WA, USA). A value of p=0.05 was a significant difference. (n - represents number of separate experiments performed in duplicates).

RESULTS

Activation of the Classical Pathway of Complement by SARS-CoV-2 Proteins

The first question we asked was: do the viral proteins trigger activation of the classical pathway of complement? As shown in **Figure 1**, preincubation of each of the viral structural proteins with normal human serum results in a dose-dependent diminished complement activity. The diminished hemolytic activity in turn, is due to consumption of complement as a result of preincubation of the viral proteins with NHS. However, since preincubation of the viral proteins with NHS could also result in inhibition of complement due to blockade of C1q or other proteins critical in complement activation, we performed two additional experiments to confirm a *bona fide* activation of the cascade. First, we showed that incubation of the viral proteins with C1q-depleted serum did not result in complement activation (not shown), suggesting that the classical pathway is activated by the SARS-CoV-2 structural proteins. Second, we confirmed that incubation of NHS to microtiter bound viral proteins results in C1q deposition (**Figure 2A**), followed by the presence of degradation fragments such as C4d (**Figure 2B**), which confirms the activation of the classical pathway of complement. The fact that the viral proteins bind to C1q in a specific and dose-dependent manner, followed by the experiment in which C1q-depleted serum did not result in complement

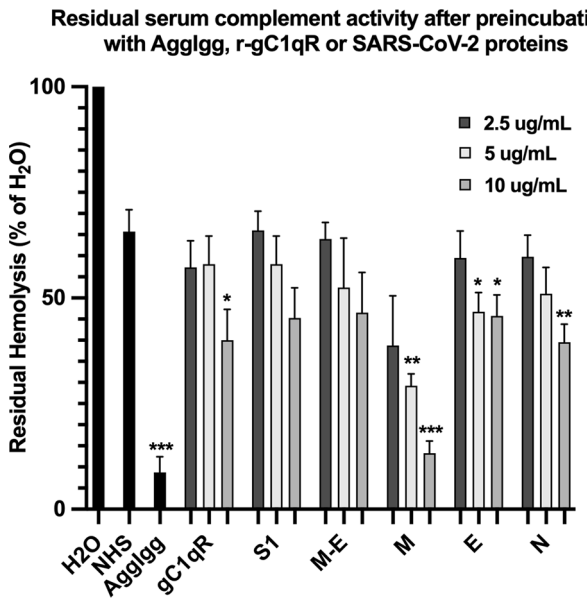


FIGURE 1 | Hemolytic assay to assess the effect of SARS-CoV-2 recombinant proteins on complement activity. Recombinant spike (S1), membrane (M), envelope (E) and nucleocapsid (N) proteins were pre-incubated with NHS in GVB^{++} (1:10 dilution) for 1 hr at 37°C. Then EAs were added and incubated continued for an additional hour. Cells were removed by centrifugation and the absorbance of the hemoglobin released into the supernatants was read at 412 nm. Positive control for complement activity is NHS incubated with EAs, and control for complement consumption is NHS pre-incubated with AgglgG and then with EAs. Data are representative of three independent experiments (n=3). Results are expressed as percent hemolysis relative to 100% (H_20). Statistical analysis unpaired T test with Welch's correction was performed in Graphpad Prism. Error bars represent 1SD. *p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001.

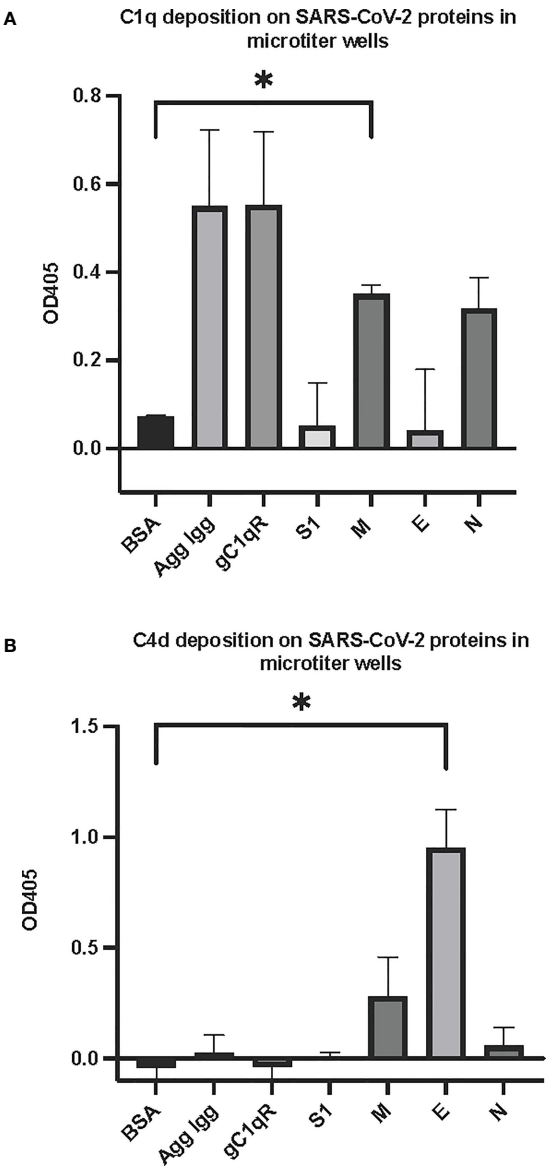


FIGURE 2 | Deposition of complement proteins **(A)** C1q and **(B)** C4d fragment on SARS-CoV-2 proteins immobilized on microtiter plates. Microtiter wells were coated with test proteins at 10 μg/mL. Following blocking with inactivated BSA, the wells were washed with GVB^{++} followed by application of NHS (1:10 dilution in GVB^{++}) and incubation at 37°C for 2 hr. After washing, goat anti human C1q antibody **(**panel **A)** or biotinylated anti C4d polyclonal antibody **(B)** was applied for 1 hr, then wells were incubated with streptavidin-AP **(A)** or rabbit anti goat AP **(B)** and proteins detected with the AP substrate pNPP. Statistical analysis on all samples compared to BSA negative control, unpaired T test with Welch's correction was performed in Graphpad Prism. Error bars represent 1SD. *p ≤ 0.05.

activation suggests that it is the classical pathway and not the MBL pathway that is activated under these conditions. This distinction is important since recent studies have also shown that the MBL pathway, which is similar to the classical pathway in its mode of activation, can be activated by SARS-CoV-2 proteins (21).

SARS-CoV-2 Proteins Bind HK, and FXII and Generate BK

One of the mechanisms by which edema formation can occur in COVID-19 patients is through the activation of the KKS and the subsequent formation of bradykinin (BK). However, for this to occur, the viral proteins would have to bind to the key components in KKS, which are HK and FXII. Therefore, we tested whether any of the viral proteins could bind to these proteins. As shown in **Figure 3**, both HK (A) and FXII (B) dose-dependently (50 ng/ml to 10 μg/ml) bind to immobilized proteins of SARS-CoV-2. Binding increases relative to the concentration of the added protein with a $p \leq 0.0045$ for HK and $p \leq 0.0001$ for FXII. More importantly, incubation of the viral proteins with normal human plasma in the presence of 50 μM $ZnCl_2$ results in the generation of BK (**Figure 4**), which in turn is dependent on the amount of viral protein added to the plasma. At the highest concentration of each viral protein added, there is a diminished BK generation. However, this may be because at high concentration, there is a rapid generation of BK, followed by rapid enzymatic degradation consistent with the half-life of BK in plasma, which is <1 min. Therefore, the degraded BK fragments may not be recognized by the antibody

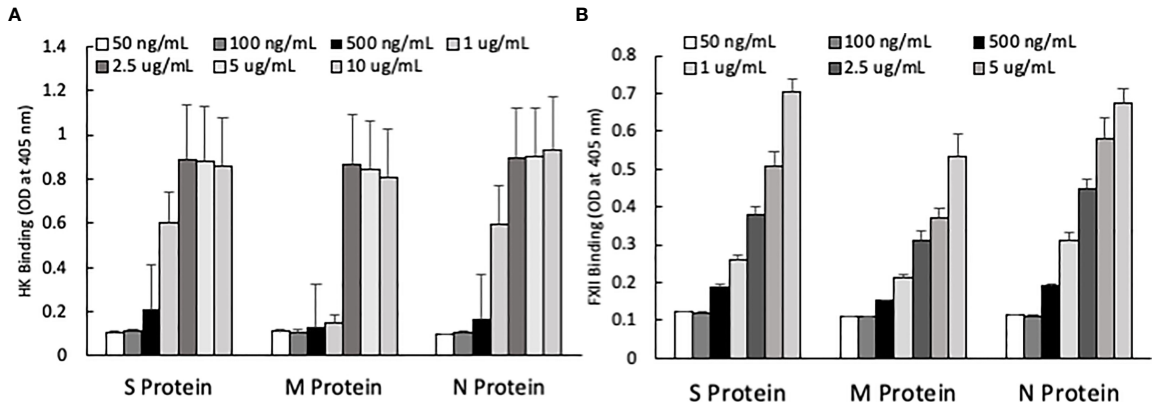


FIGURE 3 | **(A)** High molecular weight kininogen (HK) binding to immobilized S, M and N protein at different concentrations. **(B)** Factor XII (FXII) binding to immobilized S, M and N protein at different concentrations. Data is presented as mean + standard error (n=3-8 for HK binding, and n=4 for FXII binding). ANOVA indicates that the viral protein concentration has a significant effect on HK binding (*P*=0.0033 for S protein, *P*=0.0045 for M protein, and *P*=0.0163 for N protein) and FXII binding *(P*=0.0001 for all three proteins).

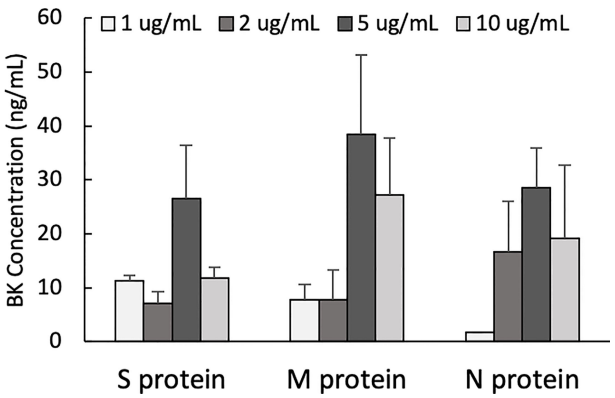


FIGURE 4 | Bradykinin assay. Bradykinin concentration (ng/mL) in normal human plasma incubated with S, M and N protein at 37°C for 1 hour. Data is presented as mean + 1SD (n=2-3.).

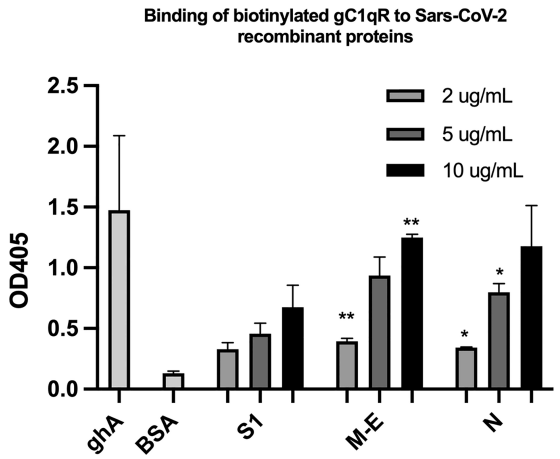


FIGURE 5 | Binding of biotinylated gC1qR to SARS-CoV-2 structural proteins immobilized on microtiter plates. Microtiter plate wells were coated with the indicated amount of test protein, and after blocking unreacted sites with BSA, biotinylated gC1qR at a concentration of 1 ug/ml was added and the bound gC1qR detected by incubation with AP-streptavidin. Positive control for binding is ghA (the A chain of the globular head of C1q) and negative control for non-specific binding is BSA. Statistical analysis comparing , each data point with the BSA negative control is the unpaired T test with Welch's correction was performed in Graphpad Prism (n=3; and *p ≤ 0.05, **p ≤ 0.01).

to BK, thereby giving the false impression that higher concentrations of viral proteins give rise to the formation of fewer molecules of BK.

Binding of SARS-CoV-2 Structural Proteins to gC1qR

Since soluble or cell surface gC1qR can activate the classical pathway of complement and the kinin-kallikrein system (KKS)–both of which play a significant role in the pathogenesis of COVID-19–we postulated that if any of the SARS-CoV-2 structural proteins bind gC1qR, then the gC1qR-decorated viral particles could potentially provide a platform for the simultaneous activation of both the complement and the KKS pathways. To test this hypothesis, we first used solid phase ELISA to test if the viral proteins bind to gC1qR. As shown in **Figure 5**, all of the SARS-CoV-2 structural proteins bind to gC1qR in a dose-dependent manner. Since gC1qR is the receptor for the globular head of the A chain of C1q, recombinant ghA was used as a positive control for gC1qR. Furthermore, the binding of the viral proteins is enhanced in the presence of 50 μM Zn^{+} (not shown) suggesting that the viral protein-gC1qR interaction may be zinc ion-dependent in a manner similar to the interaction between FXII and HK to gC1qR (16–19).

To examine the interaction between gC1qR and the viral proteins through an independent approach, we also used surface

plasmon resonance binding assay. In these experiments, purified viral proteins were injected over surfaces of either wild-type gC1qR or a deletion mutant that removed the two disordered negatively charged loops found in the gC1qR sequence (i.e., gC1qR-ΔΔ). Representative series of sensorgrams from at least three independent determinations are shown in **Figure 6**. Apparent binding affinities for each interaction are as follows: (A, B) Spike-S1 protein over gC1qR-WT (K_D=91 nM) or gC1qR-ΔΔ (K_D=370 nM), (C, D) Nucleocapsid protein over gC1qR-WT (K_D=6 μM) or gC1qR-ΔΔ (K_D=12 μM), (E, F), Membrane-Envelope Fusion protein over gC1qR-WT (K_D=410 nM) or gC1qR-ΔΔ (K_D=360 nM), and (G, H) a synthetic peptide corresponding to the gC1qR-binding region from HK (19, 20) as a positive control (over gC1qR-WT, K_D=2 mM or over gC1qR-ΔΔ, 10 mM). Together, these observations revealed that two different forms of gC1qR bind the various SARS-CoV-2 structural proteins we tested with affinities in the ~0.1-10 μM range. Furthermore, since SPR analyses were conducted using purified components, our results strongly suggest that these interactions occur directly and can take place in the absence of contributions from other proteins.

DISCUSSION

Experimental evidence available to date supports the concept that the pathogenesis induced by SARS-CoV-2 infection is largely due to the simultaneous activation of several cross-reactive immune pathways (2, 20–26) that generate in the process potent and multifunctional activation products that collectively contribute to severe inflammation, intravascular thrombosis, excessive edema, and eventually death (5, 8, 9, 11–13). One of the most consistent laboratory findings associated with the severity of COVID-19 is the elevated levels of D-dimer, which is produced when plasmin dissolves blood clots through a process called fibrinolysis (20, 21). However, in addition to its important role in fibrinolysis, plasmin has many other functions outside its conventional role including activation of FXII (27). For example, plasmin can activate proteins of the complement system and releases powerful bioactive fragments such as C3a and C5a (**Figure 7**), which in turn cause vascular permeability and recruitment of leukocytes that produce proinflammatory cytokines, thus contributing to the cytokine storm that is often associated with COVID-19 disease. In addition, the role of plasmin in activating FXII in the KKS, resulting in release of BK, has long been recognized (28, 29), and has recently been gaining more attention [reviewed in (27)].

Although advances have been made since the onset of the COVID-19 pandemic, the viral and host molecular networks that interact to trigger activation of the innate pathways (20, 21) and exacerbate the COVID-19 pathology are still poorly understood. As explained earlier, the major SARS-CoV-2 structural proteins are: the spike (S) protein, the nucleocapsid (N) protein (1, 2, 22–26, 30). the membrane (M) protein (2, 22–26, 30), and the envelope (E) protein (23, 26). Although all of these proteins are required to produce a structurally complete and highly pathogenic viral particle (1, 2), it is the S protein that is responsible for viral attachment to cells, fusion of viral and cellular membranes, and entry into cells, thus causing a full-blown SARS-CoV-2 infection (9, 10, 22). Not surprisingly therefore, this protein has been the focus of extensive studies as well as the major target for vaccine production.

Infection with SARS-CoV-2 is initiated when the S protein interacts with ACE-2 on the epithelial cell surface (9, 22). However, although the primary function of ACE-2 is to control the activity of ACE-1 in the renin-angiotensin system (RAS), another critical function of ACE-2 is the degradation of BK into non-functional peptides. Since BK generation is at the center of the edema formation in COVID-19 pathology, occupancy of ACE-2 by the viral S protein would inadvertently interfere with its ability to degrade BK thus leaving unregulated and active BK to circulate freely. Furthermore, activation of the kinin system also generates in the process multiple activation peptides including HKa, a cleavage product of HK, which binds gC1qR and promotes the release of cytokines such as TNFα, IL-1β, IL-6, and the chemokines IL-8 and MCP-1 from human mononuclear cells (31–37). Moreover, gC1qR secreted by infected cells has been shown to act as an autocrine signal to induce the expression of the high affinity receptor for HK, namely the bradykinin receptor 1 (B1R) (38). Together, these events would contribute to the exacerbation of the inflammatory processes associated with COVID-19.

Previous studies have shown that some of the SARS-CoV-2 associated proteins, and in particular the S protein, can activate the complement system (31–37) either *via* the MBL pathway (35) or the alternative pathway (37). In the present study, we show that all of the viral proteins also activate complement *via* the classical pathway, presumably by binding to the globular heads of C1q (gC1q) in a manner similar to IgG or gC1qR, which are also able to activate complement (38–41).

By virtue of its ability to bind and activate (29, 42, 43) two of the most potent inflammatory systems in plasma–the complement system and the kinin-kallikrein system (KKS)–we hypothesized that cell surface expressed or secreted gC1qR could also contribute to the rapid inflammatory response and the "cytokine storm" that is associated with COVID-19 pathology. The rationale for this postulate, in turn, is based on the fact that a diverse array of viruses such as HIV-1 (19), hepatitis C virus (44), bovine circovirus (45) Hantavirus (46), Epstein-Barr virus (47), and rubella virus (48) target gC1qR, inside and outside the cell for cellular entry and to enhance their own survival. For example, while infection with human papillomavirus (HPV) triggers gC1qR signaling and mitochondrial dysfunction and apoptosis (49), vesicular stomatitis virus induces gC1qR signaling to block retinoic acid-inducible gene I (RIG-I) activation thereby promoting its replication (50). Moreover, gC1qR expression and secretion is enhanced as a consequence of viral infection. Although it is not yet known whether SARS-CoV-2 engages gC1qR inside the cell, the ELISA and SPR binding data seem to suggest that it would.

To survive and multiply, SARS-CoV-2, like many other viruses, has a built-in strategy that takes advantage of proteins

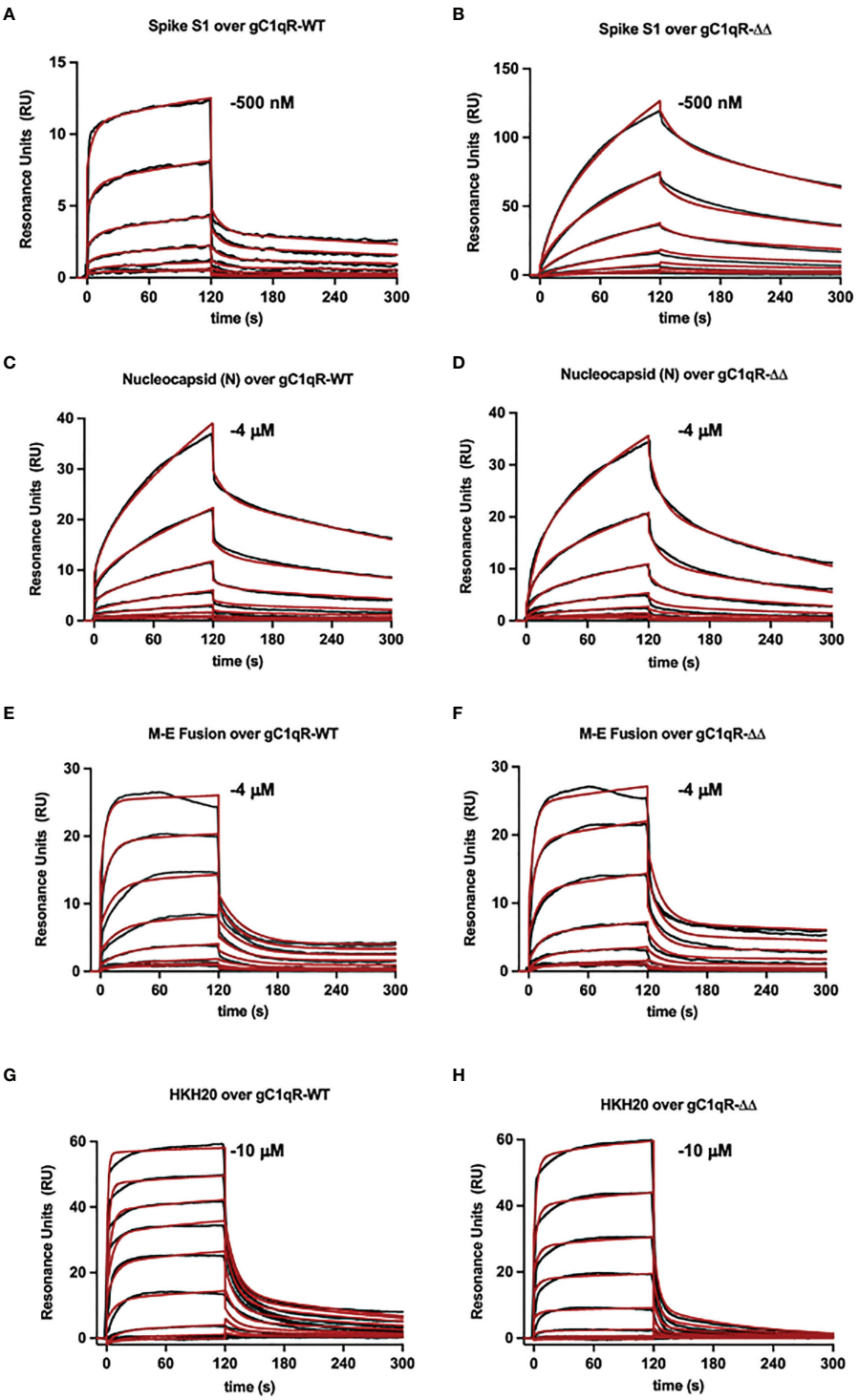


FIGURE 6 | Surface Plasmon Resonance. Surfaces were prepared by immobilizing either wild-type gC1qR (i.e. gC1qR-WT) or a gC1qR deletion mutant that removed the flexible, negatively charged loops (i.e. gC1qR-ΔΔ). A two-fold dilution series of recombinant forms of various SARS-CoV-2 structural proteins was injected over each surface, where the highest concentration of each protein used is inset. The reference-corrected sensorgrams (black traces) were fit to kinetic models (red traces) to obtain the apparent equilibrium dissociation constant for each interaction pair. Representative sensorgrams from 3 independent determinations are shown for **(A, B)** Spike-S1 protein over gC1qR-WT (K_D=91 nM) or gC1qR-ΔΔ (K_D=370 nM), **(C, D)** Nucleocapsid protein over gC1qR-WT (K_D=6 μM) or gC1qR-ΔΔ (K_D=12 μM), **(E, F)**, Membrane-Envelope Fusion protein over gC1qR-WT (K_D=410 nM) or gC1qR-ΔΔ (K_D=360 nM), and **(G, H)** a synthetic peptide corresponding to the gC1qR-binding region from HK as a positive control (K_D=2 mM and 10 mM, respectively).

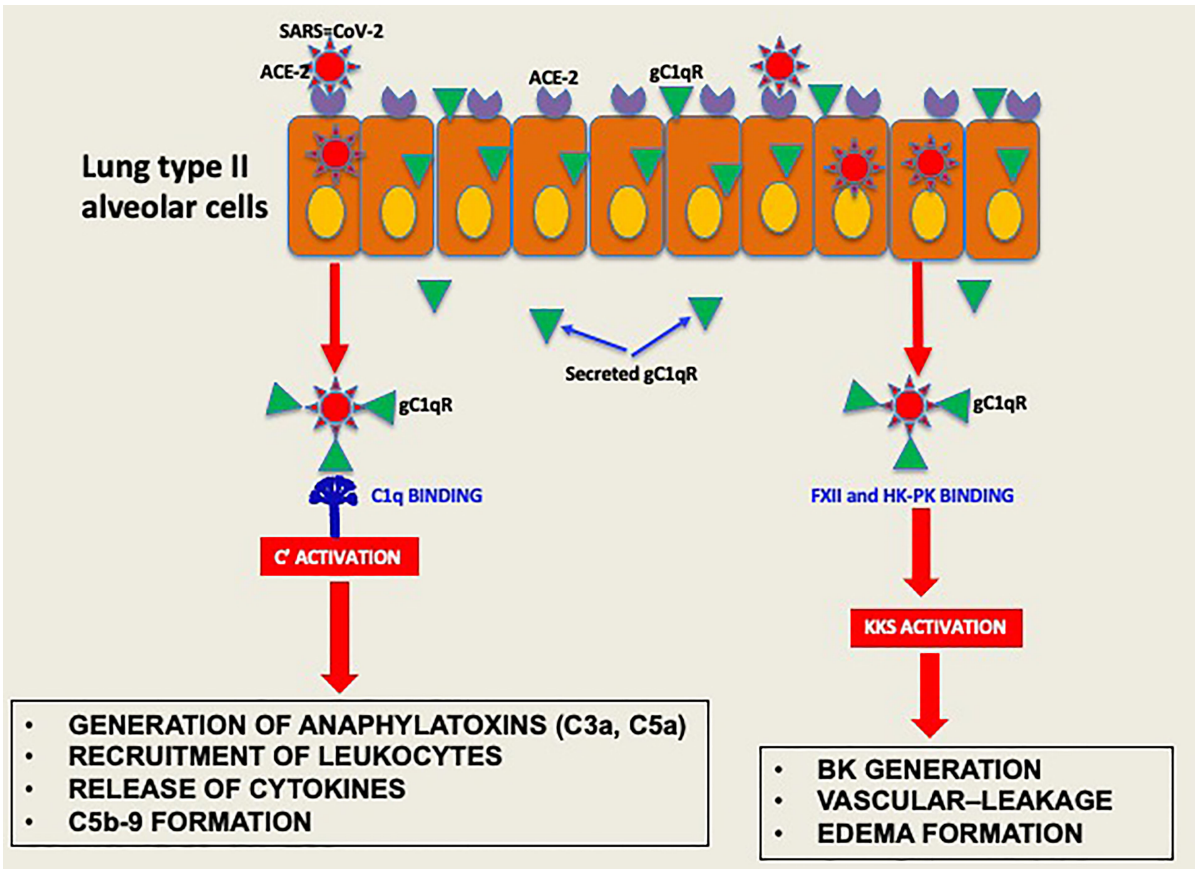


FIGURE 7 | Hypothetical role of gC1qR as an activator of complement and KKS. After the initial infection, multiple copies of the virus leave the primary cell and upon release bind to secreted gC1qR, a 'self' protein, to evade the immune system. The virus decorated with gC1qR in turn serves as an efficient platform for the activation of both the complement system and the kinin system to generate vasoactive peptides such as C3a, C5a and BK.

of the host cell, to enter the cell. However, after dividing inside the initial host cell, new viruses are released and ready to invade other cells. But first, they must escape recognition by the immune system using self-molecules as a camouflage. By virtue of its abundance and affinity for pathogenic microorganisms, intracellular or secreted gC1qR may bind SARS-CoV-2 and thus prevent it from recognition by the immune system. In addition, the gC1qR-coated virus could serve as a platform for the assembly and activation of both the complement and the KKS pathway (39). Activation of the two systems (**Figure 8**) would then result not only in the generation of bradykinin, but also generation of activation fragments from the complement system such as C3a and C5a. In addition, the secreted gC1qR itself can induce the expression of B1R, the high affinity receptor for BK, thus providing the requisite receptor for vascular leakage, recruitment of leukocytes and secretion of cytokines. In addition to C1q, the plasma proteins that bind gC1qR are mostly blood coagulation proteins and include high molecular weight kininogen [HK], Factor XII [Hageman factor], fibrinogen, thrombin [FII], and multimeric vitronectin (38, 43). Although HK and factor XII compete for binding to gC1qR (51), recent crystallization of the complexes (52) show that the simultaneous binding of both to gC1qR, which is zinc dependent (18, 52) is possible. The involvement of the bradykinin forming cascade is shown by the findings that bronchoalveolar lavage studies of COVID-19 patients reveal marked upregulation of kallikreins, HK, and bradykinin receptors with downregulation of C1 inhibitor, ACE, ACE-2 (53) and improvement in oxygenation with a B-2 receptor antagonist (18, 53). Therefore, by binding to these proteins, gC1qR can play an additional role in COVID-19 pathology by modulating the crosstalk between the complement and coagulation pathways through fibrin formation, immune injury and/or inflammation The fact that all of the major structural proteins bind gC1qR efficiently therefore suggests that SARS-CoV-2 could potentially use gC1qR as an alternate receptor for cellular entry and/or intercellular communication, thus making gC1qR a pluripotent target exploited by SARS-CoV-2. More importantly, since gC1qR is also localized intracellularly, there is a potential for intracellular interaction between gC1qR and SARS-CoV-2 proteins (54, 55).

By virtue of its significance in cellular entry, the S protein has been the major focus of anti-SARS-CoV-2 vaccine production.

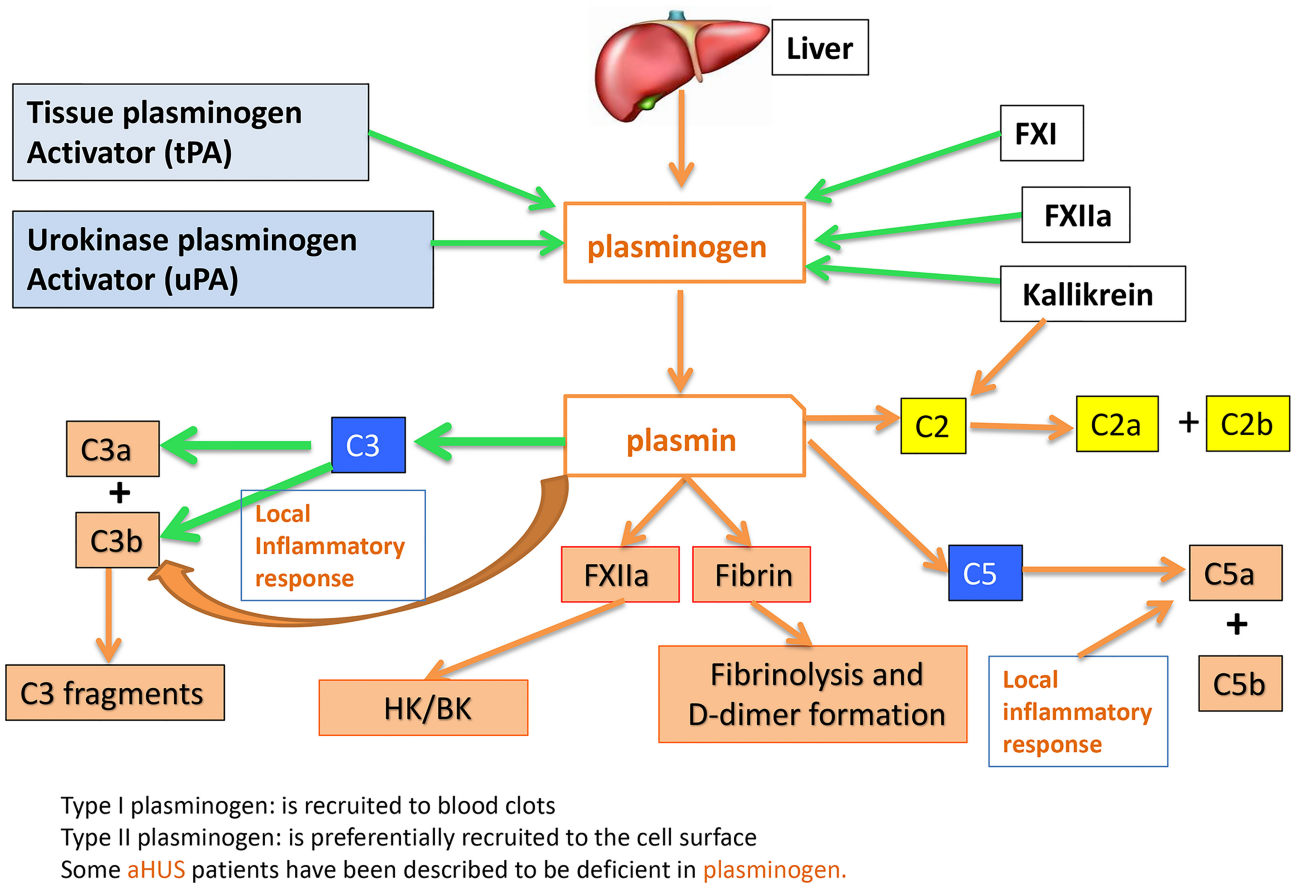


FIGURE 8 | Crosstalk between complement and coagulation enzymes: The role of plasmin.

However, the finding that all known viral structural proteins bind and activate innate pathways suggests that SARS-CoV-2 is probably one of the most efficient viruses capable of using its structural proteins for maximal damage. This in turn suggests that SARS-CoV-2 may hijack gC1qR as an alternate receptor for cellular entry, activation of innate pathways or intercellular communication and energy metabolism (54). More importantly, if the viral proteins are released and could survive in plasma or other tissues such as on platelet microparticles (56, 57), complement activation and BK generation could continue to occur even long after the virus has been cleared and severe symptoms associated with the initial infection have disappeared as is the case in the so called "long haulers". Although they test negative for the SARS-CoV-2, approximately 10% of these patients develop a myriad of post-COVID-19 lingering symptoms that include incessant coughing, shortness of breath, body aches, brain fog, headaches, and joint pain. We speculate that fragments of the virus or the viral proteins may still linger in the blood possibly bound to self-molecules such as gC1qR, which would then continuously activate the complement system, and the KKS, thus releasing vasoactive and inflammatory molecules that contribute to the multi-system inflammation of "long-haulers".

Our results not only reveal novel molecular correlates involved in the induction and/or enhancement of the "cytokine storm", vascular permeability and edema that are the hallmarks of COVID-19 pathology, but also show for the first time that *not one but all* the major structural proteins, S, M, N and E, are able to activate the complement and kinin systems. Although therapeutics that target the complement (11, 12) and the kinin systems (9) have been promising, thorough understanding of the interplay between complement and coagulation systems in COVID-19 pathophysiology will still be requisite if we are to design therapeutic interventions to treat not only active patients but also the "long-haulers". More importantly however, since SARS-CoV-2 undergoes frequent mutations to generate a new strain or a new variant, computational models and mathematical algorithms, that identify mutation-prone sites in the sequence of each of the

structural proteins, may also help in the design of vaccines ahead of an impending SARS-CoV-2-variant pandemic.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

AS, SM, and TW performed the complement experiments. WY, DR, and MF did the work on FXII and HK binding as well as the BK assay. BVG designed and HD and XX performed the SPR studies and BG designed and planned the experiments and helped with interpretation of the data. AK consulted on the kinin studies, and EP contributed with her expertise in blood coagulation and helped with the discussion and interpretation of data as well as writing the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in part by grants from the National Institutes of Allergy and Infectious Diseases R01 AI 060866, R01 AI-084178, R56-AI 1223476 (to BG), from the National Institute of General Medical Sciences R01 GM121511 and Terry C. Johnson Cancer Center (to BVG), and the NIH/NCI cancer support grant P30 CA008748 (to MSKCC).

REFERENCES

1. Fehr AR, Perlman S. Coronaviruses: An Overview of Their Replication and Pathogenesis. *Methods Mol Biol* (2015) 1282:1–23. doi: 10.1007/978-1-4939-2438-7_1
2. Masters PS. The Molecular Biology of Coronaviruses. *Adv Virus Res* (2006) 66:193–292. doi: 10.1016/S0065-3527(06)66005-3
3. Franks TJ, Chong PY, Chui P, Galvin JR, Lourens RM, Reid AH, et al. Lung Pathology of Severe Acute Respiratory Syndrome (SARS): A Study of 8 Autopsy Cases From Singapore. *Hum Pathol* (2003) 34:743–8. doi: 10.1016/s0046-8177(03)00367-8
4. Hwang DM, Chamberlain DW, Poutanen SM, Low DE, Asa SL, Butany J. Pulmonary Pathology of Severe Acute Respiratory Syndrome in Toronto. *Mod Pathol* (2005) 18:1–10. doi: 10.1038/modpathol.3800247
5. Nicholls JM, Poon LL, Lee KC, Ng WF, Lai ST, Leung CY, et al. Lung Pathology of Fatal Severe Acute Respiratory Syndrome. *Lancet* (2003) 361:1773–8. doi: 10.1016/s0140-6736(03)13413-7
6. Cui J, Li F, Shi ZL. Origin and Evolution of Pathogenic Coronaviruses. *Nat Rev Microbiol* (2019) 17:181–92. doi: 10.1038/s41579-018-0118-9
7. Cruz JL, Becares M, Sola I, Oliveros JC, Enjuanes L, Zuniga S. Alphacoronavirus Protein 7 Modulates Host Innate Immune Response. *J Virol* (2013) 87:9754–67. doi: 10.1128/JVI.01032-13
8. Ghebrehiwet B, Peerschke EI. Complement and Coagulation: Key Triggers of COVID-19-Induced Multiorgan Pathology. *J Clin Invest* (2020) 130:5674–6. doi: 10.1172/JCI142780
9. Kaplan AP, Ghebrehiwet B. Pathways for Bradykinin Formation and Interrelationship With Complement as a Cause of Edematous Lung in COVID-19 Patients. *J Allergy Clin Immunol* (2021) 147:507–9. doi: 10.1016/j.jaci.2020.10.025
10. Li F, Li W, Farzan M, Harrison SC. Structure of SARS Coronavirus Spike Receptor-Binding Domain Complexed With Receptor. *Science* (2005) 309:1864–8. doi: 10.1126/science.1116480
11. Mastaglio S, Ruggeri A, Risitano AM, Angelillo P, Yancopoulou D, Mastellos DC, et al. The First Case of COVID-19 Treated With the Complement C3 Inhibitor AMY-101. *Clin Immunol* (2020) 215:108450. doi: 10.1016/j.clim.2020.108450
12. Skendros P, Mitsios A, Chrysanthopoulou A, Mastellos DC, Metallidis S, Rafailidis P, et al. Complement and Tissue Factor-Enriched Neutrophil Extracellular Traps Are Key Drivers in COVID-19 Immunothrombosis. *J Clin Invest* (2020) 130:6151–7. doi: 10.1172/JCI141374
13. Gralinski LE, Bankhead A 3rd, Jeng S, Menachery VD, Proll S, Belisle SE, et al. Mechanisms of Severe Acute Respiratory Syndrome Coronavirus-Induced Acute Lung Injury. *mBio* (2013) 4:e00271–13. doi: 10.1128/mBio.00271-13
14. Ghebrehiwet B, Lim BL, Peerschke EI, Willis AC, Reid KB. Isolation, cDNA Cloning, and Overexpression of a 33-kD Cell Surface Glycoprotein That Binds to the Globular "Heads" of C1q. *J Exp Med* (1994) 179:1809–21. doi: 10.1084/jem.179.6.1809
15. Ghebrehiwet B, CebadaMora C, Tantral L, Jesty J, Peerschke EI. Gc1qr/P33 Serves as a Molecular Bridge Between the Complement and Contact Activation Systems and Is an Important Catalyst in Inflammation. *Adv Exp Med Biol* (2006) 586:95–105. doi: 10.1007/0-387-34134-X_7
16. Kumar R, Peerschke EI, Ghebrehiwet B. Zinc Induces Exposure of Hydrophobic Sites in the C-Terminal Domain of Gc1q-R/P33. *Mol Immunol* (2002) 39:69–75. doi: 10.1016/s0161-5890(02)00046-9
17. Pednekar L, Valentino A, Ji Y, Tumma N, Valentino C, Kadoor A, et al. Identification of the Gc1qr Sites for the HIV-1 Viral Envelope Protein Gp41 and the HCV Core Protein: Implications in Viral-Specific Pathogenesis and Therapy. *Mol Immunol* (2016) 74:18–26. doi: 10.1016/j.molimm.2016.03.016
18. Ghebrehiwet B, Jesty J, Xu S, Vinayagasundaram R, Vinayagasundaram U, Valentino A, et al. Structure-Function Studies Using Deletion Mutants Identify Domains of Gc1qr/P33 as Potential Therapeutic Targets for Vascular Permeability and Inflammation. *Front Immunol* (2011) 2:58. doi: 10.3389/fimmu.2011.00058
19. Ghebrehiwet B, Lu PD, Zhang W, Lim BL, Eggleton P, Leigh LE, et al. Identification of Functional Domains on Gc1q-R, a Cell Surface Protein That Binds to the Globular "Heads" of C1Q, Using Monoclonal Antibodies and Synthetic Peptides. *Hybridoma* (1996) 15:333–42. doi: 10.1089/hyb.1996.15.333
20. Price LC, McCabe C, Garfield B, Wort SJ. Thrombosis and COVID-19 Pneumonia: The Clot Thickens! *Eur Respir J* (2020) 56:2001608. doi: 10.1183/13993003.01608-2020
21. Yao Y, Cao J, Wang Q, Shi Q, Liu K, Luo Z, et al. D-Dimer as a Biomarker for Disease Severity and Mortality in COVID-19 Patients: A Case Control Study. *J Intensive Care* (2020) 8:49. doi: 10.1186/s40560-020-00466-z
22. Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* (2020) 181:271–80.e278. doi: 10.1016/j.cell.2020.02.052
23. Schoeman D, Fielding BC. Coronavirus Envelope Protein: Current Knowledge. *Virol J* (2019) 16:69. doi: 10.1186/s12985-019-1182-0
24. Wang C, Zheng X, Gai W, Zhao Y, Wang H, Wang H, et al. MERS-CoV Virus-Like Particles Produced in Insect Cells Induce Specific Humoural and Cellular Imminity in Rhesus Macaques. *Oncotarget* (2017) 8:12686–94. doi: 10.18632/oncotarget.8475
25. de Haan CA, Rottier PJ. Molecular Interactions in the Assembly of Coronaviruses. *Adv Virus Res* (2005) 64:165–230. doi: 10.1016/S0065-3527(05)64006-7
26. McBride R, van Zyl M, Fielding BC. The Coronavirus Nucleocapsid Is a Multifunctional Protein. *Viruses* (2014) 6:2991–3018. doi: 10.3390/v6082991
27. Maas C. Plasminflammation-An Emerging Pathway to Bradykinin Production. *Front Immunol* (2019) 10:2046. doi: 10.3389/fimmu.2019.02046

28. Kaplan AP, Austen KF. A Prealbumin Activator of Prekallikrein. II. Derivation of Activators of Prekallikrein From Active Hageman Factor by Digestion With Plasmin. *J Exp Med* (1971) 133:696–712. doi: 10.1084/jem.133.4.696
29. Kaplan AP, Ghebrehiwet B. The Plasma Bradykinin-Forming Pathways and its Interrelationships With Complement. *Mol Immunol* (2010) 47:2161–9. doi: 10.1016/j.molimm.2010.05.010
30. Klumperman J, Locker JK, Meijer A, Horzinek MC, Geuze HJ, Rottier PJ. Coronavirus M Proteins Accumulate in the Golgi Complex Beyond the Site of Virion Budding. *J Virol* (1994) 68:6523–34. doi: 10.1128/JVI.68.10.6523-6534.1994
31. Stahel PF, Barnum SR. Complement Inhibition in Coronavirus Disease (COVID)-19: A Neglected Therapeutic Option. *Front Immunol* (2020) 11:1661. doi: 10.3389/fimmu.2020.01661
32. Noris M, Benigni A, Remuzzi G. The Case of Complement Activation in COVID-19 Multiorgan Impact. *Kidney Int* (2020) 98:314–22. doi: 10.1016/j.kint.2020.05.013
33. Risitano AM, Mastellos DC, Huber-Lang M, Yancopoulou D, Garlanda C, Ciceri F, et al. Complement as a Target in COVID-19? *Nat Rev Immunol* (2020) 20:343–4. doi: 10.1038/s41577-020-0320-7
34. Ramlall V, Thangaraj PM, Meydan C, Foox J, Butler D, Kim J, et al. Immune Complement and Coagulation Dysfunction in Adverse Outcomes of SARS-CoV-2 Infection. *Nat Med* (2020) 26:1609–15. doi: 10.1038/s41591-020-1021-2
35. Ip WK, Chan KH, Law HK, Tso GH, Kong EK, Wong WH, et al. Mannose-Binding Lectin in Severe Acute Respiratory Syndrome Coronavirus Infection. *J Infect Dis* (2005) 191:1697–704. doi: 10.1086/429631
36. Holter JC, Pischke SE, de Boer E, Lind A, Jenum S, Holten AR, et al. Systemic Complement Activation is Associated With Respiratory Failure in COVID-19 Hospitalized Patients. *Proc Natl Acad Sci USA* (2020) 117:25018–25. doi: 10.1073/pnas.2010540117
37. Yu J, Yuan X, Chen H, Chaturvedi S, Braunstein EM, Brodsky RA. Direct Activation of the Alternative Complement Pathway by SARS-CoV-2 Spike Proteins is Blocked by Factor D Inhibition. *Blood* (2020) 136:2080–9. doi: 10.1182/blood.2020008248
38. Ghebrehiwet B, Geisbrecht BV, Xu X, Savitt AG, Peerschke EIB. The C1q Receptors: Focus on Gc1qr/P33 (C1qBP, P32, HABP-1)(1). *Semin Immunol* (2019) 45:101338. doi: 10.1016/j.smim.2019.101338
39. Ghebrehiwet B, Ji Y, Valentino A, Pednekar L, Ramadass M, Habiel D, et al. Soluble Gc1qr is an Autocrine Signal That Induces B1R Expression on Endothelial Cells. *J Immunol* (2014) 192:377–84. doi: 10.4049/jimmunol.1302031
40. Peerschke EI, Ghebrehiwet B. Human Blood Platelet Gc1qr/P33. *Immunol Rev* (2001) 180:56–64. doi: 10.1034/j.1600-065x.2001.1800105.x
41. Peerschke EI, Ghebrehiwet B. Platelet Receptors for the Complement Component C1q: Implications for Hemostasis and Thrombosis. *Immunobiology* (1998) 199:239–49. doi: 10.1016/S0171-2985(98)80030-2
42. Joseph K, Ghebrehiwet B, Peerschke EI, Reid KB, Kaplan AP. Identification of the Zinc-Dependent Endothelial Cell Binding Protein for High Molecular Weight Kininogen and Factor XII: Identity With the Receptor That Binds to the Globular "Heads" of C1q (Gc1q-R). *Proc Natl Acad Sci USA* (1996) 93:8552–7. doi: 10.1073/pnas.93.16.8552
43. Peerschke EI, Yin W, Ghebrehiwet B. Platelet Mediated Complement Activation. *Adv Exp Med Biol* (2008) 632:81–91. doi: 10.1007/978-0-387-78952-1_7
44. Yao ZQ, Nguyen DT, Hiotellis AI, Hahn YS. Hepatitis C Virus Core Protein Inhibits Human T Lymphocyte Responses by a Complement-Dependent Regulatory Pathway. *J Immunol* (2001) 167:5264–72. doi: 10.4049/jimmunol.167.9.5264
45. Kouokam Fotso GB CB, Bigault L, de Boisseson C, Mankertz A, Jestin A, Grasland B. The Expression Level of Gc1qr is Down Regulated at the Early Time of Infection With Porcine Circovirus of Type 2 (PCV-2) and Gc1qr Interacts Differently With the Cap Proteins of Porcine Circoviruses. *Virus Res* (2016) 220:21–32. doi: 10.1016/j.virusres.2016.04.006
46. Choi Y, Kwon YC, Kim SI, Park JM, Lee KH, Ahn BY. A Hantavirus Causing Hemorrhagic Fever With Renal Syndrome Requires Gc1qr/P32 for Efficient Cell Binding and Infection. *Virology* (2008) 381:178–83. doi: 10.1016/j.virol.2008.08.035
47. Wang Y, Finan JE, Middeldorp JM, Hayward SD. P32/TAP, a Cellular Protein That Interacts With EBNA-1 of Epstein-Barr Virus. *Virology* (1997) 236:18–29. doi: 10.1006/viro.1997.8739
48. Mohan KV, Ghebrehiwet B, Atreya CD. The N-Terminal Conserved Domain of Rubella Virus Capsid Interacts With the C-Terminal Region of Cellular P32 and Overexpression of P32 Enhances the Viral Infectivity. *Virus Res* (2002) 85:151–61. doi: 10.1016/s0168-1702(02)00030-8
49. Chen Z-l, Su Y-j, Zhang H-l, Gu P-q, Gao L-j. The Role of the Globular Heads of the C1q Receptor in HPV-16 E2-Induced Human Cervical Squamous Carcinoma Cell Apoptosis *via* a Mitochondria-Dependent Pathway. *J Translat Med* (2014) 12:286. doi: 10.1186/s12967-014-0286-y
50. Xu L, Xiao N, Liu F, Ren H, Gu J. Inhibition of RIG-I and MDA5-Dependent Antiviral Response by Gc1qr at Mitochondria. *Proc Natl Acad Sci USA* (2009) 106:1530–5. doi: 10.1073/pnas.0811029106
51. Joseph K, Tholanikunnel BG, Ghebrehiwet B, Kaplan AP. Interaction of High Molecular Weight Kininogen Binding Proteins on Endothelial Cells. *Thromb Haemost* (2004) 91:61–70. doi: 10.1267/THRO04010061[pii
52. Kaira BG, Slater A, McCrae KR, Dreveny I, Sumya U, Mutch NJ, et al. Factor XII and Kininogen Asymmetric Assembly With Gc1qr/C1QBP/P32 Is Governed by Allostery. *Blood* (2020) 136:1685–97. doi: 10.1182/blood.2020004818
53. Garvin MR, Alvarez C, Miller JI, Prates ET, Walker AM, Amos BK, et al. A Mechanistic Model and Therapeutic Interventions for COVID-19 Involving a RAS-Mediated Bradykinin Storm. *Elife* (2020) 9. doi: 10.7554/eLife.59177
54. Sunderhauf A, Raschdorf A, Hicken M, Schlichting H, Fetzer F, Brethack AK, et al. GC1qR Cleavage by Caspase-1 Drives Aerobic Glycolysis in Tumor Cells. *Front Oncol* (2020) 10:575854. doi: 10.3389/fonc.2020.575854
55. Sunderhauf A, Hicken M, Schlichting H, Skibbe K, Ragab M, Raschdorf A, et al. Loss of Mucosal P32/Gc1qr/HABP1 Triggers Energy Deficiency and Impairs Goblet Cell Differentiation in Ulcerative Colitis. *Cell Mol Gastroenterol Hepatol* (2021) 12:229–50. doi: 10.1016/j.jcmgh.2021.01.017
56. Peerschke EI, Yin W, Ghebrehiwet B. Complement Activation on Platelets: Implications for Vascular Inflammation and Thrombosis. *Mol Immunol* (2010) 47:2170–5. doi: 10.1016/j.molimm.2010.05.009
57. Peerschke EI, Petrovan RJ, Ghebrehiwet B, Ruf W. Tissue Factor Pathway Inhibitor-2 (TFPI-2) Recognizes the Complement and Kininogen Binding Protein Gc1qr/P33 (Gc1qr): Implications for Vascular Inflammation. *Thromb Haemost* (2004) 92:811–9. doi: 10.1267/THRO04040811

Conflict of Interest: BG and EP receive royalties from the sale of anti-gC1qR mAbs and gC1qR detection assay kit.

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Immunology

ORIGINAL RESEARCH
published: 18 November 2021
doi: 10.3389/fimmu.2021.767376

Persistence of High Levels of Serum Complement C5a in Severe COVID-19 Cases After Hospital Discharge

Yaiza Senent[1,2,3], Susana Inogés[3,4,5], Ascensión López-Díaz de Cerio[3,4,5], Andres Blanco[6], Arantxa Campo[7], Francisco Carmona-Torre[3,6,8], Patricia Sunsundegui[6], Antonio González-Martín[1,9], Daniel Ajona[1,2,3,10], Marcin Okrój[11], Felipe Prósper[3,4,10,12], Ruben Pio[1,2,3,10], José Ramón Yuste[3,6,8]† and Beatriz Tavira[1,3,13]†*

[1] Program in Solid Tumors, Translational Oncology Group, Cima-University of Navarra, Pamplona, Spain, [2] Department of Biochemistry and Genetics, School of Sciences, University of Navarra, Pamplona, Spain, [3] Respiratory Tract Cancer Group, Navarra Institute for Health Research (IdISNA), Pamplona, Spain, [4] Department of Immunology and Immunotherapy, Clinica Universidad de Navarra, Pamplona, Spain, [5] Area of Cell Therapy and Department of Hematology, Clinica Universidad de Navarra, Pamplona, Spain, [6] Department of Internal Medicine, Clinica Universidad de Navarra, Pamplona, Spain, [7] Pulmonary Department, Clinica Universidad de Navarra, Pamplona, Spain, [8] Division of Infectious Diseases, Clinica Universidad de Navarra, Pamplona, Spain, [9] Department of Oncology, Clinica Universidad de Navarra, Madrid, Spain, [10] Program in Respiratory Tract Tumors, Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain, [11] Department of Cell Biology and Immunology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Gdańsk, Poland, [12] Program of Regenerative Medicine, Cima-University of Navarra, Pamplona, Spain, [13] Department of Pathology, Anatomy and Physiology, School of Medicine, University of Navarra, Pamplona, Spain

Evidence supports a role of complement anaphylatoxin C5a in the pathophysiology of COVID-19. However, information about the evolution and impact of C5a levels after hospital discharge is lacking. We analyzed the association between circulating C5a levels and the clinical evolution of hospitalized patients infected with SARS-CoV-2. Serum C5a levels were determined in 32 hospitalized and 17 non-hospitalized patients from Clinica Universidad de Navarra. One hundred and eighty eight serial samples were collected during the hospitalization stay and up to three months during the follow-up. Median C5a levels were 27.71 ng/ml (25th to 75th percentile: 19.35-34.96) for samples collected during hospitalization, *versus* 16.76 ng/ml (12.90-25.08) for samples collected during the follow-up ($p<0.001$). There was a negative correlation between serum C5a levels and the number of days from symptom onset ($p<0.001$). C5a levels also correlated with a previously validated clinical risk score ($p<0.001$), and was associated with the severity of the disease ($p<0.001$). An overall reduction of C5a levels was observed after hospital discharge. However, elevated C5a levels persisted in those patients with high COVID-19 severity (i.e. those with a longest stay in the hospital), even after months from hospital discharge ($p=0.020$). Moreover, high C5a levels appeared to be associated with the presence of long-term respiratory symptoms ($p=0.004$). In conclusion, serum C5a levels remain high in severe cases of COVID-19, and are associated with the presence of respiratory symptoms after hospital discharge. These results may suggest a role for C5a in the long-term effects of COVID-19 infection.

Keywords: innate immunity, complement system, C5a, COVID-19, SARS-CoV-2, respiratory symptoms

OPEN ACCESS

Edited by:
Nicolas Stephane Merle, National Heart, Lung and Blood Institute (NHLBI), United States

Reviewed by:
Eleni Gavriilaki, G. Papanikolaou General Hospital, Greece
Dipyaman Ganguly, Indian Institute of Chemical Biology (CSIR), India
Eric Vivier, INSERM U1104 Centre d'immunologie de Marseille-Luminy (CIML), France

****Correspondence:***
Ruben Pio
rpio@unav.es

†These authors share senior authorship

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *30 August 2021*
Accepted: *29 October 2021*
Published: *18 November 2021*

Citation:
Senent Y, Inogés S, López-Díaz de Cerio A, Blanco A, Campo A, Carmona-Torre F, Sunsundegui P, González-Martín A, Ajona D, Okrój M, Prósper F, Pio R, Yuste JR and Tavira B (2021) Persistence of High Levels of Serum Complement C5a in Severe COVID-19 Cases After Hospital Discharge. Front. Immunol. 12:767376. doi: 10.3389/fimmu.2021.767376

INTRODUCTION

The current outbreak of COVID-19 constitutes a major health challenge worldwide. Most patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection develop none or mild symptoms, but it is estimated that up to 15% of patients progress to severe pneumonia and acute respiratory distress syndrome (ARDS) (1). Severe disease is characterized by an inflammatory response in the viral infected tissue associated with the release of inflammatory cytokines, the recruitment of immune cells, and the activation of coagulation and thrombosis (2, 3). These events originate in the lungs but may extend to other organs, causing a multiorgan damage (4, 5). A better understanding of the immune response that govern the severity of the disease is necessary to understand and clinically manage long-term health consequences of COVID-19.

The complement system represents a major effector of innate immunity against viruses, which mediates potent inflammatory responses (6). A number of studies have shown that deregulated complement activation contributes to the pathogenesis of inflammatory lung diseases (6). Results obtained in preclinical models support a prominent role of the complement system in the pathophysiology of SARS-CoV and other viral infections. In a mouse model of SARS-CoV-1 infection, complement C3 deficiency hampered severe lung pathology and reduced the levels of pro-inflammatory cytokines in association with decrease lung infiltration of neutrophils, monocytes, cytokines and chemokines (7). The anaphylatoxin C5a, a potent immune modulator released from C5 cleavage during complement activation, promotes the recruitment and activation of neutrophils during lung inflammation, which results in acute lung injury and ARDS (8–10). High C5a levels have been described in a range of preclinical models of acute lung injury induced by highly pathogenic viruses, such as SARS-CoV-1, H1N1, H5N1 or H7N9 (8). Moreover, blockade of the C5a/C5a receptor-1 (C5aR1) axis alleviated lung damage in hDPP4-transgenic mice infected with MERS-CoV (11).

Complement over-activation contributes to lung disease in COVID-19 (12). C4d and C5b-9 deposition has been found along the vasculature of the lungs and the skin in hospitalized patients with COVID-19 (13). Expression changes in complement genes were identified in SARS-CoV-2 infected human lung epithelial cells (14). In this study, the interferon-activated JAK1/2-STAT1 signaling pathway and NF-κB were proposed as mediators of intracellular C3 activation in infected cells (14). Another transcriptomic analysis on the peripheral blood of COVID-19 patients revealed an upregulation of C1q and C2 expression in COVID-19 patients, high levels of C5aR1 expression were found in blood and pulmonary myeloid cells, and circulating levels of C5a were associated with the severity of COVID-19 (15). In *in vitro* studies and preclinical models, knock-down or pharmacological inhibition of C5aR1 prevented epithelial destruction (16), and the activation and recruitment of myeloid cells to the lungs (15). C5aR1 blockade also attenuated platelet-mediated COVID-19-associated thrombogenicity in a process dependent on the formation of neutrophil extracellular traps (NETs) (17).

All these data suggest that C5a may be involved in the pathophysiology of COVID-19. Circulating levels of C5a are increased in hospitalized COVID-19 patients as compared with healthy donors (18–21). However, information is lacking on the evolution of C5a levels beyond the hospitalization stay. In the present study, we performed serial measurements of serum C5a levels in COVID-19 patients during the hospitalization stay and after discharge. C5a levels correlated with disease severity, as determined by a previously validated clinical risk score (22). Moreover, C5a levels were associated with the duration and outcome of the hospitalization stay. Interestingly, in those patients with a more severe disease, C5a levels remained elevated weeks after hospital discharge, and were associated with the persistence of respiratory symptoms, suggesting that modulation of complement activation may be an effective therapeutic strategy for the treatment of COVID-19 patients with long-term respiratory problems.

MATERIALS AND METHODS

Patients

Consecutive COVID-19 patients treated at Clinica Universidad de Navarra between April and July 2020 were included in this study. Thirty-two patients were admitted to hospital due to the severity of their symptoms, while 17 individuals, who went to the emergency room due to their symptoms, did not require hospitalization. COVID-19 was diagnosed by SARS-CoV-2 real-time PCR in nasopharyngeal samples. The characteristics of the patients are described in **Table 1**, **Supplementary Table S1**. The study was approved by the Ethics Committee of Clinica Universidad de Navarra (ref. 2020.090), and all patients signed an informed consent.

Serum Collection

Venous blood samples were collected in BD Vacutainer CAT tubes (ref. 367896) periodically during the hospital stay, and up to three months after being discharged from the hospital. Blood samples were immediately processed after collection. Serum was obtained by centrifugation at 3,500 rpm for 8 minutes and stored at -80°C. Those samples collected during hospitalization were classified as H1 (for samples collected between 1 and 3 days after admission; n = 24), H5 (4 to 5 days; n = 25), H8 (7 to 9 days, n = 22), and H14 (11 to 15 days; n = 9). Follow-up samples collected after hospital discharge, or from non-hospitalized patients, were categorized as F7 (ranging from 5 to 10 days after discharge; n = 29), F14 (11 to 23 days; n = 39) and F90 (69 to 123 days; n = 40).

Marker Quantification

C5a was quantified in serum samples using Luminex technology and the anti-C5a capture and detection antibodies from the Human Complement Component C5a DuoSet ELISA kit (DY2037, R&D Systems). C5b-9 levels, routine laboratory markers and pro-inflammatory cytokines IFNγ and IL1α were also determined. Details of the procedures are provided in **Supplementary Methods**. Analyses were performed blindly.

TABLE 1 | Demographic and clinical characteristics of the COVID-19 patients included in the study.

Patient characteristics	n (%)
Sex	
Female	28 (57)
Male	21 (43)
Hospitalization	
No	17 (35)
Yes	32 (65)
Comorbidities	
No	28 (57)
Yes	21 (43)
Symptoms	
No	9 (18)
Yes	40 (82)
	Median (range)
Age (years)	54.5 (45–76)
COVID risk score	102 (81–126)
Laboratory parameters	
LDH (U/l)	232 (212–312)
D-dimer (ng/ml)	530 (380–1110)
Ferritin (ng/ml)	345 (162–520)
Troponin T (ng/ml)*	6.5 (6.5-14.6)
C-reactive protein (mg/l)	2.04 (0.66-6.65)

Values below the limit of detection (LOD) were substituted by LOD/2 (6.5 ng/ml).

Statistical Analyses

Normal distribution of data was assessed by the Shapiro-Wilk test, and the density and q-q plots. Non-parametric Mann-Whitney *U* and Kruskal-Wallis tests were applied to compare two or more groups, respectively. Spearman's rank coefficient was used to measure the correlation between continuous variables. Pearson Chi-square was used to evaluate differences between categorical variables. Continuous variables were described as median (lower to upper quartile). All tests were two-sided, and a p value less than 0.05 was considered statistically significant, with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. R v4.0.2 (R Core Team, Vienna, Austria, 2020) was used for statistical analysis. Figures were produced with the R packages ggplot2 (23) and ggpubr (24). The R package tidyverse was used for data manipulation (25).

RESULTS

Characteristics of COVID-19 Patients

This study included patients who came to Clinica Universidad de Navarra presenting symptoms compatible with COVID-19 and were hospitalized or not due to the severity of these symptoms (n = 32 and n = 17, respectively). SARS-CoV-2 infection was diagnosed by real-time PCR. Demographic characteristics, co-morbidities symptoms and routine are shown in **Table 1**, **Supplementary Table S1**. Patients had a median age of 54 (25^{th} to 75^{th} percentile: 45-76) years. Gender distribution was 57% females and 43% males. Several comorbidities were diagnosed, such as diabetes (8%), hypertension (31%), cardiopathy (24%), renal insufficiency (8%) or obesity (37%). Men showed higher frequencies of hypertension (p = 0.011) and cardiopathy (p = 0.003). More than half of the patients showed fever (55%) and/or coughing (63%). Regarding SARS-CoV-2 symptoms, no gender differences were found.

Patients were stratified into five COVID severity groups according to the number of hospitalization days (low, medium or high severity), or whether they were not hospitalized (very low severity) or died during hospitalization (very high severity). No patient in our cohort required UCI admission. Low severity was considered when the hospitalization period was equal or lower than 7 days, medium when it was between 8 and 13 days, and high if it was equal or higher than 14 days. A more detailed description of the distribution of hospitalization days per group is shown in **Supplementary Table S2**. Age was significantly associated with severity: 42 (35–47), 56 (48–58), 54 (44–76), 84 (75–87) and 88 (83–92) years for very low, low, medium, high or very high severity groups, respectively ($p < 0.001$). The presence of comorbidities (p = 0.022), higher frequencies of some them, such as diabetes (p = 0.014) or cardiopathies (p = 0.024), or the presence of respiratory problems (p = 0.037) were also associated with the severity of the disease. Disease severity was also associated with the basal levels of laboratory markers known to be associated with COVID-19-related complications, such as lactate dehydrogenase (LDH; p = 0.017), D-dimer (p = 0.004), troponin T ($p < 0.001$) or C-reactive protein (CRP; p = 0.039). Viral load at admission was not associated with disease severity (p = 0.860 for E gene expression, and p = 0.740 for N gene expression).

A clinical risk score, which estimates the risk of developing critical illness among hospitalized COVID-19 patients, was calculated for hospitalized patients in our cohort. The score is based on ten variables commonly measured on admission to the hospital: abnormal chest radiography findings, age, dyspnea, hemoptysis, unconsciousness, number of comorbidities, cancer history, neutrophil-to-lymphocyte ratio, lactate dehydrogenase, and direct bilirubin (22). The median risk score was 119, with an interquartile range of 88 to 142, and minimum and maximum values of 54 and 209, respectively. The risk score was associated with the severity of the disease: 82 (80–100), 116 (90–121), 138 (134–147) and 191 (191–205) for low, medium, high and very high severity groups, respectively ($p < 0.001$).

Serum C5a Levels in COVID-19 Patients

Serum samples were collected periodically from hospitalized patients during their hospital stay and at different time points after hospital discharge. Longitudinal samples from non-hospitalized patients were also collected during the follow-up of the patients. The follow-up days for hospitalized patients ranged from 14 to 106 days, with a median of 92 (81–97) days. In the case of non-hospitalized patients, the follow-up days ranged from 14 to 123 days, with a median of 106 (96–109) days. One hundred and eighty eight serum samples were collected (80 at hospitalization and 108 at follow-up). The median of serum C5a levels was 20.45 ng/ml, with an interquartile range of 14.66 to 30.23 ng/ml and minimum and maximum values of 6.11 and 57.18 ng/ml, respectively. C5a levels along the different time points in each patient are shown in **Supplementary Figure S1**. A negative correlation ($\rho = -0.38$, $p < 0.001$) was found between C5a levels

and the number of days from symptom onset at sample collection (**Supplementary Figure S2A**). Besides, C5a levels in samples collected during hospitalization were significantly higher than in samples collected during follow-up (27.71 [19.35-34.96] *vs.* 16.76 [12.90-25.08]; $p < 0.001$; **Supplementary Figure S2B**). Viral load at admission was not associated with C5a levels ($\rho = -0.08$, $p = 0.616$, for E gene expression; and $\rho = -0.17$, $p = 0.302$, for N gene expression). As expected, C5a levels correlated with C5b-9 levels ($\rho = 0.54$, $p < 0.001$). C5a levels also correlated with basal levels of LDH ($\rho = 0.38$, $p = 0.008$), D-dimer ($\rho = 0.39$, $p = 0.006$), ferritin ($\rho = 0.54$, $p < 0.001$), troponin T ($\rho = 0.37$, $p = 0.008$) and CRP ($\rho = 0.43$, $p = 0.002$). Finally, basal levels of pro-inflammatory makers IFNγ and IL1α could be assessed in 25 hospitalized patients. C5a serum levels significantly correlated with IL1α levels, but not with IFNγ levels ($\rho = 0.75$, $p < 0.001$ and $\rho = -0.19$, $p = 0.373$, respectively). All these correlations are shown in **Supplementary Figure S3**.

Serum C5a and COVID-19 Severity During Hospitalization and Follow-up

C5a levels in the first sample collected at hospitalization correlated with the clinical risk score assigned to that patient: $\rho = 0.49$, $p = 0.004$ (**Figure 1A**). Moreover, those hospitalized patients with a more severe disease outcome showed higher serum C5a levels ($p < 0.001$; **Figure 1B**). Interestingly, this correlation was not observed for the other complement-related marker. Thus, C5b-9 levels did not correlate with the risk score ($\rho = 0.10$, $p = 0.590$), and were not associated with clinical outcome ($p = 0.850$). No other remarkable association was found between C5a levels and the clinicopathological characteristics of the patients (**Supplementary Table S3**).

To monitor the evolution of C5a levels, as detailed in Material and Methods, samples were categorized as H1, H5, H8 and H14 for those samples collected during hospital stay, and as F7, F14 and F90 for those samples collected after hospital discharge or from non-hospitalized patients. Patients who died during their hospital stay were excluded from this part of the study since they did not have follow-up samples. Persistently elevated C5a levels were observed in those patients with a longest stay in the hospital (**Figure 2**). C5a levels in patients with low or medium COVID-19 severity were similar to those in non-hospitalized patients as soon as one week after hospital discharge. The last time point of follow-up (F90) revealed a trend to decreasing C5a levels, suggesting that marker levels begin to normalize at this time

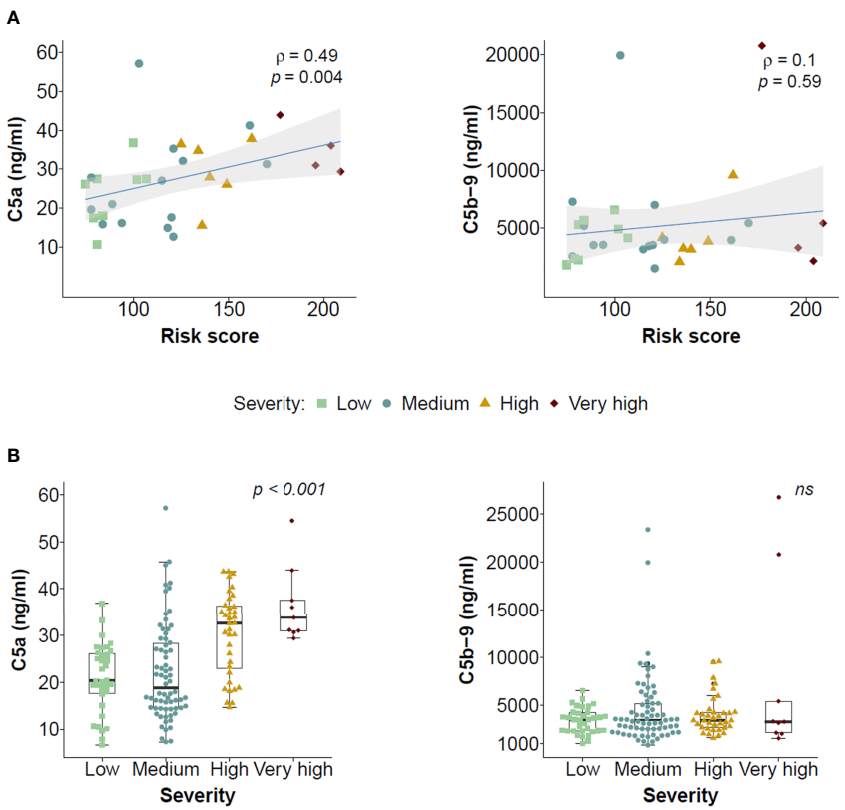


FIGURE 1 | Association of serum levels of C5a and C5b-9 with disease severity outcome in hospitalized COVID-19 patients. **(A)** Correlation between the levels of C5a or C5b-9 in the first serum sample collected after hospitalization and a clinical risk score calculated for each patient. The correlation coefficients, the statistical significances of the Spearman's test and the 95% confidence intervals (shadowed areas) are shown. The severity group at which each patient was assigned is also shown. **(B)** Association between serum C5a and C5b-9 levels in the first serum sample collected after hospitalization and severity of the disease. Statistical differences were analyzed using the Kruskal-Wallis test.

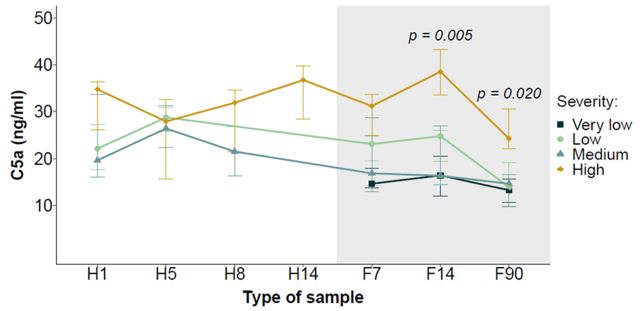


FIGURE 2 | Evolution of serum C5a levels during hospitalization and follow-up in COVID-19 patients stratified by the severity of the disease. Serial samples were collected at different time points during the hospitalization period (H1, H5, H8 and H14) and the follow-up (F7, F14 and F90). Data are presented as median ± interquartile range. The shadowed area corresponds to the follow-up period. Differences between groups at each time point were analyzed using the Kruskal-Wallis test.

point. The same trend was observed when hospitalized patients were stratified as high or low risk according to the clinical risk score (**Supplementary Figure S4**). These results suggest that complement C5a levels in serum samples collected from COVID-19 patients remain high and are associated with the severity of the disease weeks after hospital discharge.

Serum C5a and Respiratory Problems in COVID-19 Patients After Discharge

We sought to assess whether there was an association between serum C5a levels and persistent health problems in those patients discharged from the hospital after recovery from COVID-19. Symptom data were recorded during the follow-up visits at F14 and F90 in 25 and 24 discharged patients, respectively (**Supplementary Table S4**). Musculoskeletal (arthralgia and fatigue) and respiratory symptoms (cough and dyspnea) were the most frequent health problems found in the patients. At F14, no association was found between C5a levels and musculoskeletal problems. However, significantly higher C5a levels were found in patients with respiratory symptoms as compared with those in patients without respiratory symptoms (p = 0.004; **Figure 3A**). Moreover, C5a levels during hospitalization may predict which patients will present persistent respiratory symptoms after hospital discharge, as shown by the receiver operating characteristic (ROC) curve generated using C5a levels at H8 and respiratory symptoms recorded at F14 (AUC: 0.846 [0.650-1.000]; **Supplementary Figure S5**). Nevertheless, this result should be considered preliminary because only four patients showed respiratory problems at this follow-up time point. No analysis could be performed at F90 due to the scarcity of discharged patients with respiratory symptoms (e.g. only one patient with mild dyspnea).

We next evaluated the changes in C5a levels between the samples collected before hospital discharge and the samples collected at F14 in those hospitalized patients who had respiratory problems. While not all patients followed the same evolution, an overall reduction in C5a levels was observed in those patients in whom the respiratory symptoms had disappeared at F14 (p = 0.078), but not in those patients who still were experiencing respiratory symptoms (**Figure 3B**). Finally, at F90, respiratory symptoms had disappeared in two patients with respiratory problems at F14. In both cases, C5a levels decreased between the two follow-up time points (from 29.25 to 16.91 ng/ml, and from 43.65 to 30.62 ng/ml). These results suggest that elevated C5a levels in circulation may be associated with the persistence of respiratory problems in patients discharged from the hospital after recovery from COVID-19.

DISCUSSION

In this study, we report the persistent presence of high levels of circulating complement C5a, a cleavage product of terminal complement C5, in COVID-19 patients with a long hospital

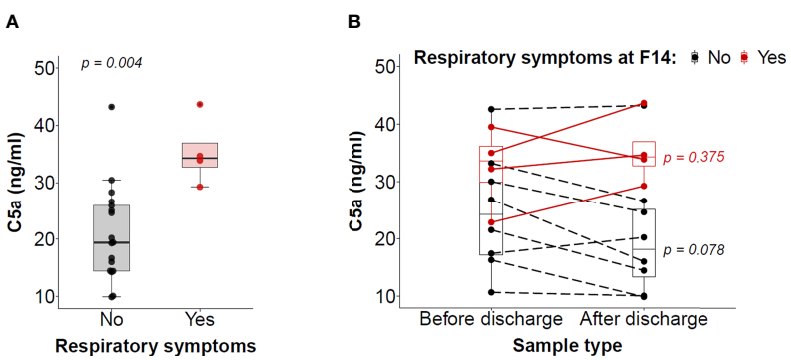


FIGURE 3 | Association between serum C5a levels and the presence of respiratory symptoms. **(A)** Association between serum C5a levels and the presence or not of respiratory symptoms two weeks after hospital discharge. **(B)** Evolution of serum C5a levels from the last sample at hospitalization and the follow-up sample at F14 in patients who suffered respiratory problems during their hospitalization. Patients are divided into patients with or without respiratory problems in the follow-up visit (red and black, respectively). Differences were analyzed with the Wilcoxon signed-rank test for paired samples.

stay, even weeks after hospital discharge. Our data also suggest that C5a is linked to the persistence of respiratory symptoms after hospital discharge. Long-term health consequences have been reported in 50 to 80% of patients discharged from the hospital after recovery from COVID-19 (26–28). Patients who are more severely ill during their hospital stay are especially susceptible to suffer for impaired pulmonary function (29), and are considered the main target population for intervention (30). However, the biological mediators responsible for the pathophysiology of long-term symptoms are mostly unknown.

Circulating levels of C5a are increased in hospitalized COVID-19 patients as compared with healthy donors (18–21), while this does not appear to be the case in non-hospitalized patients (20). About the association between C5a levels and the severity of the disease in hospitalized individuals, Carvelli et al. found higher plasma C5a levels in those hospitalized patients with lung damage (15). C5a levels were higher in severe COVID-19 patients who required intensive care unit admission (21). In another study performed with patients at intensive care unit, C5a correlated with hypoxemia (determined as PaO_2/FiO_2 ratio) at the time of admission (31). In contrast, in other studies, elevated C5a levels were found in hospitalized patients regardless of the severity of the disease (18, 19). The presence of a genetic variant linked to severe COVID-19 predisposition (rs11385942) was associated with increased circulating C5a levels on the day of admission, but these levels did not correlate with markers of inflammation or tissue damage (32). Patients with respiratory failure, either at admission or developed during hospital stay, showed significantly higher levels of soluble C5b-9 and C4d, but not of C5a (33). In our study, at the time of admission, we did not find any association between C5a levels and COVID-related symptoms. However, those patients who were going to require a longer hospital stay showed higher C5a levels. C5a levels at admission also correlated with a previously reported score that estimates the risk of developing critical illness among hospitalized COVID-19 patients (22), and with several laboratory values known to be associated with COVID-19 severity. These results suggest that the effects mediated by C5a become more evident over the course of the disease. Only in those patients in which high C5a levels persist, or are further induced, this molecule may exert more clearly its influence. In this line, in hospitalized COVID-19 patients on hemodialysis, clinical deterioration was preceded by a peak of C5a levels, suggesting that C5a may be a marker of disease progression (20). In a study that monitored the evolution of C5a levels during the hospitalization stay, in those patients who stayed less than 15 days in the hospital, C5a levels decreased from admission to day 10, whereas this reduction was not observed in patients with longer hospitalization (18). We also observed that C5a levels were maintained in those patients with a longest stay in the hospital. On the other hand, until now very little information existed about the evolution of C5a levels after hospital discharge. Cugno et al. reported a significant reduction of serum C5a levels after discharge (19). We also report now an overall reduction of C5a at the follow-up visits, with levels comparable to those from non-hospitalized patients. However, this reduction was not observed in those patients with a longer hospital stay, in whom C5a remained high in association with persistent respiratory symptoms.

Activation of different complement pathways may be responsible for the release of C5a during the acute phase of COVID-19. Holter et al. found a weak correlation between antibodies against SARS-CoV-2 and complement activation in hospitalized patients (33). Deposits of C4d, mannose binding lectin-associated serine protease-2 (MASP-2) and C5b-9 were found in the lung microvasculature of patients with severe COVID-19 (13). Ma et al. found that factor D strongly correlates with markers of endothelial injury and coagulation (21). Therefore, there is evidence for the implication of the three major pathways of complement activation in COVID-19 pathogenesis. Sustained high C5a levels implies a state of persistent production of the peptide from C5, since C5a has a very short half-life *in vivo* (it is readily captured by its receptors, or rapidly inactivated by carboxypeptidases). Interestingly, although we found a significant correlation between the levels of C5a and the terminal complement complex C5b-9, the latter marker was not associated with the clinical risk score or the severity of the disease. This result suggests the existence of alternative mechanisms of C5 activation that may be governing the long-lasting production of C5a levels. Noteworthy, C5a is not only produced by the canonical C5 convertase, but also by extrinsic factors such as thrombin (34). Since an enhanced thrombin-generating capacity has been observed in COVID-19 patients after months from hospital discharge (35), we can speculate that persistent C5a production may be caused by this abnormal hemostatic state. An unresolved prothrombotic state, maybe associated with dysfunction of the endothelium, may enhance thrombin production, which, subsequently, would lead to the cleavage of C5 into C5a. The implication of other potential mechanisms, such as a prolonged viral shedding that would maintain complement activation or a chronic state of unresolved immune response, also merit further investigation.

Growing evidence supports the role played by C5a in the pathogenesis of respiratory distress following SARS-CoV-2 infection (36, 37). C5a orchestrates a strong inflammatory response (38), and is a potent mediator of the acute lung injury induced by viral infections (8–11). C5aR1 blockade with monoclonal antibodies prevented C5a-mediated recruitment and activation of human myeloid cells, and inhibited acute lung injury in human C5aR1 knock-in mice (15). Stimulation of myeloid cells by C5a also contributes to thrombosis and tissue damage (17, 39). Excessive activation of the complement cascade, e.g. by antibodies against SARS-CoV-2 (30) or the activation of the lectin pathway (12), may lead to an over-production of C5a, resulting in cytokine storm, severe lung inflammation, infiltration of immune cells, endothelial dysfunction and thromboinflammation (40, 41). Elevated C5a levels may sustain endothelial dysfunction and an unresolved prothrombotic state, creating a vicious circle that would perpetuate the respiratory problems observed in some discharged patients. If this holds true, patients with long-term respiratory problems would benefit from therapies targeting C5a production.

From the early onset of the pandemic, complement intervention to decrease C5a levels was proposed as an anti-inflammatory strategy that may overt the excessive inflammatory response seen in severe cases of COVID-19 (36, 42). Results from early clinical studies targeting complement at the level of C3 or C5 have already been reported (15, 43–48). They included the use of avdoralimab (IPH 5401), a recombinant human monoclonal antibody that targets C5aR1; AMY-101, a peptidic C3 inhibitor; eculizumab, a humanized monoclonal antibody that inhibits C5 cleavage; or vilobelimab (IFX-1), a chimeric monoclonal antibody that binds to C5a. Some preliminary data have been obtained, showing tolerable adverse effects and potential clinical benefit associated with an improvement in respiratory function (46). While both C3 and C5 inhibition lead to sustained anti-inflammatory response, it has been suggested that C3 inhibition may have a more prominent effect on endothelial or alveolar injury (47). Results from larger controlled trials are awaiting to confirm the clinical efficacy of complement intervention, and to identify the best inhibitory strategy. Given the cost, it may be also necessary to select those patients that more likely would benefit from these treatments by using circulating markers or genetic variants linked to severe COVID-19 predisposition.

We acknowledge some limitations in our study. We have used serum samples, whereas plasma is the preferred sample type for the determination of complement factors. The number of patients included in the study is limited, which may have reduced the ability to detect differences between groups. It also limited our capacity to evaluate the diagnostic performance of C5a as a predictive marker for persistence respiratory problems. Besides, the study was conducted with participants recruited in a single center. For all these reasons, validation studies in independent cohorts are required for a generalization of our results, and for exploring their clinical application. More experiments are also required to assess if C5a levels are specific of persistent respiratory symptoms after COVID-19 infection or constitute a common feature of many persistent respiratory diseases. The correlation observed between C5a and IL1α serum levels also merit further research.

In conclusion, we report for the first time the persistence of elevated serum C5a levels in discharged COVID-19 patients who suffered a severe manifestation of the disease. Moreover, sustained high C5a levels may be associated with long-term respiratory symptoms. Complement activation may be considered as a therapeutic target for the treatment of severe COVID-19 patients with persistent respiratory complications, at least in those cases in which there is an excessive complement activation.

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 study was approved by the Ethics Committee of Clinica Universidad de Navarra (ref. 2020.090). The patients provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YS, RP, JY, and BT conceptualized the study and supervised the work. SI, AL-DC, AB, AC, FC-T, PS, FP, and JY were responsible for the collection of clinical samples, sample processing and/or data management. YS, MO, and BT carried out the experiments. YS, AG-M, JY, RP, and BT performed the analyses. YS, DA, RP, and BT wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Foundation for Applied Medical Research (FIMA), Fundación MTorres, Fundación Ramón Areces, Fondo de Investigación Sanitaria-Fondo Europeo de Desarrollo Regional "Una manera de hacer Europa" (PI20/00419), Departamento de Salud de Gobierno de Navarra (0011-3638-2020-000004), and Departamento de Desarrollo Económico y Empresarial del Gobierno de Navarra (AGATA and DESCARThES projects).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.767376/full#supplementary-material

REFERENCES

1. Cao X. COVID-19: Immunopathology and its Implications for Therapy. *Nat Rev Immunol* (2020) 20:269–70. doi: 10.1038/s41577-020-0308-3
2. Darif D, Hammi I, Kihel A, El Idrissi Saik I, Guessous F, Akarid K. The Pro-Inflammatory Cytokines in COVID-19 Pathogenesis: What Goes Wrong? *Microb Pathog* (2021) 153:104799. doi: 10.1016/j.micpath.2021.104799
3. Bikdeli B, Madhavan MV, Jimenez D, Chuich T, Dreyfus I, Driggin E, et al. COVID-19 and Thrombotic or Thromboembolic Disease: Implications for Prevention, Antithrombotic Therapy, and Follow-Up: JACC State-of-the-Art Review. *J Am Coll Cardiol* (2020) 75:2950–73. doi: 10.1016/j.jacc.2020.04.031
4. Guan W, Ni Z, Hu Y, Liang W, Ou C, He J, et al. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* (2020) 382:1708–20. doi: 10.1056/nejmoa2002032
5. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical Features of Patients Infected With 2019 Novel Coronavirus in Wuhan, China. *Lancet* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
6. Reis ES, Mastellos DC, Hajishengallis G, Lambris JD. New Insights Into the Immune Functions of Complement. *Nat Rev Immunol* (2019) 19:503–16. doi: 10.1038/s41577-019-0168-x
7. Gralinski LE, Sheahan TP, Morrison TE, Menachery VD, Jensen K, Leist SR, et al. Complement Activation Contributes to Severe Acute Respiratory

Syndrome Coronavirus Pathogenesis. *MBio* (2018) 9:e01753–18. doi: 10.1128/mBio.01753-18
8. Wang R, Xiao H, Guo R, Li Y, Shen B. The Role of C5a in Acute Lung Injury Induced by Highly Pathogenic Viral Infections. *Emerg Microbes Infect* (2015) 4:e28. doi: 10.1038/emi.2015.28
9. Bosmann M, Ward PA. Role of C3, C5 and Anaphylatoxin Receptors in Acute Lung Injury and in Sepsis. *Adv Exp Med Biol* (2012) 946:147–59. doi: 10.1007/978-1-4614-0106-3_9
10. Russkamp NF, Ruemmler R, Roewe J, Moore BB, Ward PA, Bosmann M. Experimental Design of Complement Component 5a-Induced Acute Lung Injury (C5a-ALI): A Role of CC-Chemokine Receptor Type 5 During Immune Activation by Anaphylatoxin. *FASEB J* (2015) 29:3762–72. doi: 10.1096/fj.15-271635
11. Jiang Y, Zhao G, Song N, Li P, Chen Y, Guo Y, et al. Blockade of the C5a-C5aR Axis Alleviates Lung Damage in Hdpp4-Transgenic Mice Infected With MERS-CoV Article. *Emerg Microbes Infect* (2018) 7:77. doi: 10.1038/s41426-018-0063-8
12. Bosmann M. Complement Control for COVID-19. *Sci Immunol* (2021) 6: eabj1014. doi: 10.1126/sciimmunol.abj1014
13. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement Associated Microvascular Injury and Thrombosis in the Pathogenesis of Severe COVID-19 Infection: A Report of Five Cases. *Transl Res* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
14. Yan B, Freiwald T, Chauss D, Wang L, West E, Mirabelli C, et al. SARS-CoV-2 Drives JAK1/2-Dependent Local Complement Hyperactivation. *Sci Immunol* (2021) 6:eabg0833. doi: 10.1126/sciimmunol.abg0833
15. Carvelli J, Demaria O, Vély F, Batista L, Chouaki Benmansour N, Fares J, et al. Association of COVID-19 Inflammation With Activation of the C5a–C5aR1 Axis. *Nature* (2020) 588:146–50. doi: 10.1038/s41586-020-2600-6
16. Posch W, Vosper J, Noureen A, Zaderer V, Witting C, Bertacchi G, et al. C5aR Inhibition of Nonimmune Cells Suppresses Inflammation and Maintains Epithelial Integrity in SARS-CoV-2–Infected Primary Human Airway Epithelia. *J Allergy Clin Immunol* (2021) 147:2083–97. doi: 10.1016/j.jaci.2021.03.038
17. Skendros P, Mitsios A, Chrysanthopoulou A, Mastellos DCC, Metallidis S, Rafailidis P, et al. Complement and Tissue Factor–Enriched Neutrophil Extracellular Traps Are Key Drivers in COVID-19 Immunothrombosis. *J Clin Invest* (2020) 130:6151–7. doi: 10.1172/JCI141374
18. Marcos-Jiménez A, Sánchez-Alonso S, Alcaraz-Serna A, Esparcia L, López-Sanz C, Sampedro-Núñez M, et al. Deregulated Cellular Circuits Driving Immunoglobulins and Complement Consumption Associate With the Severity of COVID-19 Patients. *Eur J Immunol* (2021) 51:634–47. doi: 10.1002/eji.202048858
19. Cugno M, Meroni PL, Gualtierotti R, Griffini S, Grovetti E, Torri A, et al. Complement Activation and Endothelial Perturbation Parallel COVID-19 Severity and Activity. *J Autoimmun* (2021) 116:102560. doi: 10.1016/j.jaut.2020.102560
20. Prendecki M, Clarke C, Medjeral-Thomas N, McAdoo SP, Sandhu E, Peters JE, et al. Temporal Changes in Complement Activation in Haemodialysis Patients With COVID-19 as a Predictor of Disease Progression. *Clin Kidney J* (2020) 13:889–96. doi: 10.1093/CKJ/SFAA192
21. Ma L, Sahu SK, Cano M, Kuppuswamy V, Bajwa J, McPhatter J, et al. Increased Complement Activation is a Distinctive Feature of Severe SARS-CoV-2 Infection. *Sci Immunol* (2021) 6:eabh2259. doi: 10.1126/sciimmunol.abh2259
22. Liang W, Liang H, Ou L, Chen B, Chen A, Li C, et al. Development and Validation of a Clinical Risk Score to Predict the Occurrence of Critical Illness in Hospitalized Patients With COVID-19. *JAMA Intern Med* (2020) 180:1081–9. doi: 10.1001/jamainternmed.2020.2033
23. Wickham H. *Ggplot2: Elegant Graphics for Data Analysis* (2016). Available at: https://ggplot2.tidyverse.org.
24. Kassambara A. *Ggpubr* (2020). Available at: https://rpkgs.datanovia.com/ggpubr/.
25. Wickham H, Averick M, Bryan J, Chang W, D'Agostino McGowan L, François R, et al. Welcome to the Tidyverse. *J Open Source Softw* (2019) 4:1686. doi: 10.21105/joss.01686
26. Carfì A, Bernabei R, Landi F. Persistent Symptoms in Patients After Acute COVID-19. *JAMA* (2020) 324:603–5. doi: 10.1001/jama.2020.12603
27. Arnold DT, Hamilton FW, Milne A, Morley AJ, Viner J, Attwood M, et al. Patient Outcomes After Hospitalisation With COVID-19 and Implications for Follow-Up: Results From a Prospective UK Cohort. *Thorax* (2021) 76:399–401. doi: 10.1136/thoraxjnl-2020-216086
28. Morin L, Savale L, Pham T, Colle R, Figueiredo S, Harrois A, et al. Four-Month Clinical Status of a Cohort of Patients After Hospitalization for COVID-19. *JAMA* (2021) 325:1525–34. doi: 10.1001/jama.2021.3331
29. Mo X, Jian W, Su Z, Chen M, Peng H, Peng P, et al. Abnormal Pulmonary Function in COVID-19 Patients at Time of Hospital Discharge. *Eur Respir J* (2020) 55:2001217. doi: 10.1183/13993003.01217-2020
30. Huang C, Huang L, Wang Y, Li X, Ren L, Gu X, et al. 6-Month Consequences of COVID-19 in Patients Discharged From Hospital: A Cohort Study. *Lancet* (2021) 397:220–32. doi: 10.1016/S0140-6736(20)32656-8
31. Dupont A, Rauch A, Staessens S, Moussa M, Rosa M, Corseaux D, et al. Vascular Endothelial Damage in the Pathogenesis of Organ Injury in Severe COVID-19. *Arterioscler Thromb Vasc Biol* (2021) 41:1760–73. doi: 10.1161/atvbaha.120.315595
32. Valenti L, Griffini S, Lamorte G, Grovetti E, Uceda Renteria SC, Malvestiti F, et al. Chromosome 3 Cluster Rs11385942 Variant Links Complement Activation With Severe COVID-19. *J Autoimmun* (2021) 117:102595. doi: 10.1016/j.jaut.2021.102595
33. Holter JC, Pischke SE, de Boer E, Lind A, Jenum S, Holten AR, et al. Systemic Complement Activation is Associated With Respiratory Failure in COVID-19 Hospitalized Patients. *Proc Natl Acad Sci USA* (2020) 117:25018–25. doi: 10.1073/pnas.2010540117
34. Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, et al. Generation of C5a in the Absence of C3: A New Complement Activation Pathway. *Nat Med* (2006) 12:682–7. doi: 10.1038/nm1419
35. von Meijenfeldt FA, Havervall S, Adelmeijer J, Lundström A, Magnusson M, Mackman N, et al. Sustained Prothrombotic Changes in COVID-19 Patients 4 Months After Hospital Discharge. *Blood Adv* (2021) 5:756–9. doi: 10.1182/bloodadvances.2020003968
36. Risitano AM, Mastellos DC, Huber-Lang M, Yancopoulou D, Garlanda C, Ciceri F, et al. Complement as a Target in COVID-19? *Nat Rev Immunol* (2020) 20:343–4. doi: 10.1038/s41577-020-0320-7
37. Ram Kumar Pandian S, Arunachalam S, Deepak V, Kunjiappan S, Sundar K. Targeting Complement Cascade: An Alternative Strategy for COVID-19. *3 Biotech* (2020) 10:479. doi: 10.1007/s13205-020-02464-2
38. Guo RF, Ward PA. Role of C5a in Inflammatory Responses. *Annu Rev Immunol* (2005) 23:821–52. doi: 10.1146/annurev.immunol.23.021704.115835
39. Ritis K, Doumas M, Mastellos D, Micheli A, Giaglis S, Magotti P, et al. A Novel C5a Receptor-Tissue Factor Cross-Talk in Neutrophils Links Innate Immunity to Coagulation Pathways. *J Immunol* (2006) 177:4794–802. doi: 10.4049/jimmunol.177.7.4794
40. Chauhan AJ, Wiffen LJ, Brown TP. COVID-19: A Collision of Complement, Coagulation and Inflammatory Pathways. *J Thromb Haemost* (2020) 18:2110–7. doi: 10.1111/jth.14981
41. Noris M, Benigni A, Remuzzi G. The Case of Complement Activation in COVID-19 Multiorgan Impact. *Kidney Int* (2020) 98:314–22. doi: 10.1016/j.kint.2020.05.013
42. Woodruff TM, Shukla AK. The Complement C5a-C5aR1 GPCR Axis in COVID-19 Therapeutics. *Trends Immunol* (2020) 41:965–7. doi: 10.1016/j.it.2020.09.008
43. Mastaglio S, Ruggeri A, Risitano AM, Angelillo P, Yancopoulou D, Mastellos DC, et al. The First Case of COVID-19 Treated With the Complement C3 Inhibitor AMY-101. *Clin Immunol* (2020) 215:108450. doi: 10.1016/j.clim.2020.108450
44. Annane D, Heming N, Grimaldi-Bensouda L, Frémeaux-Bacchi V, Vigan M, Roux AL, et al. Eculizumab as an Emergency Treatment for Adult Patients With Severe COVID-19 in the Intensive Care Unit: A Proof-of-Concept Study. *EClinicalMedicine* (2020) 28:100590. doi: 10.1016/j.eclinm.2020.100590
45. Diurno F, Numis FG, Porta G, Cirillo F, Maddaluno S, Ragozzino A, et al. Eculizumab Treatment in Patients With COVID-19: Preliminary Results From Real Life ASL Napoli 2 Nord Experience. *Eur Rev Med Pharmacol Sci* (2020) 24:4040–7. doi: 10.26355/EURREV_202004_20875
46. Vlaar APJ, de Bruin S, Busch M, Timmermans SAMEG, van Zeggeren IE, Koning R, et al. Anti-C5a Antibody IFX-1 (Vilobelimab) Treatment Versus Best Supportive Care for Patients With Severe COVID-19 (PANAMO): An

Exploratory, Open-Label, Phase 2 Randomised Controlled Trial. *Lancet Rheumatol* (2020) 2:e764–73. doi: 10.1016/S2665-9913(20)30341-6
47. Mastellos DC, Pires da Silva BGP, Fonseca BAL, Fonseca NP, Auxiliadora-Martins M, Mastaglio S, et al. Complement C3 vs C5 Inhibition in Severe COVID-19: Early Clinical Findings Reveal Differential Biological Efficacy. *Clin Immunol* (2020) 220:108598. doi: 10.1016/j.clim.2020.108598
48. Giudice V, Pagliano P, Vatrella A, Masullo A, Poto S, Polverino BM, et al. Combination of Ruxolitinib and Eculizumab for Treatment of Severe SARS-CoV-2-Related Acute Respiratory Distress Syndrome: A Controlled Study. *Front Pharmacol* (2020) 11:857. doi: 10.3389/fphar.2020.00857

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Immunology

ORIGINAL RESEARCH
published: 25 November 2021
doi: 10.3389/fimmu.2021.791429

Complement Mediated Hemolytic Anemias in the COVID-19 Era: Case Series and Review of the Literature

Bruno Fattizzo[1,2], *Raffaella Pasquale*[2], *Valentina Bellani*[1,2], *Wilma Barcellini*[1] *and Austin G. Kulasekararaj*[3]*

[1] Hematology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, [2] Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy, [3] Hematology Unit, King's College Hospital, London, United Kingdom

The complex pathophysiologic interplay between SARS-CoV-2 infection and complement activation is the subject of active investigation. It is clinically mirrored by the occurrence of exacerbations of complement mediated diseases during COVID-19 infection. These include complement-mediated hemolytic anemias such as paroxysmal nocturnal hemoglobinuria (PNH), autoimmune hemolytic anemia (AIHA), particularly cold agglutinin disease (CAD), and hemolytic uremic syndrome (HUS). All these conditions may benefit from complement inhibitors that are also under study for COVID-19 disease. Hemolytic exacerbations in these conditions may occur upon several triggers including infections and vaccines and may require transfusions, treatment with complement inhibitors and/or immunosuppressors (i.e., steroids and rituximab for AIHA), and result in thrombotic complications. In this manuscript we describe four patients (2 with PNH and 2 with CAD) who experienced hemolytic flares after either COVID-19 infection or SARS-Cov2 vaccine and provide a review of the most recent literature. We report that most episodes occurred within the first 10 days after COVID-19 infection/vaccination and suggest laboratory monitoring (Hb and LDH levels) in that period. Moreover, in our experience and in the literature, hemolytic exacerbations occurring during COVID-19 infection were more severe, required greater therapeutic intervention, and carried more complications including fatalities, as compared to those developing after SARS-CoV-2 vaccine, suggesting the importance of vaccinating this patient population. Patient education remains pivotal to promptly recognize signs/symptoms of hemolytic flares and to refer to medical attention. Treatment choice should be based on the severity of the hemolytic exacerbation as well as of that of COVID-19 infection. Therapies include transfusions, complement inhibitor initiation/additional dose in the case of PNH, steroids/rituximab in patients with CAD and warm type AIHA, plasma exchange, hemodialysis and complement inhibitor in the case of atypical HUS. Finally, anti-thrombotic prophylaxis should be always considered in these settings, provided safe platelet counts.

Keywords: paroxysmal nocturnal hemoglobinuria, cold agglutinin disease, SARS-CoV-2, COVID19 vaccine, hemolytic uremic syndrome, autoimmune hemolytic anemia

OPEN ACCESS

Edited by:
Nicolas Stephane Merle, National Heart, Lung, and Blood Institute (NHLBI), United States

Reviewed by:
Ronald Paul Taylor, University of Virginia, United States
Marie-Agnes Dragon-Durey, Université Paris Descartes, France

***Correspondence:**
Austin G. Kulasekararaj
austin.kulasekararaj@nhs.net

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *08 October 2021*
Accepted: *29 October 2021*
Published: *25 November 2021*

Citation:
Fattizzo B, Pasquale R, Bellani V, Barcellini W and Kulasekararaj AG (2021) Complement Mediated Hemolytic Anemias in the COVID-19 Era: Case Series and Review of the Literature. Front. Immunol. 12:791429. doi: 10.3389/fimmu.2021.791429

INTRODUCTION

There is an increasing interest in the relationship between COVID-19 infection and complement activation. Several reports highlighted that the virus is able to induce an over inflammatory state encompassing the activation of various pathways such as coagulation (namely thrombo-inflammation) and the complement cascade (1–3). The latter is a complex system which may be activated by three different ways: the classical, the lectin and the alternative pathway. The former two require the presence of a specific trigger, either an immune-complex or an infectious agent. The complement alternative pathway is homeostatically active and its functioning may be amplified in the presence of triggers. Complement-mediated hematologic diseases include paroxysmal nocturnal hemoglobinuria (PNH), autoimmune hemolytic anemia (AIHA), particularly cold agglutinin disease (CAD), and hemolytic uremic syndrome (HUS). PNH is caused by the acquisition of somatic mutation of *PIG-A* gene resulting in the loss of glycosylphosphatidylinositol-anchored proteins, including the complement inhibitors CD55 and CD59. PNH cells are therefore subject to complement-mediated destruction that mainly involves erythrocytes with consequent hemolytic anemia. PNH is managed successfully with terminal complement inhibitors targeting C5 (eculizumab or ravulizumab) with good control of intravascular hemolysis (IVH), but potentially inducing iatrogenic extravascular hemolysis (EVH) which is targeted by novel C3 inhibitors (4). CAD is an autoimmune hemolytic anemia caused by cold reactive anti-erythrocyte autoantibodies usually of the IgM class that are intrinsically able to fix complement with consequent positivity of the direct antiglobulin test (DAT) for C3d (5). This may result in both IVH and EVH due to either terminal activation of complement and membrane attack complex (MAC) formation, or C3b deposition on erythrocytes and reticulo-endothelial phagocytosis. CAD is mainly treated with immunosuppressants (steroids and the anti-CD20 monoclonal antibody rituximab) and complement inhibitors are in clinical trials with promising results (6). Complement activation may be observed even in warm type AIHA (wAIHA), particularly in case of high levels of IgG anti-erythrocyte autoantibodies, resulting in DAT positivity for IgG+C. IgG+C wAIHAs are generally more severe and show higher relapse frequency. Clinical trials with complement inhibitors in wAIHA are also ongoing (7). HUS is a thrombotic microangiopathy characterized by the formation of platelet microthrombi in arterioles and capillaries, causing platelet consumption, nonimmune hemolytic anemia, and acute renal injury. Typical HUS is caused by Shiga-like toxin (verotoxin) produced by Escherichia coli (O157: H7) and Shiga toxin by Shigella dysenteriae. The atypical form may be secondary to bacteria, medication, or immune processes capable of endothelial damage, or to congenital or acquired conditions inducing widespread complement activation, such as atypical familial HUS. In the latter cases C5 inhibitors are employed with clinical benefit (8). All these complement-mediated hemolytic conditions may experience exacerbations upon several triggers including infections, surgery, traumas (4, 5). Particularly, hemolytic flares in PNH patients on complement inhibitors are denominated breakthrough hemolysis (BTH) (4). COVID19 infection and its vaccine are also possible triggers through various mechanisms including lectin- and alternative pathway activation by Nucleocapside and Spike viral proteins, molecular mimicry and autoantibody production with activation of the classical pathway, etc (1). In this manuscript we describe four patients who experienced PNH BTH and CAD exacerbation after either COVID infection or SARS-Cov2 vaccine and we review the most recent literature.

METHODS

We report four patients, 2 with PNH and 2 with CAD, diagnosed according to the current guidelines (4, 5) and regularly followed at a tertiary university hospital in Milan, Italy, who experienced hemolytic flares during COVID-19 infection or after SARS-CoV-2 vaccine.

The study was conducted according the Declaration of Helsinki and approved by the local Ethical Committee. Patients signed informed consent.

We conducted a review of the literature about complement mediated hemolytic anemias and COVID-19 infection and SARS-CoV-2 vaccine by searching for indexed articles and published abstracts until September 2021 in MEDLINE *via* PubMed and the National Library of Medicine. The following keywords were used: paroxysmal nocturnal hemoglobinuria; cold agglutinin disease; SARS-CoV-2; COVID19 vaccine; hemolytic uremic syndrome; autoimmune hemolytic anemia; and complement. We selected only studies including human subjects with a clear diagnosis of PNH, CAD, AIHA, HUS, and reporting data on treatment and outcome of COVID-19 infection and of the hemolytic flare.

RESULTS

PNH Flares During COVID-19 Infection

Case Description

A 27-year-old man was diagnosed with PNH in 2010 (granulocyte clone size 93%) associated with transfusion dependent anemia (Hb 6.8 g/dL, LDH 7.5 x upper limit of normality, ULN, PLT 121x10^9/L, neutrophils 1.8 x10^9/L, reticulocytes 121x10^9/L) and symptoms of hemolysis with abdominal pain. He was immediately started on eculizumab 900 mg fortnightly with good response (Hb 11.1 g/dL, LDH 1.1xULN, reticulocytes 51x10^9/L). In March 2021, a nasopharyngeal swab for SARS-Cov-2 performed for screening purposes was positive and the patient was initially asymptomatic. Two days later, he presented to the emergency room complaining of shortness of breath, asthenia, and dark urine. Hb was 6.1 g/dL and LDH increased to 10.7 x ULN. A CT scan revealed bilateral interstitial pneumonia and he was admitted to hospital and treated with antibiotics, dexamethasone, and low molecular weight heparin (LMWH), as well as an earlier dose of eculizumab (10 days after the previous one). During admission he also required red blood cells (RBC) transfusion (3 units) and progressively recovered.

Literature Review

Table 1 summarizes a literature review of PNH cases experiencing hemolytic flares after COVID-19 infection. Since March 2020, we found eight case reports describing a total of 19 patients, 15 with classic hemolytic PNH and 4 with PNH in the context of an aplastic anemia (9–16). Patients were mostly women (63% females, 37% males), with a wide age range (19 to 77 years). 15 patients were on active therapy with a complement inhibitor, and four were treatment naïve. The great majority (89.4%) experienced a mild to moderate COVID-19 infection, whilst only two subjects needed intensive care unit admission and one died of respiratory failure (11, 14). PNH flares had heterogenous severity with some patients requiring RBC transfusions and additional doses of complement inhibitors, and other experiencing only mild Hb reduction with slight LDH elevation. Importantly, no thrombotic episodes were reported. Specifically, Pike et al. reported the experience of the Leeds Paroxysmal Nocturnal Hemoglobinuria national service. Within their PNH cohort, they observed COVID-19 infection in 4 patients on anti-complement therapy (3 eculizumab, 1 ravulizumab); 75% experienced a BTH and one patient died from respiratory failure after being intubated, despite additional doses of eculizumab for ongoing haemolysis (14). Similarly, Barcellini et al. analyzed the experience of eight Italian PNH reference centers and reported 4 patients who had positive SARS-Cov-2 on routine surveillance SARS-Cov-2 nasal-pharyngeal swab. Only two subjects developed mild respiratory symptoms and only one experienced BTH. None of them required hospitalization (16). Additionally, Kulasekararaj et al. reported the clinical course, degree of intravascular hemolysis and outcomes of COVID-19 in four PNH patients, and showed a beneficial effect of complement inhibition both in controlling the intravascular haemolysis and in dampening the hyperinflammatory lung damage due to COVID-19 (13).

Our case report along with the review of the literature suggest that PNH hemolytic flares may occur during COVID-19 infection and may be handled with transfusion support and additional doses of complement inhibitors. The latter, rather than increasing the risk of a more severe COVID-19 infection, appear to dampen the over-inflammatory state induced by SARS-CoV-2 (16, 17). Additionally, the role of complement activation in both early (18) and late stage (19) COVID-19 infection and the potential to target them with ravulizumab is currently being studied in two randomized controlled trials. The latter trial was interrupted early due to futility on interim analysis. The fact that no thrombotic events were observed during these acute hemolytic episodes suggest a further protective role of complement inhibition and may also be in keeping with the high level of medical surveillance/patient education in PNH patients.

PNH Flares After SARS-CoV-2 Vaccination

Case Description

A 17-year-old man was diagnosed with classic hemolytic PNH in a low-income Country in 2019 and was managed with vitamin and iron support, warfarin prophylaxis, and RBC transfusions during hemolytic crises. In March 2021, he came to our center and was started on eculizumab with optimal control of intravascular hemolysis and warfarin was stopped. In July

TABLE 1 | Hemolytic flares in patients with paroxysmal nocturnal hemoglobinuria during COVID-19 infection.

PNH type	Therapy	N° of patients	COVID outcome	PNH outcome	Reference
Classic PNH	Eculizumab	1	Resolved	Clinical remission	*Schuller et al., Annals of Hematology 2021* (9)
Classic PNH	LMWH, dexamethasone, cefuroxime for COVID At the time of diagnosis of COVID19 the patient was waiting for approval of eculizumab	1	Resolved (test negative after 3 months)	Clinical remissionPNH clone did not change.	*Sokol et al., Case Rep Med 2021* (10)
Classic PNH	Antibiotics, Hydroxychloroquine Lopinavir/ritonavir Tocilizumab for COVID Eculizumab	1	Resolved	Clinical remission	*Genthon et al., Leukemia & Lymphoma 2021* (11)
AA PNH	Eculizumab (2 doses) then ravulizumab (2 doses)	1	Resolved	Persistence of pancytopenia	*Otieno et al., Case Rep Hematol 2021* (12)
Classic PNH	1) Ravulizumab 2) Eculizumab, blood transfusions 3) naïve to complement inhibitor treatment, warfarin 4) naïve to complement inhibitor treatment, warfarin	4	1)Resolved 2)Resolved 3)Resolved 4)Resolved	1)Resolved 2)Resolved 3)Resolved 4)Readmitted with worsening symptoms	*Kulasekararaj et al., Br J Haematol 2020* (13)
1) Classic PNH 2) AA PNH 3) AA PNH 4) AA PNH	1) Ravulizumab 2) Eculizumab, antibiotics 3) Eculizumab, 4 units packed red cells, antibiotics 4) Eculizumab, 4 units packed red cells, antibiotics	4	1)Resolved 2)Resolved 3)Death 4)Resolved	1)Resolved 2)Resolved 3) Death 4)Resolved	*Pike A et al., Br J Haematol 2020* (14)
Classic PNH	1) Eculizumab 2) Eculizumab 3) Eculizumab	3	1)Resolved 2)Resolved 3)Resolved	1)Resolved 2)Resolved 3)Resolved	*Araten et al., Am J Case Reports 2020* (15)
Classic PNH	1)Treatment naïve 2)Ravulizumab 3)Ravulizumab 4)Eculizumab	4	1)Resolved 2)Resolved 3)Resolved 4)Resolved	1)No BTH 2)No BTH 3)Resolved 4)No BTH	*Barcellini W et al., Br J Haematol 2021* (16)

PNH, paroxysmal nocturnal hemoglobinuria; AA, aplastic anemia; BTH, breakthrough hemolysis; RBC, red blood cells; rhEPO, recombinant human erythropoietin.

2021, 5 days after the first dose of Pfizer mRNA vaccine (14 days after prior eculizumab dose), he experienced a decrease of 1.5 g/dL Hb (from 12.6 to 11.1 g/dL) associated with an increase in unconjugated bilirubin (1.9 to 3.2 mg/dL) and LDH levels (from normal to 2 x ULN). He complained of mild abdominal pain and urine darkening in the last 2 days. He received eculizumab treatment and symptoms progressively recovered soon after therapy. One-week later laboratory parameters were normalized.

Literature Review

mRNA-based vaccines act by inducing the expression of SARS-Cov-2 Spike protein in order to elicit immune recognition and antibody response. Yu et al. recently illustrated that Spike protein is able to activate complement alternative pathway by competing with complement factor H for binding heparin sulfate, thus representing a possible trigger for PNH reactivation (20). However, Gerber et al. recently showed that the addition of Spike protein didn't increase hemolysis of PNH erythrocytes *ex vivo*, suggesting that hemolysis isn't due to the direct effect of Spike protein on red blood cells but rather to the activation of the classic pathway after antibody generation (21). Clinically, 8 PNH patients experiencing hemolytic flares after SARS-Cov-2 vaccine have been reported, including 6 BTH and 2 exacerbations in treatment naïve subjects (**Table 2**) (21–23). Gerber et al. reported six patients with PNH, who received either Moderna or Pfizer-BioNTech vaccine. Four, all on intravenous ravulizumab and one in combination therapy with D inhibitor danicopan, showed signs of BTH from the vaccine day up to 5 days later, 2 with severe anemia, and 1 requiring transfusion support. Interestingly, only 1 patient who was therapy naïve experienced a likely thrombotic episode. Five days after the second dose of the Pfizer-BioNTech vaccine, he developed abdominal pain and CT scan showed peripancreatic fat stranding indicating possible small bowel microvascular thrombosis; D-dimer was elevated, and the patient was initiated on ravulizumab with signs and symptoms recovery (21). We previously described another case of PNH on treatment with subcutaneous ravulizumab displaying severe BTH the day after the second dose of Moderna vaccine. She was handled with supportive treatment and recombinant erythropoietin with progressive recovery. The latter, along with that reported in the present report, were the only 2 BTH episodes observed after SARS-CoV-2 vaccine in our cohort of 13 PNH patients on active complement inhibition who received mRNA SARS-CoV-2 vaccine from March 2021 at our center (23).

These experiences indicate that hemolytic exacerbations may occur in PNH patients after SARS-Cov-2 vaccine so that monitoring of blood counts and hemolytic markers in the week after vaccine doses is advisable. Patient education to recognize signs/symptoms of PNH activity is pivotal, particularly in naïve patients who may be at higher risk of developing thrombotic complications. Although Gerber et al. observed BTH episodes in patients receiving COVID-19 vaccine more than 4 weeks after last ravulizumab dose, hemolytic flares may occur as soon as the day after the last complement inhibitor administration, so that it is difficult to suggest a specific timing, although it would be advisable to give vaccinations closer to anti-complement therapy (21). Importantly, transfusion requirement was rarer after COVID-19 vaccine than during infection, and no fatalities were reported in PNH after vaccination, confirming that vaccine expected benefits still outweigh the risks. Additionally, the risk of complement activation after Neisseria meningitis vaccination, and leading to thrombosis in patients with PNH has been reported (24).

CAD Exacerbations During COVID-19 Infection

Case Description

A 68-year-old female diagnosed with CAD in 2017 previously treated with steroids and rituximab with response, was admitted to hospital in April 2020 due to fever and shortness of breath. She complained of dark urine and jaundice during the previous 5 days. Swab and CT scan revealed SARS-CoV-2 pneumonia and laboratory tests showed severe anemia (Hb 5.1 g/dL) with increased LDH 2.1 xULN. Direct antiglobulin test was confirmed positive for complement at high titer and consumption of C3 and C4 was noted (C3 <77 mg/dL, C4 undetectable). The patient was managed with oxygen support, hydroxychloroquine, antibiotics, steroids, and LMWH for COVID-19. For CAD reactivation, the patient received a total of 6 RBC transfusions during a period of 27 days. The clinical picture progressively improved and hemolytic anemia recovered, thereby allowing steroid tapering (from 50 mg day of prednisone reduced by 10 mg week until 25 mg day, then by 5 mg week until stop in about 8 weeks).

Literature Review

Available literature regarding CAD development or reactivation during COVID-19 infection is summarized in **Table 3** (25–42). From March 2020, we found 20 reports of patients experiencing cold agglutinin mediated hemolysis after COVID-19 infection (11 males, 9 females, aged from 17 to 77 years). Only 2 patients had a previous history of CAD (30, 42) whilst the others were firstly diagnosed after SARS-CoV-2 infection. All but 2 patients experienced mild to severe respiratory involvement, thereby requiring steroids, hydroxychloroquine, antibiotics, antiviral, tocilizumab, and intubation. Concerning CAD treatment, 12 patients received RBC transfusions, 8 high dose steroids, and 3 rituximab. Two patients received plasma exchange, one for CAD and one for COVID-19 itself. Four patients died despite medical interventions. One fatality occurred in a patient who experienced severe COVID-19 disease with deep venous thrombosis of upper extremities and cerebrovascular disease (33). Given the known risk of thrombosis in patients with CAD, the authors speculated that this may have contributed to thrombosis and to the unfavorable outcome. Accordingly, other investigators have suggested that the development of autoimmune hemolysis may represent a risk factor for worse outcome in COVID-19 patients. Specifically, Algassim et al. reported that 14.7% of intensive care unit (ICU) patients with COVID-19, and 9% of non-ICU patients, had AIHA (46). These patients had significantly more severe courses with longer hospital stay as compared with anemic patients without AIHA. Although anemia and increased levels of LDH and other hemolytic markers may be nonspecifically observed during severe infections, autoimmune hemolysis should be suspected in the context of an unexplained or persistent anemia in patients with COVID-19. From a pathogenic point of view, molecular mimicry

TABLE 2 | Hemolytic flares in patients with paroxysmal nocturnal hemoglobinuria after SARS-CoV-2 vaccine.

PNH type	Therapy	N° of patients	Vaccine	Time to BTH	Therapy for BTH	Outcome	Ref.
1)Classic PNH 2)AA PNH 3)AA 4)Classic PNH 5)Classic PNH 6)Classic PNH	1)treatment naïve 2)Ravulizumab 3)Ravulizumab and Danicopan (oral complement factor D inhibitor) 4)Ravulizumab 5)Ravulizumab 6)Ravulizumab and Danicopan	6	1)Pfizer 2)Pfizer 3)Moderna 4)Moderna 5)Pfizer 6)Pfizer	1)5 days after second dose 2)same day of first dose 3)same day of second dose 4)same day of second dose 5)No BTH 6)No BTH	1)Ravulizumab, LMWH 2)Ravulizumab 3)Ravulizumab, RBC transfusion 4)Ravulizumab 5)Ravulizumab 6)Ravulizumab	1) microvascular bowel thrombosis 2)Resolved 3)Resolved 4)Resolved 5) resolved 6) resolved	*Gerber et al., Blood 2021* (21)
Classic PNH	treatment naïve	1	Moderna	One day after receiving the second dose	Eculizumab + methylprednisolone + RBC transfusion ➔ Switched to Ravulizumab in the outpatient setting	Resolved	*Portuguese et al., Blood advances 2021* (22)
Classic PNH	Ravulizumab	1	Moderna	One day after receiving the second dose	Ravulizumab, antibiotics, rhEPO, LMWH	Resolved	*Fattizzo B et al., Am J Hematol 2021* (23)

PNH, paroxysmal nocturnal hemoglobinuria; AA, aplastic anemia; BTH, breakthrough hemolysis; RBC, red blood cells; rhEPO, recombinant human erythropoietin.

among SARS-CoV-2 antigens and red cells seem the prominent mechanism, as also described for CAD developing after Mycoplasma infection (47). Additionally, the hyperinflammation triggered by the virus itself may result in cross activation of the immune system against self-antigens in an "*innocent bystander*" fashion. This includes the complement cascade that may be activated through both the classic and lectin pathway. Furthermore, the oxidative stress against erythrocyte and platelet membranes typical of the septic state, may favor the exposure of phosphatidyl serine (PS) and consequent complement deposition, thus causing blood cell consumption (47–49).

Overall, the association between CAD development/reactivation and COVID-19 infection has been reported with more than half cases showing severe anemia requiring transfusions. Interestingly, the proportion of COVID-19-associated CAD is greater than what generally observed with CAD among all AIHAs, where CAD represent about 20% of cases (5). Most cases resolved with steroids and along with resolution of COVID-19 infection, but thrombotic episodes may occur and require prophylaxis (as also suggested for COVID-19 infection itself). Finally, it is still debated whether rituximab can be safely administered during septic state, and in most cases this treatment was deferred to allow recovery of active infection (50).

CAD Exacerbations After SARS-CoV-2 Vaccination

Case Description

A 59-year-old male suffering from CAD since 2019, and previously treated with rituximab, had an Hb drop from 10.1 to 6.8 g/dL and LDH elevation (1.8 xULN) 5 days after the second dose of Moderna vaccine. The levels of complement fractions were also decreased, with C3 55 mg/dL (normal values >77 mg/dL) and undetectable C4. He had also experienced fever and pain at the injection site and complained of dark urine and jaundice starting the day after vaccination. He received steroids (1 mg/Kg day prednisone) and recombinant erythropoietin (40,000 UI/week of epoetin alpha subcutaneously) and Hb stabilized at about 8.2 g/dL. Two weeks later, rituximab was administered with progressive and complete recovery, allowing steroid tapering.

Literature Review

As shown in **Table 4**, only 4 cases of CAD or mixed form of AIHA (i.e., direct anti-globulin test positive for IgG plus C at high titer) developing (N=2) or reactivating (N=2) after SARS-CoV-2 vaccination have been reported (23, 28, 51, 52). Al Aoun et al. reported a 45-year-old female patient developing severe CAD 3 days after the first dose of Pfizer vaccine. The patient was treated with blood transfusion and rituximab, achieving complete remission 8 weeks later (51). Brito et al. reported an 88-year-old woman who developed very severe AIHA (Hb 4.5 g/dL) complicated by acute kidney injury two days after the second dose of an mRNA vaccine (not specified). The DAT revealed very high titers of anti-erythrocyte IgG autoantibodies and high anti-C3d titers. The patient was treated with methylprednisolone 1 g boluses and was transfused with subsequent Hb stabilization and amelioration of hemolytic markers (52). As regards exacerbations of pre-existing CAD, Lamas et al. reported a 57-year-old female who experienced shortness of breath, jaundice and mild hemoglobinuria 2 days after the first dose of an mRNA vaccine (not specified); hb had decreased to 8.6 g/dL and the patient received prednisone 20 mg daily with progressive improvement. The same episode occurred 2 days after the second dose of the same vaccine. Anti-Spike IgG testing showed that the patient had efficiently seroconverted (55). Interestingly, our group reported the results of a prospective monitoring of 58 individuals with AIHA, including 15 CAD during SARS-CoV-2 vaccination campaign. This consisted in testing Hb and LDH levels the week before and after each vaccine dose. We detected 3 warm AIHA and 1 CAD reactivation, all effectively managed with steroids and/or rituximab (23).

The case described and data from literature show that SARS-CoV-2 vaccination may be associated with CAD development as

TABLE 3 | Hemolytic flares in patients with cold agglutinin disease (CAD) and warm autoimmune hemolytic anemia (wAIHA) during COVID-19 infection.

Study type	Population of CAD	N°	Time to AIHA	Clinical presentation	Covid treatment	Covid outcome	CAD treatment	CAD outcome	Ref
Case report	Adult (54y, M)	1	–	Pneumonia	Hydroxychloroquine, tocilizumab, plasma exchange	Resolved	Steroids, plasma exchange	Resolved	Ramos-Ruperto et al., SN Compr Clin Med 2021 (25)
Case series	Adult (62y, F) Adult (69y, F) Adult (61y, M)	3	4 days 10 days 11 days	Severe pneumoniae Moderate pneumoniae Mild pneumoniae	Not reported	Resolved	Steroid, rituximab steroids transfusion	Remission Remission Still active hemolysis	Lazarian et al., Br J Haematol 2020 (26)
Case report	Adult (49y, F)	1	Not reported	Severe pneumonia	Not reported	Not reported	Not reported	Not reported	Ahmadnezhad et al., Hematol Transfus Cell Ther 2020 (27)
Case report	Adult (46y, F)	1	Not reported	Pneumonia	Not reported	Death	Not reported	Death	Zagorski Br J Haematol 2020 (28)
Case report	Adult (51y, F)	1	Concomitant	Pulmonary embolism	Heparin	Resolved	transfusion	Remission	Patil et al., Hematol Oncol Stem Cell Ther 2020 (29)
Case report	Adult (70y, M)	1	Concomitant	Not reported	steroids	Resolved	Transfusion, plasma exchange	Remission	Ahmed et al., BMJ Case Rep 2021 (30)
Case report	Adult (24y, F)	1	3 days	Pneumonia	Favipiravir, darunavir/ritonavir, azithromycin	Resolved	None	Remission, spontaneous	Moonla et al., Clin Case Rep 2020 (31)
Case report	Adult (77y, M)	1	9 days	Pneumonia	Intravenous steroids, antibiotics, hydroxychloroquine	Death	Transfusion	NR	Gupta et al., Eur J Case Rep Intern Med 2021 (32)
Case report	Adult (48y, M)	1	Concomitant	DVT, cerebrovascular disease	ND	Death	Transfusion	NR	Maslov et al., TH Open 2020 (33)
Clinical trial	Adult (33y, F)	1	Concomitant	Pneumonia	Steroids, tocilizumab	Resolved	Transfusion, steroids, rituximab 600 mg single infusion	Remission after rituximab	Jacobs et al., Transfusion 2021 (34)
Case report	Adult (62y, M)	1	16 days	Pneumonia	Intubation	Resolved	Transfusion	Remission	Capes et al., Ann Hematol 2020 (35)
Case report	Adult (69y, F)	1	20 days	Not reported	Levofloxacin, steroids	Resolved	Steroids, rituximab, Intravenous immunoglobulins	Remission	Aldaghlawi et al., Clin Case Rep 2021 (36)
Case series	Adult (43y, F) Adult (63y, M)	2	6 days	Pneumonia	Oxygen, antibiotics	Resolved	Transfusion Not reported	Remission Remission	Huscenot et al., Ann Hematol 2020 (37)
Case report	Adult (45 y, M)	1	Concomitant	Pneumonia	Not reported	Not reported	Transfusion	Not reported	Raghuwanshi, Cureus 2020 (38)
Case report	Adult (61 y, M)	1	21 days	Pneumonia	Intubation, hydroxychloroquine, azithromycin, methylprednisolone	Resolved	None	Spontaneous recovery	Kaur et al., Cureus 2020 (39)
Case report	Adult (17y, M)	1	Concomitant	Mild pneumonia	None	Resolved	Steroids, transfusion	Remission	Wahlster Pediatr Blood Cancer 2020 (40)

(Continued)

TABLE 3 | Continued

Study type	Population of CAD	N°	Time to AIHA	Clinical presentation	Covid treatment	Covid outcome	CAD treatment	CAD outcome	Ref
Case report	Adult (48y, M)	1	6 days	Severe pneumonia	mechanical ventilation, vasopressors, sedation	Resolved	Transfusion	Remission	Hassanein et al., J Med Cases 2021 (41)
Cohort study	Adult (71y, F)	1/108 CAD patients	4 days	Severe pneumonia	oxygen, hydroxychloroquine, azithromycine, lopinavir/ritonavir	Resolved	Steroids, transfusions	Remission	Barcellini et al., Front Immunol 2021 (42)
Study type	**Population of wAIHA**	**N°**	**Time to AIHA**	**Presentation**	**Covid treatment**	**Covid outcome**	**AIHA treatment**	**AIHA outcome**	**Ref**
Case report	Adult (54y, M) IgG+	1	–	Pneumonia, diabetic ketoacidosis, acute kidney injury, hematuria, and anemia	–	Resolved	Steroids	Resolved	Huda et al., Cureus 2021 (43)
Case report	Adult (33y, F) IgG+C+	1	–	Asymoptomatic	–	.	Transfusions, Steroids	Resolved	Liput et al., Cureus 2021 (44)
Case series	Adult (72y,F) IgG+ Adult (76y,F) IgG+	2/3	concomitant	Bilateral pneumonia in both	Hydroxychloroquine, dexamethasone and tocilizumab	Resolved	Transfusion, steroids	Resolved	Ramos-Ruperto et al., SN Compr Clin Med 2021 (25)
Case series	Adult (61y,M) IgG+C+ Adult (89y,F) IgG+ Adult (61y,M) IgG+ Adult (75y,M) IgG+	4/6	13 days 7 days 9 days 6 days	Moderate pneumonia Mild pneumonia Severe pneumonia Moderate pneumonia	Oxygen, Steroids, hydroxychloroquine, lopinavir and ritonavir.	Resolved	Steroids and transfusions, rituximab in 1	Not resolved at the time of publication	Lazarian et al., Br J Haematol 2020 (26)
Case report	Adult (24y,M) IgG+C AIHA	1	concomitant	Fever, myalgias and cough, pulmonary embolism, encephalitis	Steroids, anticoagulants, intubation, vasopressors, intravenous immunoglobulin	Superimposed Cryptococcus neoformans infection and death	Steroids, cyclophosphamide	Partial remission	Woldie et al., J Med Cases 2020 (45)
Retrospective study	Adult (59y,M) IgG+ Adult (78y,M) IgG+	2/139	– 3 weeks	Bilateral pneumonia and dysimmune encephalitis moderate pneumonia	intubation, steroids, hydroxychloroquine, tocilizumab, darunavir, and LMWH prophylaxis, Ivlg oxygen support, steroids, HCQ, azithromycine, full-dose LMWH	Resolved	Nothing Steroids and IVIG	Remission	Barcellini et al., Front Immunol 2020 (42)
Case report	Adult (33y, F) IgG+C+	1	concomitant	Bilateral pneumonia	Steroids, tocilizumab	Resolved	Trasnfusion, steroids, rituximab	Remisson	Jacobs et al., Transfusion 2021 (34)

well as with exacerbation in less than 10% of pre-existing CAD cases (i.e., 1/15 in our experience). CAD exacerbations are unpredictable, may occur after either after the first or the second dose, and regardless of vaccine type. Since most cases may be successfully managed with steroids and transfusion, prospective hematologic monitoring as adopted in our survey (1 week before, 1 week after the first and the second dose) appears appropriate to intercept and manage hemolytic flares. Similarly to what described for PNH, CAD episodes following COVID-19 vaccine are milder than those following infection, with no thrombotic nor fatal events, strengthening the message that the benefits of vaccination greatly outweigh the risks.

Exacerbations/Development of wAIHA After COVID-19 Infection or SARS-CoV-2 Vaccination

Eleven patients with warm type AIHA developing or reactivating during COVID-19 infection have been reported in the literature (**Table 3**) (25, 26, 34, 42, 44, 45, 54). Of these patients, 4 showed complement positivity at DAT evaluation (26, 34, 44, 45).

TABLE 4 | Hemolytic flares in patients with cold agglutinin disease (CAD) and warm autoimmune hemolytic anemia (wAIHA) after SARS-CoV-2 vaccine.

Study type	Population	N°	Vaccine	Time to event	AIHA treatment	AIHA outcome	Ref
Case report	Adult (45y, F) CAD	1	Pfizer, 1st dose	4 days	blood transfusions, rituximab	Remission	Al Aoun and Motabi (51)
Case report	Adult (88y, F) CAD	1	mRNA vaccine, 2nd dose	2 days	Transfusions, methylprednisolone 1 g bolus	Remission	Brito et al. (52)
Case report	Adult (57y, F) CAD	1	mRNA vaccine, 1st dose mRNA vaccine, 2nd dose	2 days 5 days	prednisone 20 mg day	Remission	Zagorski et al. (28)
Cohort study	Adult (77y,M) CAD	1/15 CAD patients	Moderna vaccine, 1st dose	7 days	Steroids, rituximab, recombinant erythropoietin	Remission	Fattizzo et al. (53)
Cohort study	Adult (79y,F) IgG +IgA+ wAIHA	3/41 wAIHA patients	Pfizer vaccine, 1st dose	7 days	Steroids	Remission	Fattizzo et al. (53)
	Adult (73y,M) IgG+ wAIHA		Moderna vaccine, 1st dose	7 days	Steroids		
	Adult (73y,M) IgG +wAIHA		Pfizer vaccine, 2nd dose	5 days	Steroids		
Case report	Adult (41 y, F) IgG+ wAIHA	1	Moderna, 1st dose	20 days	Transfusion, steroids, rituximab, mycophenolate mofetil, and immunoglobulins	Remission	Gadi et al. (54)
Case report	Adult (88y,F) IgG+C + wAIHA	1	mRNA vaccine, 2nd dose	2 days	Transfusions, steroids	Remission	Brito et al., Cureus 2021 (52)

WAIHA flares occurred concomitantly with- or up to 21 days after initial COVID-19 detection and were mostly managed with transfusions and steroids. Only 2 patients required rituximab and 1 received cyclophosphamide. Notably, we previously reported 2 patients out of a cohort of 139 wAIHA patients who experienced a severe COVID-19 infection (42). Only one experienced wAIHA reactivation 3 weeks after COVID-19 pneumonia and was effectively managed with steroids and intravenous immunoglobulins. Additionally, 5 cases of wAIHA (only 1 had DAT positive for IgG and C3d) developing or reactivating after mRNA SARS-CoV-2 vaccination have been described, 3 after the 1st and 2 after the 2nd dose (**Table 4**) (23, 52, 54). Three cases had a severe presentation (i.e., Hb<8 g/dL), 2 required transfusions and only one subject received therapy other than steroids (rituximab and mycophenolate). Overall, as outlined for patients with CAD, hemolytic flares after COVID-19 infection appear more severe than those occurring after vaccination and the same monitoring of blood counts and LDH should be applied.

Exacerbations/Development of HUS During COVID-19 Infection

The review of the literature showed a total of 9 patients with HUS associated with COVID-19 infection (**Table 5**) (56–59). Eight subjects developed an atypical HUS and 1 a typical HUS with bloody diarrhea in the preceding days and confirmed E. coli infection. Interestingly, most patients had mild or asymptomatic COVID-19 infection, whilst HUS presented with severe haemolytic anemia and acute kidney injury requiring transfusions, hemodialysis, and the C5 inhibitor eculizumab in 7 cases (56–59). All HUS flares occurred within 1 month from COVID-19 infection, mostly concomitantly, and all resolved. The largest cohort was that reported by El Sissy et al., who noted a sharp contrast between mild respiratory symptoms and severe renal and neurological HUS manifestations (54). Importantly, 3/5 subjects had undergone a previous renal transplant and were receiving tacrolimus/everolimus therapy that may have contributed to trigger HUS. Interestingly, patients with COVID-19- associated atypical HUS, who underwent genetic testing, were found to harbour genetic complement dysregulation, and 2 patients were positive for antibodies against complement factor H. Although limited, available data suggest that COVID-19 is a potential trigger for HUS, in accordance with previous data on complement-mediated aHUS precipitated by viral infections such as Influenza. The clinical suspicion should be acute to prompt quick diagnosis and establishment of supportive treatment (hemodialysis, transfusions and plasma exchange), along with use of anti-complement therapies.

Discussion and Conclusions

There is an association between COVID-19 and exacerbations of complement mediated hemolytic anemias. Although hemolytic flares in these diseases may be observed upon several infectious triggers (i.e., Mycoplasma pneumoniae, hepatitis, herpetic viruses, human immunodeficiency virus, Influenza virus, etc) (4, 60), and sporadically after other vaccines (i.e., Influenza vaccine) (61–63), SARS-CoV-2 seems to induce a higher and broader complement activation. Complement is clearly involved in the pathogenesis of COVID-19, namely respiratory failure, intravascular coagulopathy, and over-inflammation. Recently, it has been shown that a prominent activation of the alternative and lectin complement pathways identify a subset of severe COVID-19 patients with a higher proportion of fatalities, need of oxygen support, and ICU admission (64–67). If during COVID-19 infection alternative and lectin pathways are more involved, SARS-CoV-2 vaccine seems to mainly act through the classical pathway activation (21). As a matter of fact, complement activation has a dichotomous nature since it does contribute to the hyper-inflammatory state but may also exert a neutralizing effect against SARS-CoV-2 infection. This may account for the controversial results of ongoing trials with complement inhibitors (18, 19, 68–70). Likewise, immunosuppressive treatment in

TABLE 5 | Hemolytic flares in patients with hemolytic uremic syndrome (HUS) during COVID-19 infection.

Study type	Population	N°	Time to AIHA	Clinical presentation	Covid treatment	Covid outcome	Hemolytic treatment	hemolytic outcome	Ref
Case series	5 patients with COVID-19-associated atypical HUS	5	Concomitant to 30 days	mild respiratory symptoms renal ysfunction, severe thrombocytopenia, neurological symptoms (confusion, central facial palsy), intestinal involvement (pain, diarrhoea), intestinal capillary thrombi.	Oxygen in 1/5 patients	Resolved	Two patients underwent plasma exchanges with fresh frozen plasma, while three were treated with eculizumab. Patient 4 received two infusions of rituximab for anti-FH antibodies.	All resolved	El Sissy et al., Blood 2021 (56)
Case series	Adult (22y,F) atypical HUS	2	Concomitant	Diarrhea, vomiting, loss of taste, fatigue, severe hemolytic anemia	–	–	Hemodialysis, Transfusions, plasma exchange, eculizumab	Both cases Resolved	Kaufeld et al., Kidney Int Rep 2021 (57)
	Adult (52y,F) atypical HUS		2 days	flu-like symptoms, loss of taset, fatigue, abdominal cramps, severe hemolysis			Ttransfusions, hemodialysis, eculizumab		
Case report	Adult (28y,F) atypical HUS	1	Concomitant	Fever, dysphagia, headache, hemolytic anemia, mild thrombocytopenia, acute kidney injury	–	–	Eculizumab, penicillin prophylaxis, anticoagulation	Resolved	Ville et al., Kidney Int 2021 (58)
Case report	Adult (24y,F) typical HUS	1	Concomitant	bloody diarrhea, acute kidney injury, and focal seizures	–	–	Eculizumab, lorazepam, levetiracetam, valproic acid	Resolved	Simpson et al., Epilepsy Behav Rep 2021 (59)

immune-mediated anemias may have a dual effect in reducing hyper-inflammation and dampening immunocompetence.

Patient education is pivotal to promptly recognize signs/symptoms of hemolytic flares and to seek medical attention. The latter should be always high and should focus on the assessment of the severity of hemolytic exacerbation to prompt treatment choice (transfusions, complement inhibitor initiation/additional dose in the case of PNH, steroids/rituximab in the case of CAD and wAIHA, plasma exchange, hemodialysis and complement inhibitor in the case of aHUS) and anti-thrombotic prophylaxis. Since most episodes occurred within the first 10 days after COVID-19 infection/vaccination, laboratory monitoring in that period appears feasible and cost-effective. Finally, hemolytic exacerbations occurring during COVID-19 infection were more severe, required higher therapeutic burden, and carried more complications including fatalities, as compared to those developing after SARS-CoV-2 vaccine. This highlights the importance of vaccinating this patient population but with meticulous monitoring for complications.

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 human participants were reviewed and approved by Comitato Etico Milano Area 2. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors equally contributed to the present article.

REFERENCES

1. Ma L, Sahu SK, Cano M, Kuppuswamy V, Bajwa J, McPhatter J, et al. Increased Complement Activation Is a Distinctive Feature of Severe SARS-CoV-2 Infection. *Sci Immunol* (2021) 6:1–18. doi: 10.1126/sciimmunol.abh2259
2. Gao T, Hu M, Zhang X, Li H, Zhu L, Dong Q, et al. Highly Pathogenic Coronavirus N Protein Aggravates Lung Injury by MASP-2-Mediated Complement Over-Activation. *medRxiv*. doi: 10.1101/2020.03.29.20041962
3. Fodil S, Annane D. Complement Inhibition and COVID-19: The Story So Far. *Immunotargets Ther* (2021) 10:273–84. doi: 10.2147/ITT.S284830
4. Brodsky RA. How I Treat Paroxysmal Nocturnal Hemoglobinuria. *Blood* (2021) 137(10):1304–9. doi: 10.1182/blood.2019003812
5. Berentsen S. New Insights in the Pathogenesis and Therapy of Cold Agglutinin-Mediated Autoimmune Hemolytic Anemia. *Front Immunol* (2020) 11:590.eCollection 2020. doi: 10.3389/fimmu.2020.00590.eCollection2020
6. Röth A, Barcellini W, D'Sa S, Miyakawa Y, Broome CM, Michel M, et al. Sutimlimab in Cold Agglutinin Disease. *N Engl J Med* (2021) 384(14):1323–34. doi: 10.1056/NEJMoa2027760
7. Barcellini W, Zaninoni A, Giannotta JA, Fattizzo B. New Insights in Autoimmune Hemolytic Anemia: From Pathogenesis to Therapy. *J Clin Med* (2020) 9:3859. doi: 10.3390/jcm9123859
8. Jokiranta TS. HUS and Atypical HUS. *Blood* (2017) 129(21):2847–56. doi: 10.1182/blood-2016-11-709865
9. Schüller H, Klein F, Lübbert M, Prager EP. Hemolytic Crisis in a Patient Treated With Eculizumab for Paroxysmal Nocturnal Hemoglobinuria

Possibly Triggered by SARS-CoV-2 (COVID-19): A Case Report. *Ann Hematol* (2021) r100(3):841–2. doi: 10.1007/s00277-020-04318-6
10. Sokol J, Nehaj F, Mokan M, Lisa L, Stasko J. COVID19 Infection in a Patient With Paroxysmal Nocturnal Hemoglobinuria: A Case Report. *Case Rep Med (Baltimore)* (2021) 100(20):e25456. doi: 10.1097/MD.0000000000025456
11. Genthon A, Chiarabini T, Baylac P, Valin N, Ubrina T, Pakanowski J, et al. Severe COVID-19 Infection in a Patient With Paroxysmal Nocturnal Hemoglobinuria on Eculizumab Therapy. *Leuk Lymphoma* (2021) 62 (6):1502–5. doi: 10.1080/10428194.2020.1869963
12. Otieno SB, Altahan A, Kaweeta F, Karri S, Alnoor F, Johnson R. Severe Hemolysis in a COVID-19 Patient With Paroxysmal Nocturnal Hemoglobinuria. *Case Rep Hematol* (2021) 2021:6619177. doi: 10.1155/2021/6619177
13. Kulasekararaj AG, Lazana I, Large J, Posadas K, Eagleton H, Lord Villajim J, et al. Terminal Complement Inhibition Dampens the Inflammation During COVID-19. *Br J Haematol* (2020) 190(3):e141–3. doi: 10.1111/bjh.16916
14. Pike A, Muus P, Munir T, Mitchell L, Arnold L, Riley K, et al. Griffin M COVID-19 Infection in Patients on Anti-Complement Therapy: The Leeds National Paroxysmal Nocturnal Hemoglobinuria Service Experience. *Br J Haematol* (2020) 191(1):e1–4. doi: 10.1111/bjh.17097
15. Araten DJ, Belmont HM, Schaefer-Cutillo J, Iyengar A, Mattoo A. Reddy R Mild Clinical Course of COVID-19 in 3 Patients Receiving Therapeutic Monoclonal Antibodies Targeting C5 Complement for Hematologic Disorders. *Am J Case Rep* (2020) 21:e927418. doi: 10.12659/AJCR.927418
16. Barcellini W, Fattizzo B, Quattrocchi L, Giannotta JA, Aydin S, Barone F, et al. Covid-19 Infection in Patients With PNH: An Italian Multicenter Survey. *Br J Haematol* (2021) 194(5):854–6. doi: 10.1111/bjh.17558
17. Risitano AM, Mastellos DC, Huber-Lang M, Yancopoulou D, Garlanda C, Ciceri F, et al. Complement as a Target in COVID-19? *Nat Rev Immunol* (2020) 20(6):343–4. doi: 10.1038/s41577-020-0320-7
18. Kulkarni S, Fisk M, Kostapanos M, Banham-Hall E, Bond S, Hernan-Sancho E, et al. Repurposed Immunomodulatory Drugs for Covid-19 in Pre-ICu Patients - mulTi-Arm Therapeutic Study in Pre-ICu Patients Admitted With Covid-19 - Repurposed Drugs (TACTIC-R): A Structured Summary of a Study Protocol for a Randomised Controlled Trial. *Trials* (2020) 21(1):626. doi: 10.1186/s13063-020-04535-4
19. *Efficacy and Safety Study of IV Ravulizumab in Patients With COVID-19 Severe Pneumonia*. Available at: https://clinicaltrials.gov/ct2/show/NCT04369469 (Accessed 6th October 2021).
20. Yu J, Yuan X, Cjhen H, Chaturvedi S, Braunstein EM. Brodsky RA Direct Activation of the Alternative Complement Pathway by SARS-CoV-2 Spike Proteins Is Blocked by Factor D Inhibition. *Blood* (2020) 136(18):2080–9. doi: 10.1182/blood.2020008248
21. Gerber GF, Yuan X YUJ, Cher BAY, Braunstein EM, Chaturvedi S, Brodsky RA. COVID-19 Vaccines Induce Severe Hemolysis in Paroxysmal Nocturnal Hemoglobinuria. *Blood* (2021) 137(26):3670–3. doi: 10.1182/blood.2021011548
22. Portuguese AJ, Sunga C, Kruse-Jarres R, Gernsheimer T, Abkowitz JN. Autoimmune- and Complement-Mediated Hematologic Condition Recrudescence Following Sars-Cov-2 Vaccination. *Blood Adv* (2021) 5 (13):2794–8. doi: 10.1182/bloodadvances.2021004957
23. Fattizzo B, Giannotta JA, Cecchi N, Barcellini W. Sars-CoV2-Vaccination Induces Breakthrough Hemolysis in PNH on Complement Inhibitor. *Am J Hematol* (2021) 96(9):E344–6. doi: 10.1002/ajh.26262
24. Arnold L, Kelly R, Munir T, Muus P, Pike A, Riley K, et al. 1684 Thrombotic Events With Neisseria Meningitidis Vaccination in Patients With Paroxysmal Nocturnal Hemoglobinuria, UK Experience. *Blood* (2020) 136(suppl 1):35–6. doi: 10.1182/blood-2020-141769
25. Ramos Ruperto L, Garcìa Pérez R, Hernández-Maraver D, Kerguelén-Fuentes A, Viejo-Llorente A, Robles-Marhuenda Á, et al. A 3-Case Series of Autoimmune Haemolytic Anaemia and COVID-19: Is Plasma Exchange an Alternative? *SN Compr Clin Med* (2021) 3:1420–3. doi: 10.1007/s42399-021-00884-6
26. Lazarian G, Quinquenel A, Bellal M, Siavellis J, Jacquy C, Re D, et al. Autoimmune Haemolytic Anaemia Associated With COVID-19 Infection. *Br J Haematol* (2020) 190(1):29–31. doi: 10.1111/bjh.16794
27. Ahmadnezhad M, Mosleh M, Ferdowsi S, Mohammadi S, Eshghi P, Oodi A. Cold Agglutinin Associated With COVID-19 Infection in a Thalassemia Patient With Multiple Alloantibodies: A Case of Cold Hemagglutinin Disease (CAD) With Complex Antibody Detection. *Hematol Transfus Cell Ther* (2021) 43(3):361–3. doi: 10.1016/j.htct.2021.04.005
28. Zagorski E, Pawar T, Rahimian S, Forman D. Cold Agglutinin Autoimmune Haemolytic Anaemia Associated With Novel Coronavirus (COVID-19). *Br J Haematol* (2020) 190(4):e183–4. doi: 10.1111/bjh.16892
29. Patil NH, Herc ES, Girgis M. Cold Agglutinin Disease and Autoimmune Hemolytic Anemia With Pulmonary Embolism as a Presentation of COVID-19 Infection. *Hematol Oncol Stem Cell Ther* (2020) S1658-3876(20)30116-3. doi: 10.1016/j.hemonc.2020.06.005
30. Ahmed Y, Khandelwal A, Walker L. Cold Agglutinin Disease and COVID-19 Requiring Therapeutic Plasma Exchange. *Case Rep* (2021) 14(7):e244227. doi: 10.1136/bcr-2021-244227
31. Moonla C, Suwanpimolkul G, Paitoonpong L, Jantarabenjaku W, Chanswangphuwana C, Polprasert C, et al. Cold Agglutinin Disease Following SARS-CoV-2 and Mycoplasma Pneumoniae Co-Infections. *Clin Case Rep* (2020) 8(12):2402–5. doi: 10.1002/ccr3.3152
32. Gupta R, Singh S, Anusim N, Gupta S, Gupta S, Huben M, et al. Coronavirus Disease 2019 and Cold Agglutinin Syndrome: An Interesting Case. *Case Rep Intern Med* (2021) 8(3):2387. doi: 10.12890/2021_002387.eCollection2021
33. Maslov DV, Simenson V, Jain S, Badari A. COVID-19 and Cold Agglutinin Hemolytic Anemia. *TH Open* (2020) 4(3):e175–7. doi: 10.1055/s-0040-1715791.eCollection2020Jul
34. Jacobs J, Eichbaum Q. COVID-19 Associated With Severe Autoimmune Hemolytic Anemia. *Transfusion* (2021) 61(2):635–40. doi: 10.1111/trf.16226
35. Capes A, Bailly S, Hantson P, Gerard L, Laterre PF. COVID-19 Infection Associated With Autoimmune Hemolytic Anemia. *Ann Hematol* (2020) 99 (7):1679–80. doi: 10.1007/s00277-020-04137-9
36. Aldaghlawi F, Shammah A, Kio E. SARS-CoV-2 Infection Complicated With Cold Agglutinin Disease and Myositis. *Clin Case Rep* (2021) 9(4):2196–9. doi: 10.1002/ccr3.3981.eCollection2021Apr
37. Huscenot T, Galland J, Ouvrat M, Rossignol M, Mouly S, Sène D, et al. SARS-CoV-2-Associated Cold Agglutinin Disease: A Report of Two Cases. *Ann Hematol* (2020) 99(8):1943–4. doi: 10.1007/s00277-020-04129-9
38. Raghuwanshi B. Serological Blood Group Discrepancy and Cold Agglutinin Autoimmune Hemolytic Anemia Associated With Novel Coronavirus Cureus. *Cureus* (2020) 12(11):e11495. doi: 10.7759/cureus.11495
39. Kaur J, Mogulla S, Khan R, Krishnamoorthy G, Garg S. Transient Cold Agglutinins in a Patient With COVID-19. *Cureus* (2021) 13(1):e12751. doi: 10.7759/cureus.12751
40. Wahlster L, Weichert-Leahey N, Trissal M, Grace RF. Sankaran VG.COVID-19 Presenting With Autoimmune Hemolytic Anemia in the Setting of Underlying Immune Dysregulation. *Pediatr Blood Cancer* (2020) 67(9): e28382. doi: 10.1002/pbc.28382
41. Hassanein H, Hajdenberg J. High Thermal Amplitude Red Blood Cell Agglutinating Cold Type Autoantibodies in a Case of Severe Acute Respiratory Syndrome Coronavirus 2 Pneumonia and Multiorgan Failure. *J Med Cases* (2021) 12(1):16–7. doi: 10.14740/jmc3608
42. Barcellini W, Giannotta JA, Fattizzo B. Are Patients With Autoimmune Cytopenias at Higher Risk of COVID-19 Pneumonia? The Experience of a Reference Center in Northern Italy and Review of the Literature. *Front Immunol* (2021). doi: 10.3389/fimmu.2020.609198
43. Huda Z, Jahangir A, Sahra S, Rafay Khan Niazi M, Anwar S, Glaser A, et al. A Case of COVID-19-Associated Autoimmune Hemolytic Anemia With Hyperferritinemia in an Immunocompetent Host. *Cureus* (2021) 13(6): e16078. doi: 10.7759/cureus.16078
44. Liput JR, Jordan K, Patadia R, Kander E. Warm Autoimmune Hemolytic Anemia Associated With Asymptomatic SARS-CoV-2. *Infect Cureus* (2021) 13(3):e14101. doi: 10.7759/cureus.14101
45. Woldie IL, Brown IG, Nwadiaro NF, Patel A, Jarrar M, Quint E, et al. Med Cases. *J Med Cases* (2020) 11(11):362–5. doi: 10.14740/jmc3575
46. Algassim AA, Elghazaly AA, Alnahdi AS, Mohammed-Rahim OM, Alanazi AG, Aldhuwayhi NA, et al. Prognostic Significance of Hemoglobin Level and Autoimmune Hemolytic Anemia in SARS-CoV-2 Infection. *Ann Hematol* (2021) 100(1):37–43. doi: 10.1007/s00277-020-04256-3
47. Angileri F, Legare S, Marino Gammazza A, Conway de Macario E, Jl Macario A, Cappello F. Molecular Mimicry may Explain Multi-Organ Damage in COVID-19. *Autoimmun Rev* (2020) 19(8):102591. doi: 10.1016/j.autrev.2020.102591

48. Chauhan N, Jaggi M, Chauhan SC, Yallapu MM. COVID-19: Fighting the Invisible Enemy With microRNAs. *Expert Rev Anti Infect Ther* (2021) 19 (2):137–45. doi: 10.1080/14787210.2020.1812385
49. Kubánková M, Hohberger B, Hoffmanns J, Fürst J, Herrmann M, Guck J, et al. Physical Phenotype of Blood Cells Is Altered in COVID-19. *Biophys J* (2021) 120(14):2838–47. doi: 10.1016/j.bpj.2021.05.025
50. Fattizzo B. Evans Syndrome and Infections: A Dangerous Cocktail to Manage With Caution. *Blood Transfus* (2021) 19(1):5–8. doi: 10.2450/2021.0351-20
51. Al Aoun S, Motabi I. Cold Agglutinin Disease After COVID-19 Vaccine. *Br J Haematol* (2021). doi: 10.1111/bjh.17674
52. Brito S, Ferreira N, Mateus S, Bernardo M, Pinto B, Lourenço A, et al. A Case of Autoimmune Hemolytic Anemia Following COVID-19 Messenger Ribonucleic Acid Vaccination. *Cureus* (2021) 13(5):e15035. doi: 10.7759/cureus.15035
53. Fattizzo B, Giannotta JA, Cecchi N, Barcellini W. SARS-CoV-2 Vaccination in Patients With Autoimmune Cytopenias: The Experience of a Reference Center. *Am J Hematol* (2021) 96(11):E413–6. doi: 10.1002/ajh.26345
54. Gadi SRV, Brunker PAR, Al-Samkari H, Sykes DB, Saff RR, Lo J, et al. Severe Autoimmune Hemolytic Anemia Following Receipt of SARS-CoV-2 mRNA Vaccine. *Transfusion* (2021) 61(11):3267-71. doi: 10.1111/trf.16672
55. Pérez-Lamas L, Moreno-Jiménez G, Tenorio-Núñez MC, Velázquez-Kennedy K, Jiménez-Chillón C, Astibia-Mahillo B, et al. Hemolytic Crisis Due to Covid- 19 Vaccination in a Woman With Cold Agglutinin Disease. *Am J Hematol* (2021) 96(8):E288–91. doi: 10.1002/ajh.26214
56. El-Sissy C, Saldman A, Zanetta G, Vieira-Martins P, Poulain C, Cauchois R, et al. COVID-19 as a Potential Trigger of Complement-Mediated Atypical HUS. *Blood* (2021) 138(18):1777–82. doi: 10.1182/blood.2021012752
57. Kaufeld JK, Reinhardt M, Schröder C, Bräsen JH, Wiech T, Brylka P, et al. Atypical HUS Triggered by Infection With SARS-Cov2. *Kidney Int Rep* (2021) 6(10):2709–12. doi: 10.1016/j.ekir.2021.07.004
58. Ville S, Le Bot S, Chapelet-Debout A, Blancho G, Fremeaux-Bacchi V, Deltombe C, et al. Atypical HUS Relapse Triggered by COVID-19. *Kidney Int* (2021) 99(1):267–8. doi: 10.1016/j.kint.2020.10.030
59. Simpson HD, Johnson E, Britton J, Braksick S. Alternating Hemiparesis in the Context of Hemolytic Uremic Syndrome and COVID-19 Positivity. *Epilepsy Behav Rep* (2021) 16:100468. doi: 10.1016/j.ebr.2021.100468
60. Barcellini W, Giannotta J, Fattizzo B. Autoimmune Hemolytic Anemia in Adults: Primary Risk Factors and Diagnostic Procedures. *Expert Rev Hematol* (2020) 13(6):585–97. doi: 10.1080/17474086.2020.1754791
61. Montagnani S, Tuccori M, Lombardo G, Testi A, Mantarro S, Ruggiero E, et al. Autoimmune Hemolytic Anemia Following MF59-Adjuvanted Influenza Vaccine Administration: A Report of Two Cases. *Ann Pharmacother* (2011) 45(1):e8. doi: 10.1345/aph.1P480
62. Gani I, Hinnant G, Kapoor R, Savage N. Autoimmune Hemolytic Anemia in a Renal Transplant Patient Following Seasonal Influenza Vaccination. *Case Rep Hematol* (2019) 2019:3537418. doi: 10.1155/2019/3537418
63. Green H, Eliakim-Raz N, Zimra Y, Gafter-Gvili A. Paroxysmal Nocturnal Hemoglobinuria Diagnosed After Influenza Vaccine: Coincidence or Consequence? *Isr Med Assoc J* (2014) 16(2):122–4.
64. Defendi F, Corentin L, Epaulard O, Clavarino G, Vilotitch A, Le MArechal M, et al. Complement Alternative and Mannose-Binding Lectin Pathway Activation Is Associated With COVID- 19 Mortality. *Front Immunol* (2021) 12:742446. doi: 10.3389/fimmu.2021.742446
65. Lipcsey M, Persson B, Eriksson O, Blom AM, Fromell K, Hultström M, et al. The Outcome of Critically Ill COVID-19 Patients Is Linked to Thromboinflammation Dominated by the Kallikrein/Kinin System. *Front Immunol* (2021) 12:627579. doi: 10.3389/fimmu.2021.627579
66. Cugno M, Meroni PL, Gualtierotti R, Griffini S, Grovetti E, Torri A, et al. Complement Activation in Patients With Covid-19: A Novel Therapeutic Target. *J Allergy Clin Immunol* (2020) 146:215–7. doi: 10.1016/j.jaci.2020.05.006
67. Eriksson O, Persson B, Lipcsey M, Ekdahl K, Nilsson B. Mannose-Binding Lectin Is Associated With Thrombosis and Coagulopathy in Critically Ill COVID-19 Patients. *Thromb Haemostasis* (2020) 120(12):1720–4. doi: 10.1055/s-0040-1715835
68. *Eculizumab (Soliris) in Covid-19 Infected Patients (SOLID-C19)*. Available at: https://clinicaltrials.gov/ct2/show/NCT04288713 (Accessed 6th October 2021).
69. *Conestat Alfa in the Prevention of Severe SARS-CoV-2 Infection in Hospitalized Patients With COVID-19*. Available at: https://clinicaltrials.gov/ct2/show/NCT04414631 (Accessed 6th October 2021).
70. *A Study of the C3 Inhibitor AMY-101 in Patients With ARDS Due to COVID-19 (SAVE)*. Available at: https://clinicaltrials.gov/ct2/show/NCT04395456 (Accessed 6th October 2021).

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Immunology

ORIGINAL RESEARCH
published: 02 February 2022
doi: 10.3389/fimmu.2022.835156

Complement Activation *via* the Lectin and Alternative Pathway in Patients With Severe COVID-19

*Janina Niederreiter[1], Christine Eck[1], Tajana Ries[1], Arndt Hartmann[2], Bruno Märkl[3], Maike Büttner-Herold[1], Kerstin Amann[1] and Christoph Daniel[1]**

[1] Department of Nephropathology, University Hospital Erlangen, Friedrich-Alexander-University (FAU) Erlangen-Nürnberg, Erlangen, Germany, [2] Institute of Pathology, University Hospital Erlangen, Friedrich-Alexander-University (FAU) Erlangen-Nürnberg, Erlangen, Germany, [3] General Pathology and Molecular Diagnostics, Medical Faculty Augsburg, University Augsburg, Augsburg, Germany

Complement plays an important role in the direct defense to pathogens, but can also activate immune cells and the release of pro-inflammatory cytokines. However, in critically ill patients with COVID-19 the immune system is inadequately activated leading to severe acute respiratory syndrome (SARS) and acute kidney injury, which is associated with higher mortality. Therefore, we characterized local complement deposition as a sign of activation in both lungs and kidneys from patients with severe COVID-19. Using immunohistochemistry we investigated deposition of complement factors C1q, MASP-2, factor D (CFD), C3c, C3d and C5b-9 as well as myeloperoxidase (MPO) positive neutrophils and SARS-CoV-2 virus particles in lungs and kidneys from 38 patients who died from COVID-19. In addition, tissue damage was analyzed using semi-quantitative scores followed by correlation with complement deposition. Autopsy material from non-COVID patients who died from cardiovascular causes, cerebral hemorrhage and pulmonary embolism served as control (n=8). Lung injury in samples from COVID-19 patients was significantly more pronounced compared to controls with formation of hyaline membranes, thrombi and edema. In addition, in the kidney tubular injury was higher in these patients and correlated with lung injury (r=0.361*). In autopsy samples SARS-CoV-2 spike protein was detected in 22% of the lungs of COVID-19 patients but was lacking in kidneys. Complement activation was significantly stronger in lung samples from patients with COVID-19 *via* the lectin and alternative pathway as indicated by deposition of MASP-2, CFD, C3d and C5b9. Deposits in the lung were predominantly detected along the alveolar septa, the hyaline membranes and in the alveolar lumina. In the kidney, complement was significantly more deposited in patients with COVID-19 in peritubular capillaries and tubular basement membranes. Renal COVID-19-induced complement activation occurred *via* the lectin pathway, while activation of the alternative pathway was similar in both groups. Furthermore, MPO-positive neutrophils were found in significantly higher numbers in lungs and kidneys of COVID-19 patients and correlated with local MASP-2 deposition. In conclusion, in patients who died from SARS-CoV-2 infection complement was activated in both lungs and kidneys indicating that complement might be involved in systemic worsening of the inflammatory response.

OPEN ACCESS

Edited by:
Zoltán Prohászka, Semmelweis University, Hungary

Reviewed by:
Wilhelm Johannes Schwaeble, University of Leicester, United Kingdom
Heinz Regele, Medical University of Vienna, Austria

***Correspondence:**
Christoph Daniel
Christoph.Daniel@uk-erlangen.de

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *14 December 2021*
Accepted: *11 January 2022*
Published: *02 February 2022*

Citation:
Niederreiter J, Eck C, Ries T, Hartmann A, Märkl B, Büttner-Herold M, Amann K and Daniel C (2022) Complement Activation via the Lectin and Alternative Pathway in Patients With Severe COVID-19. Front. Immunol. 13:835156. doi: 10.3389/fimmu.2022.835156

Complement inhibition might thus be a promising treatment option to prevent deregulated activation and subsequent collateral tissue injury in COVID-19.

Keywords: complement deposition, COVID-19, kidney, lung, autopsy, lectin pathway

INTRODUCTION

Since December 2019 the new coronavirus SARS-CoV-2 has spread around the world creating a worldwide pandemic. Despite vaccination campaigns in progress, we continue to see severe cases of COVID-19 with patients requiring intensive care treatment with frequent fatal outcome. The lungs are the primary manifestation of COVID-19 with patients experiencing fever, cough and dyspnea and in severe cases acute respiratory distress syndrome (ARDS) (1, 2). Histopathological alterations include diffuse alveolar damage, necrosis of pneumocytes, inflammatory infiltrate and formation of hyaline membranes (3). Due to the ability of SARS-CoV-2 to bind to the ACE2 receptor expressed by a large range of cells multiple other organs have been reported to be affected by COVID-19 (4, 5). Particularly, the kidney is involved in many patients with a considerable proportion of hospitalized patients experiencing primarily acute kidney injury (AKI). In addition, the occurrence of AKI in severe COVID-19 is associated with a higher mortality (6, 7). Some studies suggest direct viral infection of glomerular and tubular cells through the ACE2-receptor possibly causing acute proximal tubular injury and detachment of podocytes (7–10). However, other findings implicate indirect pathomechanisms being responsible for kidney injury including hemodynamic effects through sepsis-related factors, microangiopathy, cytokine storm and excessive immune response causing inflammation and triggering tissue damage (11–13). One pathway potentially involved in the SARS-CoV-2 driven inflammatory cytokine overproduction is activation of the complement system (12). This system, which belongs to the innate immune system, acts as a crucial component in the defense against infection by opsonizing pathogens or damaged cells, attracting and activating leukocytes or directly lysing bacteria or cells through the membrane attack complex. Initiation of the complement cascade can be done by three different pathways, the classical pathway, the lectin pathway, and the alternative pathway (14, 15). The classical pathway is activated by immune complexes and many other self and non-self molecules binding to C1q, leading to a conformational change, and activating serine proteases C1s and C1r (16). To activate the lectin pathway plasma-circulating lectins (collectins like mannan-binding lectin and ficolins) recognize carbohydrate patterns on the surface of microorganisms, called pathogen-associated molecular patterns (PAMPs). By binding dimers of mannan-binding lectin-associated serine protease 2 (MASP-2) these pattern-recognition molecules (PRM) form a complex which leads to the cleavage of C4 and C2 (17, 18). In contrast, the alternative pathway is constantly active at a low level and is initiated by spontaneous hydrolysis of C3. Hydrolysed C3 binds to factor B (CFB) which acts as a substrate to serine protease factor D (CFD), resulting in formation of a C3 convertase (15, 19). In the end, all 3 complement pathways can lead to C3 convertase activation, followed by activation of C5 convertase triggering the formation of the membrane attack complex (MAC) by C5b-9. MAC consists of different complement factors, binds to the cells and causes cell lysis by producing pores in the plasma membrane (15). Cleavage products C3a and C5a are potent inflammatory peptides capable of inducing the release of cytokines, thus contributing to the development of a cytokine storm, and recruitment of inflammatory cells (12, 15, 20). Meta-analysis of studies investigating systemic complement activation using serum samples of patients with COVID-19 demonstrated that lower C3 and C4 serum levels were significantly associated with higher COVID-19 severity and mortality (21). However, evidence of COVID-19-induced complement activation in organs has only been provided in a few studies using small sample numbers to date (22–25). In an earlier study, we could demonstrate complement activation in renal samples from patients with COVID-19 (24). However, this study was limited by a small sample size, the wide range in severity of COVID-19 disease, and the inclusion of patients with various renal diseases. In this study, we extended the evaluation of complement activation in the kidney to the lung as the organ of primary injury, included more patients and focused on severe COVID-19 cases by using autopsy material from patients who died of COVID-19. After exclusion of samples with severe tissue autolysis, 38 patients have been included for analysis of complement deposition in various compartments in lungs and kidneys. A group of 8 postmortem organ samples was selected as control.

MATERIALS AND METHODS

Human Renal Tissue Specimens

To evaluate the relevance of complement in mediating severe lung and renal pathology during COVID-19 infection tissue was collected in a standardized manner during autopsy from patients who died due to SARS-CoV-2 infection (26, 27). The diagnosis of COVID-19 was confirmed by RT-PCR analysis. We included two cohorts, one from Pathology Institutes University Medical Center Augsburg (n=36) and a second from University Erlangen-Nürnberg (n=15). In a first step, we pre-evaluated the quality of all available autopsy material and excluded all samples with severe autolysis. Finally, we included 25 samples from the Augsburg collective and 13 samples from the Erlangen cohort. Autopsy material of patients who died from cerebral hemorrhage (n=2), pulmonary embolism (n=2) and cardiovascular disease (n=4) collected from the archive of the Department of Pathology at FAU served as controls. Analysis was approved by the local Ethics committees (Ethics committee of the Friedrich-Alexander University (FAU) Erlangen-Nürnberg, reference number 4415 and internal review board of the Medical Center-Augsburg (BKF No. 2020–18) and the ethics

committee of the University of Munich (Project number 20–426, COVID-19 registry of the University Hospital Augsburg). Patients' characteristics are described in **Table 1** and controls in **Table 2**.

Immunohistochemistry

For immunohistochemical stainings formalin-fixed paraffin-embedded (FFPE) lung and kidney samples were cut into 2 µm sections, deparaffinized and rehydrated. Antigen retrieval was performed using pronase E (Sigma Aldrich, Taufkirchen, Germany) digestion for 30-45 minutes at 37°C (C1q, C3c, C5b-9) or cooking in target retrieval solution pH 6 (DAKO Deutschland GmbH, Hamburg, Germany) for 2.5 minutes (C3d, C4d, CFD, MASP-2, COVID-19 spike protein, MPO). Endogenous peroxidase was blocked with 3% H_2O_2 and unspecific antigens with Avidin-Biotin (Vector laboratories, Burlingame, CA, USA) and normal goat or horse serum in blotto (1:5). The following primary antibodies were diluted in 50 mM Tris pH 7.4 and incubated over-night at 4°C: C1q, a rabbit polyclonal antibody against human C1q (A0136; DAKO Deutschland GmbH); C3c, a rabbit polyclonal antibody against human C3c (A0062; DAKO Deutschland GmbH); C3d, a rabbit monoclonal antibody against human C3d (ab136916; Abcam, Cambridge, UK); C4d, a rabbit polyclonal antibody against human C4d (RBK039-05; Zytomed Systems GmbH, Berlin, Germany); C5b-9, a mouse monoclonal antibody against human C5b-9 (M0777; DAKO Deutschland GmbH); complement factor D (CFD), a rabbit polyclonal antibody

TABLE 1 | Clinical findings in patients with COVID-19 infection.

Pt	Age	Sex	Duration of illness [days]	Comorbidities							Acute kidney injury
				Cardiovascular	Hypertension	Cancer	Diabetes	Obesity	Chronic respiratory disease	Chronic kidney disease	
1	64	M	25	n	n	y	n	n	n	n	y
2	83	M	8	y	n	n	n	n	n	n	n
3	85	M	6	y	y	n	n	n	n	n	n
4	90	F	1	y	y	n	n	n	n	n	y
5	85	M	8	n	y	y	n	n	n	y	n
6	69	M	5	y	y	n	y	y	n	y	y
7	83	M	10	y	n	n	y	y	n	n	y
8	60	M	30	n	n	n	n	n	n	n	y
9	61	F	22	n	y	n	n	y	n	n	y
10	89	M	36	y	y	n	n	n	n	y	n
11	64	M	9	y	y	n	y	y	y	n	y
12	83	M	18	y	y	n	n	n	n	n	y
13	87	M	6	y	y	n	n	n	y	n	n
14	51	F	6	y	n	n	n	y	n	y	n
15	66	M	7	y	y	n	n	n	n	y	y
16	65	M	30	n	n	y	n	n	n	n	y
17	62	M	44	n	n	n	n	n	n	n	y
18	70	M	6	y	n	n	y	n	n	y	y
19	85	M	2	y	y	n	n	n	n	y	y
20	79	F	13	n	n	n	n	n	n	n	n
21	77	M	18	y	y	n	y	n	y	n	y
22	74	M	3	y	y	n	y	y	n	y	n
23	90	F	14	y	y	y	n	n	y	n	n
24	92	F	16	y	n	y	n	n	y	n	n
25	73	M	64	y	y	y	n	n	y	n	y
26	76	F	8	n	y	n	n	n	n	y	y
27	92	F	17	n	n	n	n	n	n	y	y
28	92	F	5	y	n	n	n	n	n	n	n
29	83	F	unknown	y	y	y	n	n	n	n	y
30	59	M	60	y	n	n	n	n	n	n	n
31	89	F	unknown	y	y	n	y	n	n	n	y
32	82	M	4	y	y	n	n	n	n	n	unknown
33	82	F	23	y	y	n	n	n	y	n	n
34	74	M	38	y	y	y	y	n	n	n	n
35	70	M	6	y	n	y	n	n	n	n	y
36	77	M	8	y	y	n	n	n	n	n	unknown
37	74	M	3	y	n	y	n	n	y	n	y
38	58	M	39	y	y	n	n	n	n	n	y
	76.2 ± 11.3	**26:12**	**17.2 ± 16.1**	**29/38**	**23/38**	**10/38**	**8/38**	**6/38**	**8/38**	**10/38**	**22/38**

Pt, patient; M, male; F, female; blue shading = patients from the Augsburg collective; no shading = patients from the Erlangen collective.
Bold values represent mean+/-SD or ratios.

TABLE 2 | Cause of death in control patients.

Pt	Age	Sex	Cause of death
1	56	M	Congestive heart failure with cerebral hemorrhage
2	71	F	Hemodynamic shock with acute bowel ischemia
3	49	M	Heart failure with gastric cancer
4	61	F	Pulmonary embolism
5	59	M	Cerebral compression with cerebral hemorrhage
6	48	F	Cardiac arrhythmia
7	57	F	Acute myocardial infarction with aortic valve replacement
8	75	F	Pulmonary embolism
	59.5 ± 9.5	**3:5**	

Pt, patient; M, male; F, female.
Bold values represent mean+/-SD or ratios.

against rat cross-reactive to human CFD (PA5-79035; Thermo Fisher Scientific, Carlsbad, CA, USA); MASP-2, a rabbit polyclonal antibody against human Mannan-binding lectin serine peptidase 2 [HPA029313; Sigma Aldrich; specificity of the antibody was supported by positive staining in liver (**Supplementary Figure 1**)]; MPO a rabbit polyclonal antibody against human myeloperoxidase (ab9535; Abcam, Cambridge, UK), and SARS-CoV-2 spike protein, a mouse monoclonal antibody against SARS-CoV-2 spike protein (clone 224.2, kindly provided by M.-H. Jäck, Department of Molecular Immunology, FAU Erlangen-Nürnberg). After washing with 50 mM Tris pH 7.4, sections were incubated with biotinylated secondary goat anti-rabbit IgG (BA-1000; Vector laboratories) or horse anti-mouse IgG (BA-2001, Vector laboratories). Detection of bound antibodies was conducted using ABC-Kit and DAB-Impact as a substrate (both from Vector laboratories), while nuclei were counter stained with hemalaun. For negative controls primary antibody was substituted by antibody dilution buffer (50 mM Tris pH 7.4).

Semi-Quantitative Evaluation of Complement in Lung and Renal Samples

Immunohistochemical staining in pulmonary and renal tissue was graded in different vascular and non-vascular compartments of the lung (i.e. alveolar septa, alveolar lumen, alveolar infiltrate, vessels and bronchioli) and the kidney (i.e. in glomeruli, peritubular capillaries, arteries, tubular basement membrane) using a semi-quantitative score (score 0-2 and 0-3, respectively), which described the distribution and intensity of the staining signal. For MPO staining, score 0 was defined as no positive neutrophilic in the respective compartment, score 1 was defined as single or few scattered positive neutrophil cells in a compartment and score 2 was defined as the clusters of positive neutrophils. For the components of the complement system, score 0 was chosen for samples showing no specific reactivity in the respective compartment, score 1 represented samples showing low reactivity in single small patches. Score 2 was defined as reactivity in larger patches scattered throughout the sample and score 3 was defined as reactivity in large patches making up the majority of the sample. For compartments showing pronounced staining reactivity [i.e. peritubular capillaries and tubular basement membrane (TBM)] multiple fields of view were graded and mean values representing the distribution of reactivity in a sample were formed. In order to display the total staining of a sample, sum scores that included all individual scores for each compartment were generated.

Injury Scores

An additional qualitative score was used to assess lung injury, which included common pathological changes in COVID-19 such as edema, thrombi, inflammation, hyaline membranes and necrosis. The occurrence of each of these changes was assigned a point score, resulting in a maximum score of 5. Renal damage was evaluated using semi-quantitative scores to assess glomerulosclerosis (GSI, including glomerular matrix accumulation) and tubulointerstitial injury (TSI, including tubular interstitial fibrosis and inflammation) as described previously (28).

Statistical Analyses

After testing for normal distribution of values using Kolmogorov-Smirnov test, data were analyzed using Mann-Whitney test. Results of the evaluated immunohistochemistry were correlated with clinical data using Spearman test. In all tests $p<0.05$ was accepted as statistically significant. Statistical analyses were performed using GraphPad Prism 8 for Windows software (version 8.3, GraphPad software Inc., San Diego, CA, USA) and SPSS Statistics 28 (IBM, Armonk, NY, USA).

RESULTS

Patients With Severe COVID-19 Showed Lung and Kidney Damage

In total 38 patients who died of COVID-19 with a proportion of 26 males and 12 females with a mean age of 76.2± 11.3 years were included in the study (**Table 1**). The average time between first diagnosis and death was 17.2 ± 16.1 days. More than half of the Covid-19 patients had cardiovascular changes and hypertension as comorbidities (**Table 1**). Further comorbidities were cancer (10/38), diabetes (8/38), obesity (6/38), chronic respiratory disease (8/38) and chronic kidney disease (10/38) (**Table 1**). In 22 of 38 patients acute kidney injury was observed during COVID-19 disease (**Table 1**). In the control group we included eight patients, three males and five females with an average age of 59.5 ± 9.5 years who died of cardiac diseases including heart failure, cardiac arrhythmia or myocardial infarction or pulmonary embolism or hemodynamic shock with acute bowel ischemia (**Table 2**). Lungs from control patients showed mild changes with little alveolar infiltrate (**Figure 1A**), while changes in lungs of patients with COVID-19 showed signs of different stages of diffuse alveolar damage (DAD) with hyaline membranes, thickening af the alveolar septa and interstitial fibroblastic proliferation (**Figure 1B**) as well as mononuclear inflammatory infiltrates (**Figure 1C**). Lung injury in patients with COVID-19 was significantly stronger compared to controls, as assessed by qualitative scoring (**Figure 1D**). Most kidney sections of controls showed at least mild changes in glomeruli and within the tubulointerstitium (**Figure 1E**). In contrast,

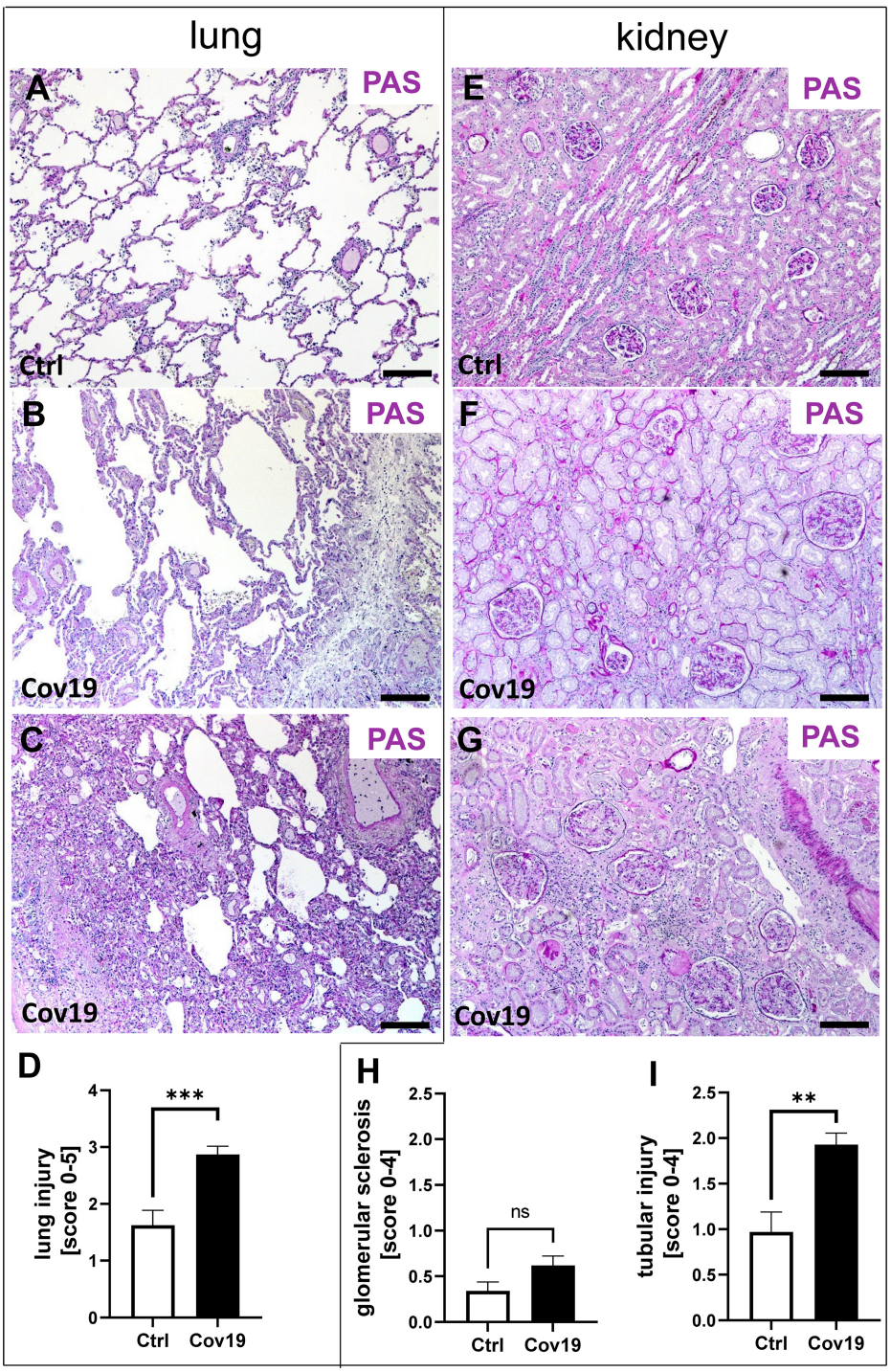


FIGURE 1 | Pulmonary and renal injury in patients with COVID-19. Representative pictures of PAS-stained lung and kidney specimens from a control patient **(A, E)** and patients with COVID-19 **(B, C, F, G)**. Lungs of a control patient showing mild changes with little alveolar accumulation of inflammatory cells **(A)**, while changes in lung samples from patients with COVID-19 ranged from mild pulmonary injury including accumulation of alveolar inflammatory cells and interstitial edema **(B)** to severe pulmonary injury with collapsed alveoli, dense alveolar infiltrate, and congested capillaries **(C)**. The extent of lung injury in control patients (Ctrl, n = 8) versus COVID-19 patients (Cov19, n = 38) was analyzed using PAS-stained lung samples and semi-quantitative scoring **(D)**. While kidneys in controls showed only minor pathological changes **(E)**, kidney samples from COVID-19 patients exhibited mild **(F)** to more severe **(G)** kidney injury. Renal injury was determined using semi-quantitative scoring of glomerular sclerosis **(H)** and acute and chronic tubular injury **(I)**. ns, non-significant; Scale bar = 200μm; $^{**}p < 0.01$; $^{***}p < 0.001$.

changes of kidney tissue from COVID-19 patients ranged mild (**Figure 1F**) to severe (**Figure 1G**) including tubular injury, tubular necrosis, mild glomerular sclerosis, interstitial infiltrates, and interstitial fibrosis. However, while glomerular injury was comparably low in both groups (**Figure 1H**), tubular injury was significantly higher in samples from COVID-19 patients (**Figure 1I**). Interestingly, kidney injury positively correlated with lung injury score (r=0.364*, p=0.013). SARS-CoV-2 virus could be detected in 22% of all investigated lungs and none of the kidney samples from COVID-19 patients (**Figures 2A, B**).

Deposition of Classical Complement Factor C1q Was Weak in Lungs and Kidneys of Patients With COVID-19

First, we investigated whether C1q, which is involved in activation of the classical pathway, was detectable in the lungs and kidneys of patients with COVID-19. C1q was barely detectable in the lungs and kidneys of controls (**Figure 3A**), but was also absent in both lungs (**Figures 3B, C**) and kidneys (**Figures 3G–I**) of COVID-19 patients which was reflected by sum scores below 1 (**Figures 3C, I**). Similarly, the detection of C3c, a stable cleavage product of C3, was detected only slightly more intensely than C1q in lung (**Figures 3D–F**) and kidney (**Figures 3J–L**). Here too, compared to the control group, the deposition of C3c was similar in COVID-19 patients in both lung (**Figure 3D**) and kidney (**Figure 3J**).

Lectin Pathway Activator Mannan-Binding Lectin-Associated Serine Protease 2 (MASP-2) Was Markedly Deposited in Lungs and Kidneys From Patients With COVID-19

In contrast, MASP-2 as an activator of the lectin pathway was significantly more abundant in the lungs of patients with COVID-19 compared to controls (**Figures 4A–C**). Compartment-specific analysis showed that MASP-2 was detectable in the bronchioli, the larger vessels, and within alveolar infiltrates in controls and COVID-19 cases in comparable quantities (**Figure 4D**). In contrast, in the alveolar septa (AS) and in the lumina of the alveoli (A lumen) it was almost exclusively present in the samples from patients with COVID-19 (**Figures 4D–J**). In particular, hyaline membranes in the lungs of patients with COVID-19 showed strong MASP-2 deposition (**Figure 4F**). We detected smaller amounts of MASP-2 in the kidneys compared to the lungs (**Figures 4K, L**). However, the MASP-2 sum score for the kidneys of patients with COVID-19 was on average more than 3-fold higher (**Figure 4M**). MASP-2 deposition in the kidneys of COVID-19 patients was seen in peritubular capillaries (PTC) and especially in tubular basement membranes (TBM), with almost 10 times higher levels than in the control group (**Figures 4N–T**). In contrast, we could hardly find MASP-2 in the renal arteries (Art) and glomeruli (Glom) (**Figure 4N**).

Alternative Pathway Activator Factor D (CFD) Was Markedly Increased in Lungs From Patients With COVID-19, But Not in Kidneys

To investigate additional complement activation *via* the alternative pathway, we analyzed the activator factor D (CFD). Interestingly, this factor was also significantly more abundant in the lungs of patients with COVID-19 compared to controls (**Figures 5A–C**). Compartment-specific analysis showed that CFD was detectable in alveolar infiltrates, bronchioles and the larger vessels in controls and COVID-19 cases in comparable quantities (**Figure 5D**). Similar to the staining pattern of MASP-2, CFD was significantly more deposited in the area of the alveolar septa (**Figure 5D**), especially the hyaline membranes (**Figures 5E–G**), and in the lumina of the alveoli (**Figures 5H–J**). In contrast, the sum score for CFD in the kidneys of COVID-19 patients was comparable to that of controls (**Figures 5K–M**). Compartment-specific analysis for CFD showed slightly

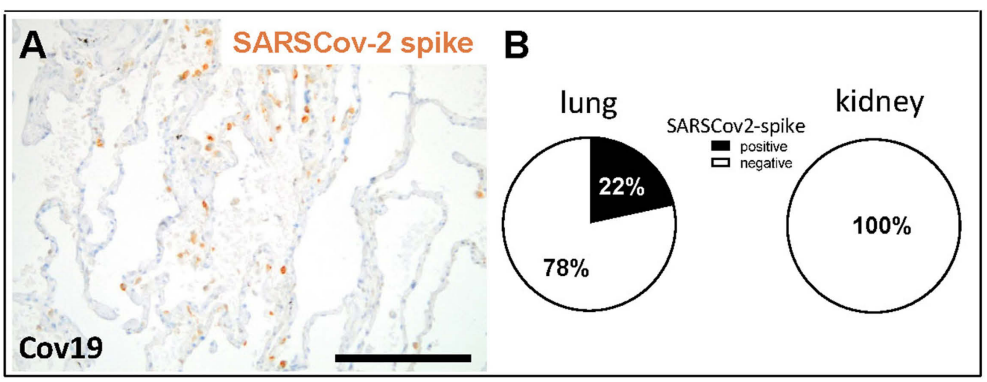


FIGURE 2 | Detection of SARS-CoV-2 spike protein in lung and kidney samples. SARS-CoV-2 spike protein was detected in lung samples from patients with COVID-19, as shown in a representative micrograph of immunohistochemistry (**A**, brown staining). Lung and kidney samples from COVID-19 patients were analyzed for positive staining for SARSCov-2 spike protein and the percentage of positive samples are shown **(B)**. Scale bar = 200µm.

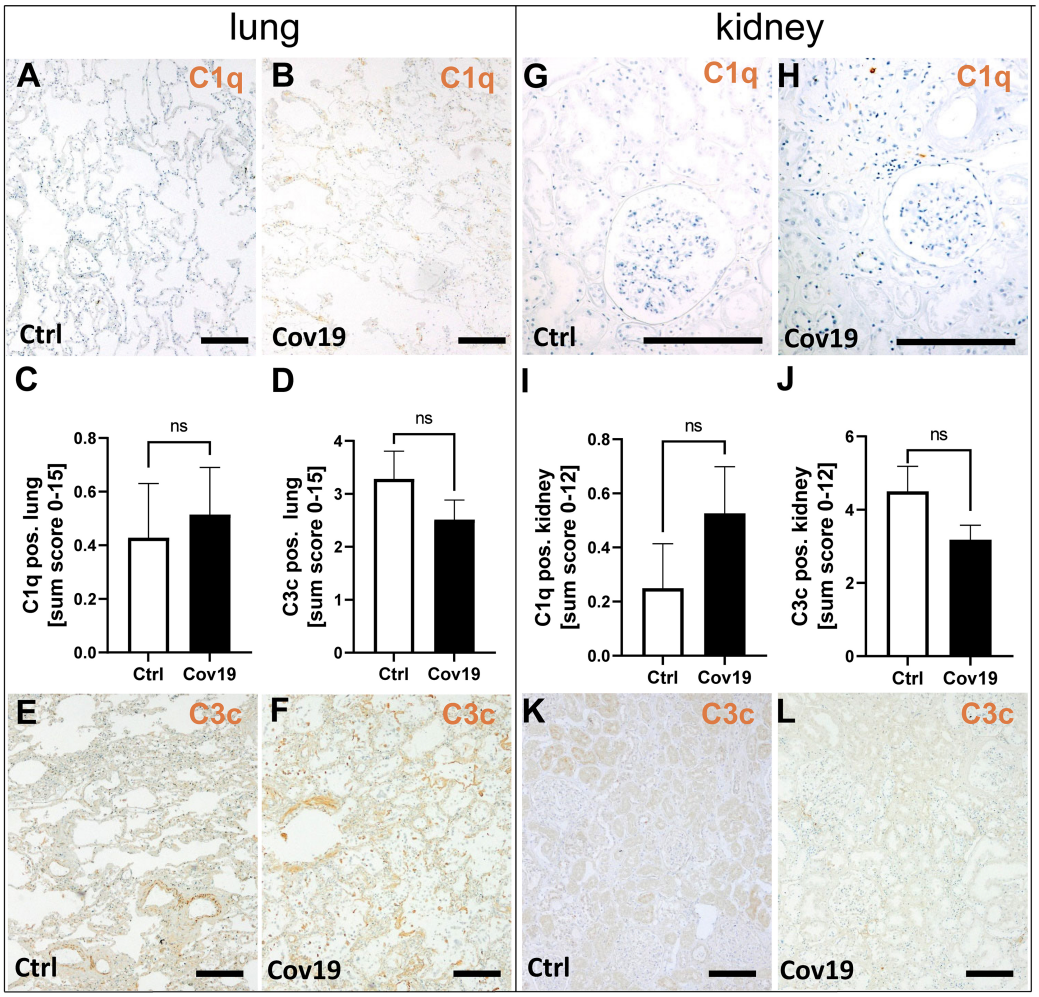


FIGURE 3 | Detection of the classical pathway component C1q and the stable complement cleavage product C3c in samples from patients with COVID-19. Representative micrographs of immunohistochemical staining for C1q in lung **(A, B)** and kidney **(G, H)** samples of a control patients **(A, G)** and a with COVID-19 patient **(B, H)** are shown. Sum scores of semi-quantitative evaluation of C1q **(C)** and C3c **(D)** deposition in lung demonstrate weak staining in both investigated groups. Representative pictures of immunohistochemical staining for C3c in lung **(E, F)** and kidney **(K, L)** samples of control patients **(E, K)** and patients with COVID-19 **(F, L)** are shown. In the kidney sum scores for semi-quantitative evaluation for C1q **(I)** and C3c **(J)** were low. ns, non-significant. Scale bar = 200 µm.

increased deposition in peritubular capillaries, but did not reach the significance level (**Figures 5N–Q**) and CFD-positive TBM were comparable in both groups (**Figures 5R–T**).

C3d Deposition Occurs in Both Lungs and Kidneys of Patients With COVID-19

Next, we stained lung and kidney samples for C3d, the final degradation product of C3. In the lungs, as in MASP-2, significantly higher C3d amounts were observed looking at the sum scores in samples from patients with COVID-19 (**Figures 6A–C**). Striking, again, was the strong staining of the hyaline membranes for C3d which lie on top of the pneumocytes in the lungs of these patients (**Figure 6F**), which is absent in the controls (**Figures 6E, G**). In addition, C3d was found to a lesser extent in alveolar infiltrates and bronchioli (**Figure 6D**). The mean C3d deposition was more than doubled in the alveolae of COVID-19 patients, but did not reach the significance level compared with the control group (**Figures 6H–J**). Larger vessels also showed deposition of C3d, but this was comparable in both groups (**Figure 6D**). In the autopsy kidneys of both groups, C3d was well detectable and was significantly more prominent in the COVID-19 group compared with controls using the sum score, although the scores were only one third higher on average (**Figures 6K–M**). In the compartment-specific analysis, C3d deposition in the peritubular capillaries (**Figures 6N–Q**; PTC) and tubular basement membrane (**Figures 6N, R–T**; TBM) was significantly increased in the COVID-19 group, reaching levels approximately 3 times higher than the controls. In contrast, there was little difference in C3d deposition in glomeruli and arterial vessels (**Figure 6N**).

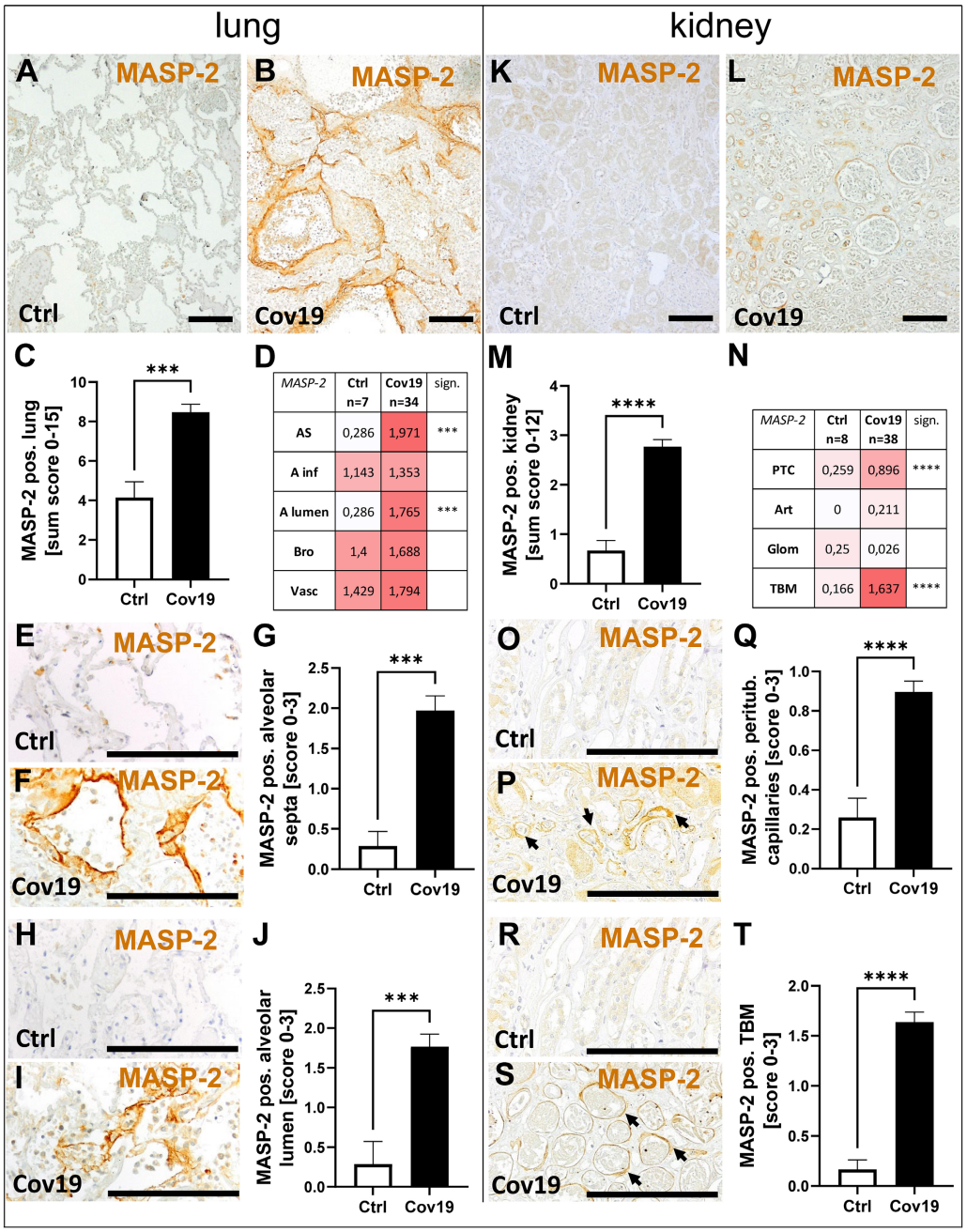


FIGURE 4 | Detection of the lectin pathway component MASP-2 in lung and kidney samples from patients with COVID-19. Overview of immunohistochemical staining for mannose-binding protein-associated serine protease 2 (MASP-2) in lung samples of a control patient **(A)** and a patient with COVID-19 **(B)** was shown. Results of semi-quantitative scoring in multiple compartments for MASP-2 deposition in the lung were summarized in a sum score **(C)** and overview of the mean values of compartment-specific analyses is given in a heat map **(D)**. Representative MASP-2 stainings in alveolar septa of a control patient **(E)** and a COVID-19 patient **(F)** and comparative analyses are shown **(G)**. MASP-2 staining in alveolar lumina in a control patient **(H)** and a patient with COVID-19 **(I)** and statistical comparison of both groups **(J)**. Overview of MASP-2 staining in kidney samples of a control patient **(K)** and a patient with COVID-19 **(L)**. Results of semi-quantitative scoring in multiple compartments for MASP-2 deposition in the kidney are summarized in a sum score **(M)** and overview of the mean values of compartment-specific analyses is given in a heat map **(N)**. Representative MASP-2 staining in peritubular capillaries of a control patient **(O)** and a COVID-19 patient (**P**, arrows) and a comparative analyses **(Q)**. MASP-2 staining of tubular basement membrane in a control patient **(R)** and a patient with COVID-19 (**S**, arrows) and comparison of both groups **(T)**. AS, alveolar septa; A inf, accumulation of alveolar inflammatory cells; A lumen, alveolar lumen; Bro, bronchioles; Vasc, vascular; sign., significance; PTC, peritubular capillaries; Art, arterial; Glom, glomerular; TBM, tubular basement membrane; BM, basement membrane. Scale bar = 200μm; ***$p < 0.001$; ****$p < 0.0001$.

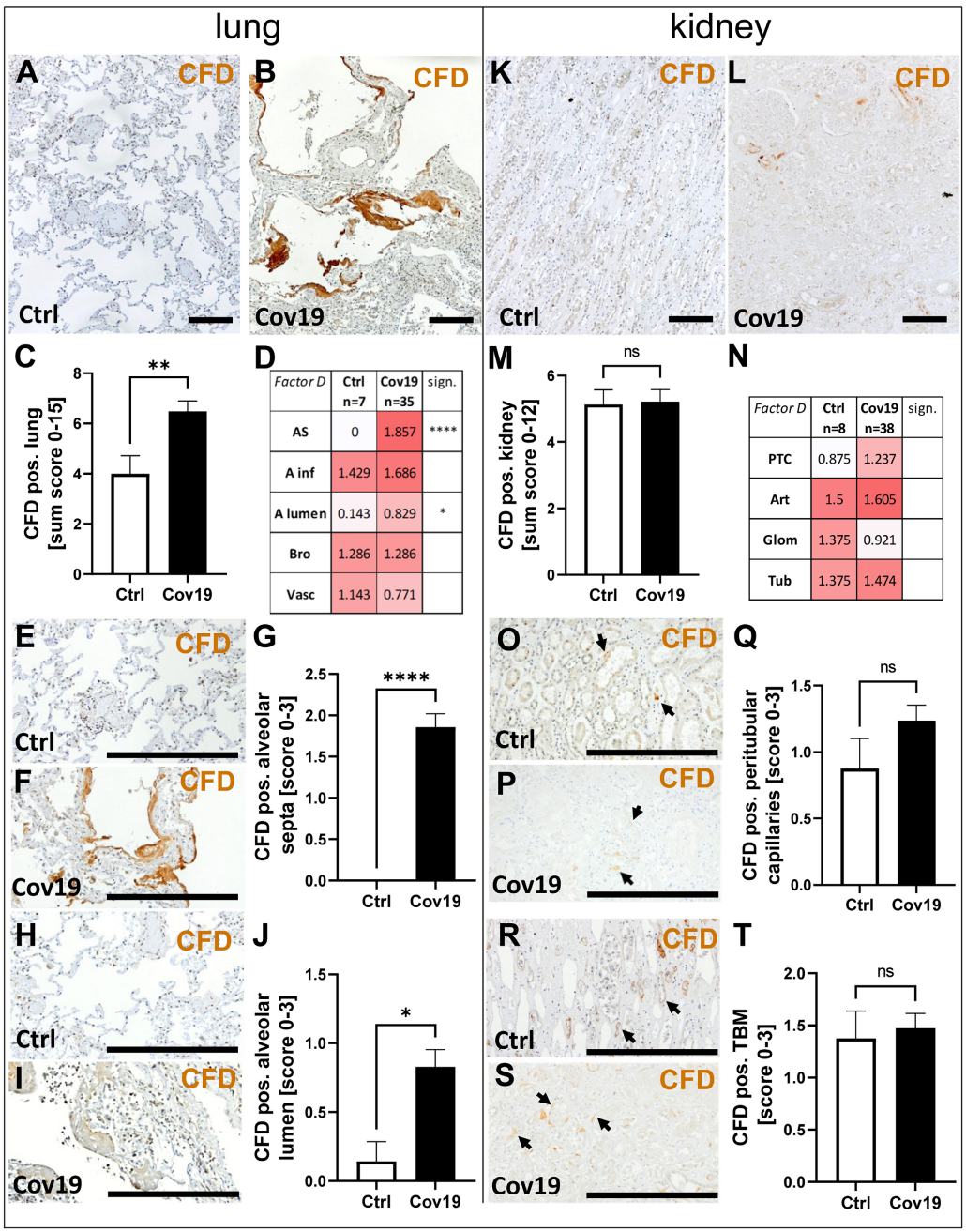


FIGURE 5 | Detection of the alternative pathway component CFD in lung and kidney samples from patients with COVID-19. Overview of immunohistochemical staining for complement factor D (CFD) in lung samples of a control patient **(A)** and a patient with COVID-19 **(B)** was shown. Results of semi-quantitative scoring in multiple compartments for CFD deposition in the lung were summarized in a sum score **(C)** and overview of the mean values of compartment-specific analyses is given in a heat map **(D)**. Representative CFD stainings in alveolar septa of a control patient **(E)** and a COVID-19 patient **(F)** and comparative analyses are shown **(G)**. CFD staining in alveolar lumina in a control patient **(H)** and a patient with COVID-19 **(I)** and statistical comparison of both groups **(J)**. Overview of CFD staining in kidney samples of a control patient **(K)** and a patient with COVID-19 **(L)**. Results of semi-quantitative scoring in multiple compartments for CFD deposition in the kidney are summarized in a sum score **(M)** and overview of the mean values of compartment-specific analyses is given in a heat map **(N)**. Representative CFD staining in peritubular capillaries of a control patient **(O)** and a COVID-19 patient (**P**, arrows) and a comparative analyses **(Q)**. CFD staining of tubular basement membrane in a control patient **(R)** and a patient with COVID-19 (**S**, arrows) and comparison of both groups **(T)**. AS, alveolar septa; A inf, accumulation of alveolar inflammatory cells; A lumen, alveolar lumen; Bro, bronchioles; Vasc, vascular; sign., significance; ns, non-significant; PTC, peritubular capillaries; Art, arterial; Glom, glomerular; TBM, tubular basement membrane; BM, basement membrane. Scale bar = 200μm; *p < 0.05; **p < 0.01; ****p < 0.0001.

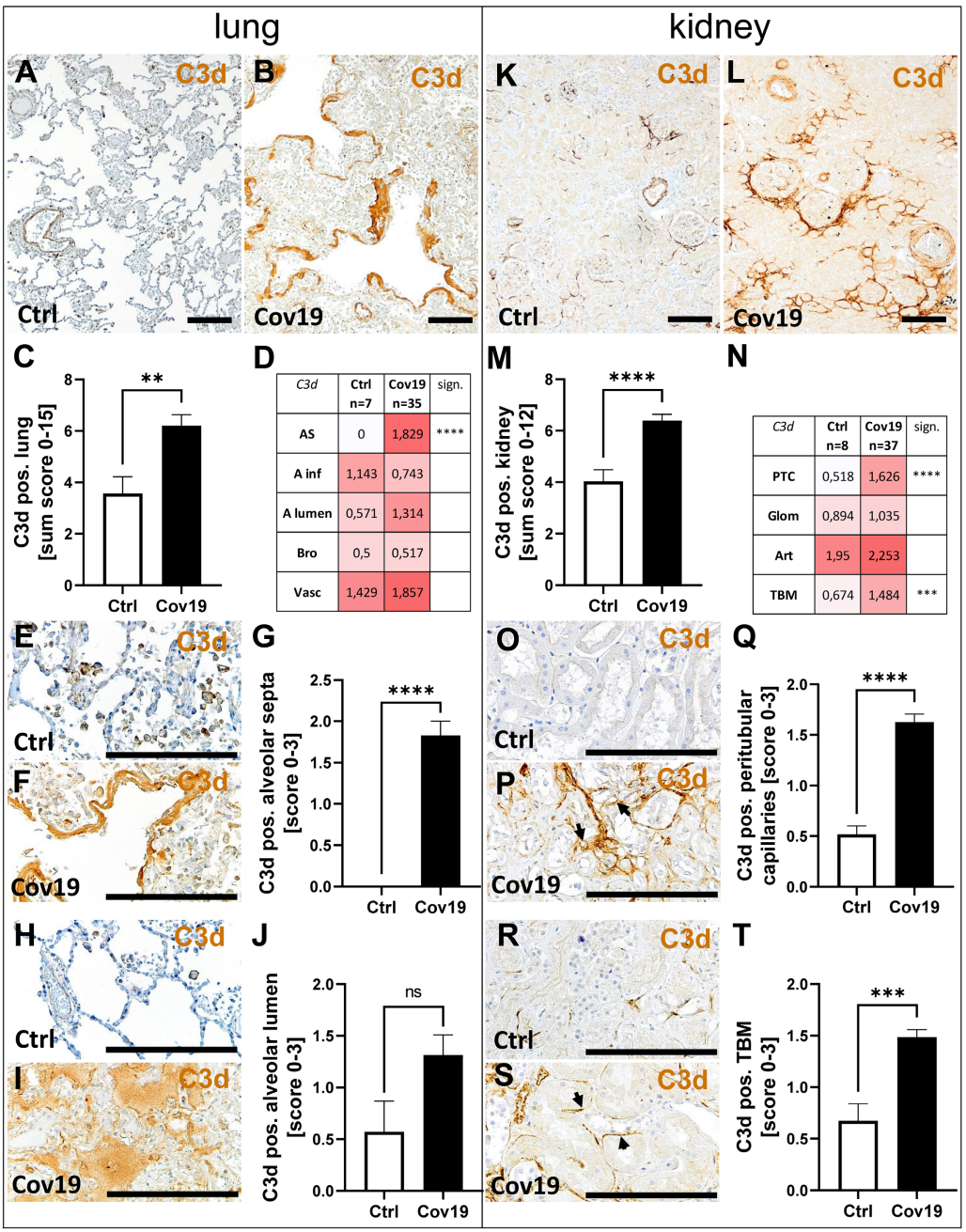


FIGURE 6 | Detection of C3d in lung and kidney samples from patients with COVID-19. Overview of immunohistochemical staining for C3d in lungs of a control patient **(A)** and a patient with COVID-19 **(B)**. Results of semi-quantitative scoring in multiple compartments for C3d deposition in the lung summarized in a sum score **(C)** and overview of the mean values of compartment-specific analyses given in a heat map **(D)**. Representative C3d staining in alveolar septa of a control patient **(E)** and hyaline membranes in a COVID-19 patient **(F)** and statistical comparison of both groups **(G)**. C3d staining in alveolar lumina in a control patient **(H)** and a patient with COVID-19 **(I)** statistical comparison of both groups **(J)**. Overview of C3d staining in kidney biopsies of a control patient **(K)** and a patient with COVID-19 **(L)**. Results of semi-quantitative scoring in multiple compartments for C3d deposition in the kidney summarized in a sum score **(M)** and overview of the mean values of compartment-specific analyses given in a heat map **(N)**. Representative C3d staining in peritubular capillaries of a control patient **(O)** and a COVID-19 patient (**P**, arrows) and statistical comparison of both groups **(Q)**. C3d staining of tubular basement membrane in a control patient **(R)** and a patient with COVID-19 (**S**, arrows) and statistical comparison of both groups **(T)**. AS, alveolar septa; A inf, alveolar infiltrate; A lumen, alveolar lumen; Bro, bronchioles; Vasc, vascular; sign., significance; ns, non-significant; PTC, peritubular capillaries; Art, arterial; Glom, glomerular; TBM, tubular basement membrane; BM, basement membrane. Scale bar = 200μm; **p < 0.01; ***p < 0.001; ****p < 0.0001.

The Correlation of MASP-2 and CFD With C3d and C5b-9 Deposition Suggests Complement Activation *via* the Lectin Pathway in Lungs and Kidneys and Additionally *via* the Alternative Pathway in Lungs of COVID-19 Patients

To investigate the terminal complement pathway, the deposition of C5b-9 in lung and kidney was analyzed. The sum score for this complement factor, however, was not significantly different between the samples of patients with COVID-19 and the control group (**Figure 7C**). In parallel to the localization of the other complement factors C3d and MASP-2, we detected C5b-9 in the alveolar septa significantly more frequently in the COVID-19 group (**Figures 7D–G**). Surprisingly, however, C5b-9 was found more frequently in the control group in the area of the alveolar inflammatory cells (**Figure 7D**) when compared to COVID-19 patients. In the pulmonary vessel walls, C5b-9 was equally frequently detectable in both groups (**Figures 7A, B, D**). In contrast, C5b-9 was barely detectable in the lumina of the alveoli and completely absent in the bronchioli (**Figure 7D**). Similar to the lungs, no significant difference could be found in the overview pictures (**Figure 7H, I**) between the study groups and with regard to the sum score of C5b-9 in the kidneys (**Figure 7J**). Nevertheless, increased deposition in the peritubular capillaries (PTC) and tubular basement membranes (TBM) was recorded in the samples of COVID-19 patients, similar to MASP-2 and C3d (**Figures 7K–Q**). Since immunohistochemical staining of complement factors requires different antigen retrieval techniques, double staining for co-localization studies was not successful. However, correlation analyses for MASP-2 and CFD with C3d and C5b-9 as well as analysis of the same areas confirm that these four complement factors are deposited in the same way in corresponding compartments in lungs (**Figure 8**), indicating complement activation *via* the lectin and alternative pathways. Co-localization of MASP-2, C3d and C5b-9 in the lung was confirmed by staining of serial sections (**Figure 8**, **Supplementary Figure 2**). However, in kidneys only MASP-2 correlated with C3d and C5b-9, indicating that COVID-19 mediated complement activation is here restricted to the lectin pathway (**Figure 8**).

Myeloperoxidase (MPO) Positive Neutrophils Were Increased in Lungs and Kidneys From Patients With COVID-19

Since neutrophils express the C5a receptor 1 on their surface in high density, we evaluated the occurrence of myeloperoxidase (MPO) positive neutrophils as potential C5a-stimulated inflammatory cells. MPO-positive neutrophils were found in large numbers in both groups (**Figures 9A, B**), but were twice as frequent in the sum score in the COVID-19 group compared to controls (**Figure 9C**). In the compartment-specific analysis of neutrophils, infiltration was significantly increased in the alveolar septa (**Figures 9D–G**). Although the number of neutrophils in the alveolar infiltrates and bronchioli of COVID-19 patients was also more than two times higher on average than in the control group, the significance level was not reached due to the large variance observed (**Figures 9D, H–J**). Comparable amounts of MPO-positive cells were found in the large vessels of both groups (**Figure 9D**, Vasc). In the kidney, the infiltration with neutrophils was lower compared to the lung (**Figures 9K, L**). The comparison of both groups in the sum score only showed a tendency towards increased numbers in the COVID-19 group (**Figure 9M**). In contrast, the number of neutrophils in the glomeruli and in the lumina of the tubules was significantly increased in the COVID-19 group (**Figures 9N–T**). Correlation analysis with MASP-2 also showed a significant correlation in both alveolar septa and tubules with the number of MPO-positive cells (**Figure 8**), suggesting that these cells may be locally activated by the complement system. In addition, alveolar MASP-2 and CFD correlated with lung injury and MASP-2 deposition in TBM correlated with tubular injury (**Figure 8**).

In summary, strong deposition of complement factors in lung and kidney samples of patients who died of COVID-19 underlined the potential importance of complement in COVID-19-mediated pathology.

DISCUSSION

This study to date investigates the largest series of lung and kidney specimens for local deposition of complement components in patients who died of severe COVID-19. The lung is considered the primary site of damage in COVID-19 disease, but there are numerous reports that damage also occurs in other organs (5). In particular, acute tubular injury has been described in the kidney after severe COVID-19 and increase of proteinuria was associated with poor prognosis and increased mortality (6, 7). Here, we therefore investigated local complement over-activation as a common pathomechanism in COVID-19-mediated injury in lung and kidney. We also tried to differentiate the complement pathway operational by which complement was activated by analyzing C1q for the classical and MASP-2 for the lectin pathway and CFD for the alternative pathway. The lectin pathway was activated in both lungs and kidneys of patients with severe COVID-19 in our study. MASP-2 was detectable in the lung mainly in the alveolar septa, the hyaline membranes but also in the lumina of the alveoli. Activation of the lectin pathway was supported by staining for C4d in the same localization as described for MASP-2 (**Supplementary Figure 3**). MASP-2 was strongly expressed in the liver (**Supplementary Figure 1**), while renal and lung MASP-2 expression in cellular components was very low or lacking, as supported by our recent study investigating complement in renal transplants (29) and RNA expression data provided in the web database "Human Protein Atlas" (30). This indicates that extrahepatic expression of MASP-2 is not required for lectin pathway activation. In contrast, other complement factors like C1 and C3 were locally expressed by renal and inflammatory cells (29) and its expression might be important in pathogenesis of complement-mediated injury. Complement activation *via* the

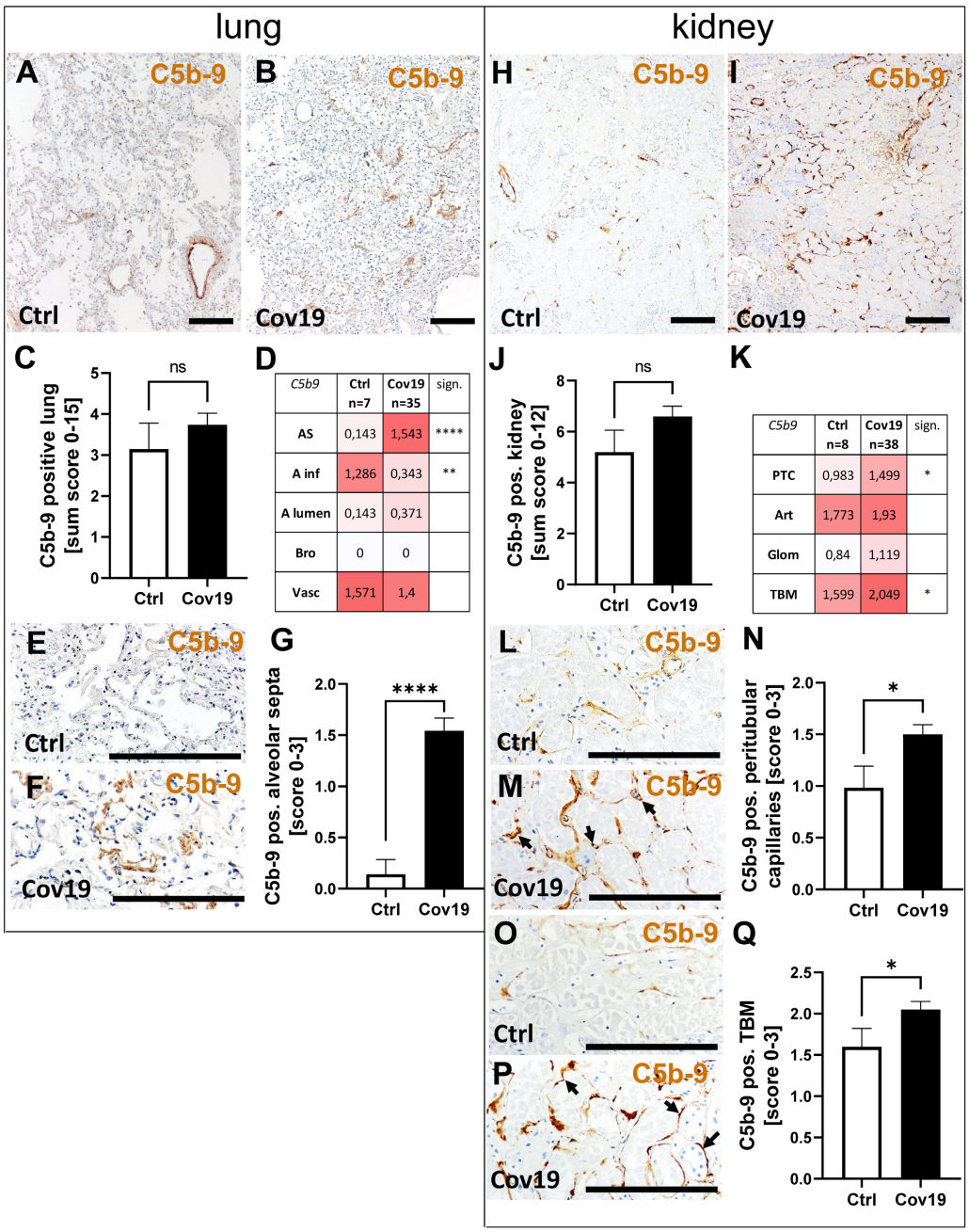


FIGURE 7 | Detection of C5b-9 in lung and kidney specimens of COVID-19 patients. Overview of immunohistochemical stainings for C5b-9 in lungs of a control patient **(A)** and a patient with COVID-19 **(B)**. Results of semi-quantitative scoring in multiple compartments for C5b-9 deposition in the lung summarized in a sum score **(C)** and overview of the mean values of compartment-specific analyses given in a heat map **(D)**. Representative C5b-9 staining in alveolar septa of a control patient **(E)** and a COVID-19 patient **(F)** and statistical comparison of both groups **(G)**. Overview of C5b-9 staining in kidneysof a control patient **(H)** and a patient with COVID-19 **(I)**. Results of semi-quantitative scoring in multiple compartments for C5b-9 deposition in the kidney summarized in a sum score **(J)** and overview of the mean values of compartment-specific analyses given in a heat map **(K)**. Representative C5b-9 staining in peritubular capillaries of a control patient **(L)** and a COVID-19 patient (M, arrows) and statistical comparison of both groups **(N)**. C5b-9 staining of tubular basement membrane in a control patient **(O)** and a patient with COVID-19 (P, arrows) and statistical comparison of both groups **(Q)**. AS, alveolar septa; A inf, alveolar infiltrate; A lumen, alveolar lumen; Bro, bronchioles; Vasc, vascular; sign., significance; ns, non-significant; PTC, peritubular capillaries; Art, arterial; Glom, glomerular; TBM, tubular basement membrane; BM, basement membrane. Scale bar = 200µm; *p < 0.05; **p < 0.01; ****p < 0.0001.

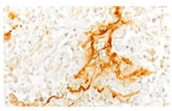
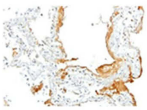
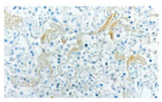
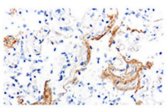
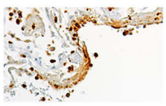
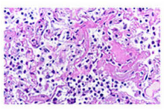
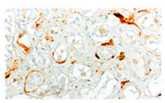
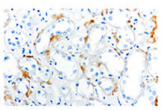
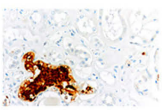
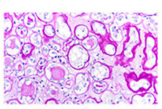
	Lung MASP-2 AS [score 0-4] 	Lung CFD AS [score 0-3] 	Kidney MASP-2 TBM [score 0-3] 
C3d AS [score 0-3] 	[r] 0.686**** [p] <0.0001 [n] 41	[r] 0.673**** [p] <0.0001 [n] 42	
C5b-9 AS [score 0-3] 	[r] 0.670**** [p] <0.0001 [n] 41	[r] 0.628**** [p] <0.0001 [n] 42	
MPO AS [score 0-3] 	[r] 0.447** [p] 0.003 [n] 41	[r] 0.307* [p] 0.048 [n] 42	
Lung injury [score 0-5] 	[r] 0.313* [p] 0.046 [n] 41	[r] 0.412** [p] 0.007 [n] 42	
C3d TBM [score 0-3] 			[r] 0.491** [p] 0.001 [n] 45
C5b-9 TBM [score 0-3] 			[r] 0.541*** [p] 0.0001 [n] 46
MPO tub. [score 0-3] 			[r] 0.307* [p] 0.038 [n] 46
Tub. injury [score 0-4] 			[r] 0.432** [p] 0.003 [n] 46

FIGURE 8 | Correlation of MASP-2 and CFD deposition with C3d, C5b-9, MPO-positive cells and tissue injury. Scores for MASP-2 as well as CFD-positive alveolar septa in lungs and MASP-2-positive tubular basement membrane in the kidney correlated with scores of C3d, C5b-9, MPO staining in the same compartment, respectively, and with lung or tubular injury score using Spearman test. AS, alveolar septa; TBM, tubular basement membrane; Tub, tubular lumen; r = correlation coefficient; p = p-value (significance level), n = sample size. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

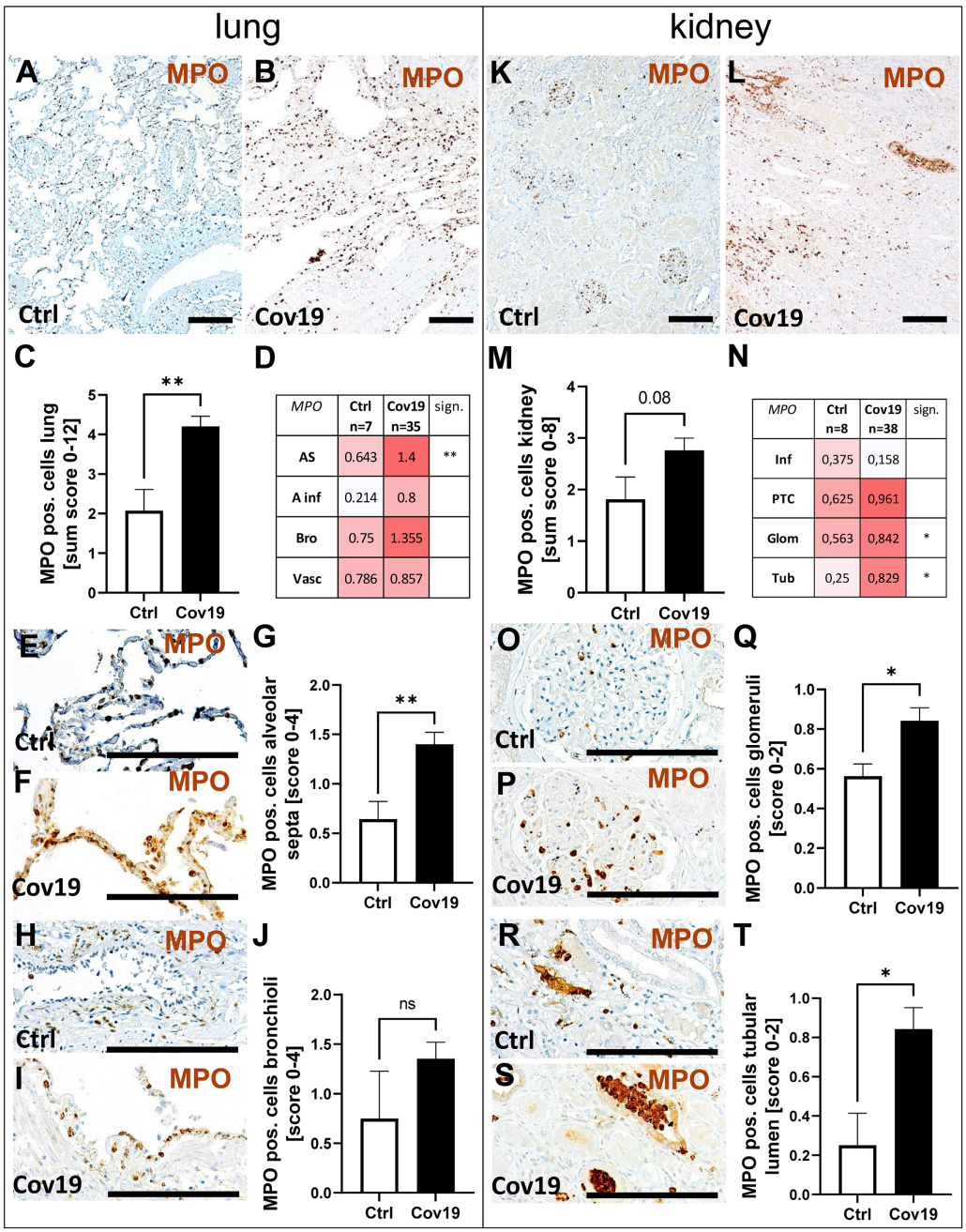


FIGURE 9 | Detection of myeloperoxidase (MPO)-positive cells in lung and kidney specimens of COVID-19 patients. Overview of immunohistochemical staining for MPO staining in lungs of a control patient **(A)** and a patient with COVID-19 **(B)**. Results of semi-quantitative scoring in multiple compartments for MPO-positive cells in the lung summarized in a sum score **(C)** and overview of the mean values of compartment-specific analyses given in a heat map **(D)**. Representative MPO staining in alveolar septa of a control patient **(E)** and a COVID-19 patient **(F)** and statistical comparison of both groups **(G)**. MPO staining in bronchioli in a control patient **(H)** and a patient with COVID-19 **(I, J)**. Overview of MPO staining in kidneys of a control patient **(K)** and a patient with COVID-19 **(L)**. Results of semi-quantitative scoring in multiple compartments for MPO-positive cells in the kidney summarized in a sum score **(M)** and overview of the mean values of compartment-specific analyses given in a heat map **(N)**. Representative MPO staining in glomeruli of a control patient **(O)** and a COVID-19 patient **(P)** and statistical comparison of both groups **(Q)**. MPO positive cells in the lumen of tubuli in a control patient **(R)** and a patient with COVID-19 (**S**, arrows) and statistical comparison of both groups **(T)**. AS, alveolar septa; A inf, alveolar infiltrate; Bro, bronchioles; Vasc, vascular; sign., significance; ns, non-significant; Inf, infiltrate; PTC, peritubular capillaries; Glom, glomerular; Tub, tubular lumen; Scale bar = 200μm; *p < 0.05; **p < 0.01.

lectin pathway was confirmed in an earlier study by Magro et al. investigating local complement activation in small sample numbers of lungs and skin from patients with COVID-19, showing MASP-2 and C4d deposition in intraalveolar septa (23). In addition, C4d staining co-localized with detection of SARS-CoV-2 spike glycoprotein (23), suggesting direct complement activation by SARS-CoV-2 virus in the lung. In experiments *in vitro*, SARS-CoV-2 spike and nucleocapsid protein bind to the recognition components of the lectin pathway namely mannose-binding lectin (MBL), ficolin 2, and collectin-11, whereas C1q does not (17). MASP-2 can then activate protein C4 after binding of these SARS-CoV-2 protein/recognition molecule complexes but also by direct interaction with the nucleocapsid protein (17). Expression of MBL and ficolin-3, both recognition molecules of the lectin pathway, were shown to be upregulated in lungs of patients with COVID-19 (31). While the complement system contributes to a reduction of the viral load in the early phase of the disease by opsonizing the viruses, the complement system can become over-activated during the course of the disease, which then exerts its harmful effects by unleashing a cytokine storm, by direct cell damage *via* the membrane attack complex or by interacting with the coagulation cascade (32). The interaction with the coagulation cascade can promote thrombus formation which is an important feature of COVID-19 pathology, which we mainly observed in the lungs but also in the kidneys of patients with COVID-19, although at much lower rates. The coagulation cascade and the complement cascade are closely linked (33) and the cross talk between the complement system and the coagulation cascade in COVID-19 patients may lead to an enhanced rate of coagulopathies (34). For example, MASP-2 can cleave prothrombin to thrombin (35) and factor XIII and fibrinogen is cleaved by MASP-1 but with significantly lower turnover than observed for the enzymes of the coagulation cascade (36). In addition to activation *via* the lectin pathway in lungs of patients with COVID-19, we established complement activation *via* the alternative pathway by detecting CFD. This is in line with reports from others who have also detected CFD in lungs from patients severely affected by COVID-19 (22, 25). However, in our study COVID-19-induced activation of the alternative pathway was restricted to the lungs, while CFD deposition was comparable in kidneys of both investigated groups. In contrast to our results, these two studies described complement activation *via* the classical pathway in addition (22, 25) and detected activation of the alternative pathway in both lungs and kidneys (22). It is possible that the detection of pathway-specific complement factors is significantly dependent on the antibodies used and the condition and fixation of the tissues. Since antigens are not masked in frozen tissue, they are easier to detect, but non-specific staining also frequently occurs. With C1q established for diagnostic purposes, we would have expected stronger staining for C1q if the classical pathway was to play an important role in COVID-19-mediated damage. Thrombus formation, as a hallmark in COVID-19 pathology, is induced by neutrophil extracellular traps (NETs) formed by activated neutrophils (37). In our study numbers of neutrophils were increased in lung and kidney and correlated with deposition of MASP-2 in both organs. Neutrophils activated by complement anaphylatoxins C5a induce expression of tissue factor, NET formation and thrombosis and could be inhibited by treatment with a C5aR1 inhibitor (38). C3a and C5a cleavage products are formed after activation of all complement pathways, bind to its receptors on inflammatory cells, e.g. neutrophils and macrophages and have a key role in recruiting and activating these cells (39). Serum levels of C5a, indicating complement activity, increased with severity in patients with COVID-19 and hereby inducing expression of cytokines that can elicit a cytokine storm (39). In our study we could confirm positive correlation of complement activation with severity of COVID-19 on organ level since lung injury scores as well as tubular injury scores were associated with increased activation of MASP-2. Blood analyses in patients with COVID-19 suggest that the outcome of the disease depends on the activated complement pathway. Accordingly, a group of patients in whom the alternative and lectin pathways were activated showed more complications and higher mortality than another group in which mainly activity of the classical pathway was detected (40). Complement activation in the kidney was lower compared to the lung and restricted to the lectin pathway. This difference may also be due to the fact that in the lung direct complement activation by the SARS-CoV-2 virus occurs because of a higher virus load. In contrast, in kidneys virus was not (11, 13) or only sporadically (7, 9) detected. Similar findings were made using our autopsy material of patients with severe COVID-19 showing no SARS-CoV-2 spike protein detection in any of the kidneys and in the lungs in only 22% of the cases. This unexpected low virus detection rate might be explained by the different disease durations of the individual patients or by the relatively poor preservation of the autopsy tissue. In the kidney we suggest that complement activation occurs indirectly as a consequence of tissue damage and cell death. Due to the relatively poor preservation of the autopsy tissue, an assessment of acute ischemic damage is not possible, so that we can only speculate whether complement activation is causative of or results secondary from renal injury in severe COVID-19 disease. Complement activation also occurs after hypoxic injury in the kidney as shown for ischemia/reperfusion (41, 42). In earlier studies, we investigated early post transplant biopsies from patients with delayed graft function, showing increased complement activation compared to zero-time biopsies *via* different activation pathways (43). However, it is questionable, if ischemia/reperfusion after transplantation is comparable to COVID-19-induced injury. However, direct complement activation by the virus at an earlier time point before death of the patients cannot be excluded. Our own and data of others underline that complement activation might be an important pathomechanism of lung but also kidney damage in COVID-19 opening the possibility of treatment with complement inhibitors. Currently, only anecdotal reports of the successful treatment of seriously ill patients with COVID-19 employing the MASP-2 inhibitor Narsoplimab (44), the C5 inhibitor Eculizumab (45–47) or the C3 inhibitor AMY-101 (48) have been published. However, more clinical studies using complement inhibitors in patients with COVID-19 evaluating more patients will show the efficiency of this treatment.

LIMITATION OF THE STUDY

Albeit we excluded material with major autolytic changes our study is limited by the quality of the autopsy material, so that some analyses, such as the investigation of capillarization, were not possible. Furthermore, the patients studied had a variety of comorbidities, such as chronic kidney disease, which could possibly also have influenced complement activation, at least in the kidney. Detection of MASP-2 is limited by the fact that the used antibody also recognizes the MASP2 splice variant MAp19, lacking the catalytic domain. Therefore we cannot distinguish between complement activating MASP-2 and MAp19.

In conclusion, in lungs we observed marked complement activation *via* the lectin and alternative pathway in patients who died on COVID-19, while in kidney COVID-19-induced complement activation was restricted to the lectin pathway. Complement deposition was located primarily in capillaries and hyaline membranes in the lung and in peritubular capillaries and on tubular basement membranes in the kidney of COVID-19 patients. Therefore, we speculate that complement is involved in vascular but also epithelial cell damage in both organs of patients with COVID-19. To prevent deregulated complement activation and subsequent collateral tissue injury specific complement inhibition might thus be a promising treatment option.

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

Analysis was approved by the local Ethics committees (Ethics committee of the Friedrich-Alexander University (FAU) Erlangen-Nürnberg, reference number 4415 and internal review board of the Medical Center-Augsburg (BKF No. 2020–18) and the ethics committee of the University of Munich (Project number 20–426, COVID-19 registry of the University hospital Augsburg). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JN analyzed data and wrote the manuscript. TR carried out immunohistochemistry staining. CE collected tissue samples and clinical data and revised the manuscript. CD, MB-H, KA, BM, and AH conceived and designed the study, analyzed data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), project number 387509280, SFB 1350 and supportet by the DEFEAT Pandemics autopsy platform of the Federal Ministry of Education and Research (BMBF).

ACKNOWLEDGMENTS

The technical assistance of Stefan Söllner, Sina Dassler, Carolin Hamann, Iris Scheitacker and Miriam Reutelshöfer is gratefully acknowledged. The authors are thankful to Lukas Borcherding for compiling the cases from the Augsburg Autopsy Series.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.835156/full#supplementary-material

REFERENCES

1. Fu D, Yang B, Xu J, Mao Z, Zhou C, Xue C. COVID-19 Infection in a Patient With End-Stage Kidney Disease. *Nephron* (2020) 144(5):245–7. doi: 10.1159/000507261
2. Guan W-J, Ni Z-Y, Hu Y, Liang W-H, Ou C-Q, He J-X, et al. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* (2020) 382(18):1708–20. doi: 10.1056/NEJMoa2002032
3. Carsana L, Sonzogni A, Nasr A, Rossi RS, Pellegrinelli A, Zerbi P, et al. Pulmonary Post-Mortem Findings in a Series of COVID-19 Cases From Northern Italy: A Two-Centre Descriptive Study. *Lancet Infect Dis* (2020) 20(10):1135–40. doi: 10.1016/s1473-3099(20)30434-5
4. Gavriatopoulou M, Korompoki E, Fotiou D, Ntanasis-Stathopoulos I, Psaltopoulou T, Kastritis E, et al. Organ-Specific Manifestations of COVID-19 Infection. *Clin Exp Med* (2020) 20(4):493–506. doi: 10.1007/s10238-020-00648-x
5. Gupta A, Madhavan MV, Sehgal K, Nair N, Mahajan S, Sehrawat TS, et al. Extrapulmonary Manifestations of COVID-19. *Nat Med* (2020) 26(7):1017–32. doi: 10.1038/s41591-020-0968-3
6. Jewell PD, Bramham K, Galloway J, Post F, Norton S, Teo J, et al. COVID-19-Related Acute Kidney Injury; Incidence, Risk Factors and Outcomes in a Large UK Cohort. *BMC Nephrol* (2021) 22(1):359. doi: 10.1186/s12882-021-02557-x
7. Puelles VG, Lütgehetmann M, Lindenmeyer MT, Sperhake JP, Wong MN, Allweiss L, et al. Multiorgan and Renal Tropism of SARS-CoV-2. *N Engl J Med* (2020) 383(6):590–2. doi: 10.1056/NEJMc2011400
8. Farkash EA, Wilson AM, Jentzen JM. Ultrastructural Evidence for Direct Renal Infection With SARS-CoV-2. *J Am Soc Nephrol* (2020) 31(8):1683–7. doi: 10.1681/asn.2020040432
9. Su H, Yang M, Wan C, Yi LX, Tang F, Zhu HY, et al. Renal Histopathological Analysis of 26 Postmortem Findings of Patients With COVID-19 in China. *Kidney Int* (2020) 98(1):219–27. doi: 10.1016/j.kint.2020.04.003

10. Soleimani M. Acute Kidney Injury in SARS-CoV-2 Infection: Direct Effect of Virus on Kidney Proximal Tubule Cells. *Int J Mol Sci* (2020) 21(9):1–6. doi: 10.3390/ijms21093275
11. Kudose S, Batal I, Santoriello D, Xu K, Barasch J, Peleg Y, et al. Kidney Biopsy Findings in Patients With COVID-19. *J Am Soc Nephrol* (2020) 31(9):1959–68. doi: 10.1681/asn.2020060802
12. Mahmudpour M, Roozbeh J, Keshavarz M, Farrokhi S, Nabipour I. COVID-19 Cytokine Storm: The Anger of Inflammation. *Cytokine* (2020) 133:155151. doi: 10.1016/j.cyto.2020.155151
13. Santoriello D, Khairallah P, Bomback AS, Xu K, Kudose S, Batal I, et al. Postmortem Kidney Pathology Findings in Patients With COVID-19. *J Am Soc Nephrol* (2020) 31(9):2158–67. doi: 10.1681/asn.2020050744
14. Walport MJ. Complement. First of Two Parts. *N Engl J Med* (2001) 344 (14):1058–66. doi: 10.1056/nejm200104053441406
15. Noris M, Remuzzi G. Overview of Complement Activation and Regulation. *Semin Nephrol* (2013) 33(6):479–92. doi: 10.1016/j.semnephrol.2013.08.001
16. Kojouharova M, Reid K, Gadjeva M. New Insights Into the Molecular Mechanisms of Classical Complement Activation. *Mol Immunol* (2010) 47 (13):2154–60. doi: 10.1016/j.molimm.2010.05.011
17. Ali YM, Ferrari M, Lynch NJ, Yaseen S, Dudler T, Gragerov S, et al. Lectin Pathway Mediates Complement Activation by SARS-CoV-2 Proteins. *Front Immunol* (2021) 12:714511. doi: 10.3389/fimmu.2021.714511
18. Świerzko AS, Cedzyński M. The Influence of the Lectin Pathway of Complement Activation on Infections of the Respiratory System. *Front Immunol* (2020) 11:585243. doi: 10.3389/fimmu.2020.585243
19. Pangburn MK, Schreiber RD, Müller-Eberhard HJ. Formation of the Initial C3 Convertase of the Alternative Complement Pathway. Acquisition of C3b-Like Activities by Spontaneous Hydrolysis of the Putative Thioester in Native C3. *J Exp Med* (1981) 154(3):856–67. doi: 10.1084/jem.154.3.856
20. Wang R, Xiao H, Guo R, Li Y, Shen B. The Role of C5a in Acute Lung Injury Induced by Highly Pathogenic Viral Infections. *Emerg Microbes Infect* (2015) 4(5):e28. doi: 10.1038/emi.2015.28
21. Zinellu A, Mangoni AA. Serum Complement C3 and C4 and COVID-19 Severity and Mortality: A Systematic Review and Meta-Analysis With Meta-Regression. *Front Immunol* (2021) 12:696085. doi: 10.3389/fimmu.2021.696085
22. Macor P, Durigutto P, Mangogna A, Bussani R, De Maso L, D'Errico S, et al. Multiple-Organ Complement Deposition on Vascular Endothelium in COVID-19 Patients. *Biomedicines* (2021) 9(8):1–15. doi: 10.3390/biomedicines9081003
23. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement Associated Microvascular Injury and Thrombosis in the Pathogenesis of Severe COVID-19 Infection: A Report of Five Cases. *Transl Res* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
24. Pfister F, Vonbrunn E, Ries T, Jäck HM, Überla K, Lochnit G, et al. Complement Activation in Kidneys of Patients With COVID-19. *Front Immunol* (2020) 11:594849. doi: 10.3389/fimmu.2020.594849
25. Satyam A, Tsokos MG, Brook OR, Hecht JL, Moulton VR, Tsokos GC. Activation of Classical and Alternative Complement Pathways in the Pathogenesis of Lung Injury in COVID-19. *Clin Immunol* (2021) 226:108716. doi: 10.1016/j.clim.2021.108716
26. von Stillfried S, Acker T, Aepfelbacher M, Baretton G, Bülow RD, Bürrig KF, et al. Cooperative Approach of Pathology and Neuropathology in the COVID-19 Pandemic: German Registry for COVID-19 Autopsies (DeRegCOVID) and German Network for Autopsies in Pandemics (DEFEAT PANDEMIcs). *Pathologe* (2021) 42(Suppl 1):69–75. doi: 10.1007/s00292-020-00897-3
27. Schaller T, Hirschbühl K, Burkhardt K, Braun G, Trepel M, Märkl B, et al. Postmortem Examination of Patients With COVID-19. *Jama* (2020) 323 (24):2518–20. doi: 10.1001/jama.2020.8907
28. Hainz N, Thomas S, Neubert K, Meister S, Benz K, Rauh M, et al. The Proteasome Inhibitor Bortezomib Prevents Lupus Nephritis in the NZB/W F1 Mouse Model by Preservation of Glomerular and Tubulointerstitial Architecture. *Nephron Exp Nephrol* (2012) 120(2):e47–58. doi: 10.1159/000334955
29. Vonbrunn E, Ries T, Söllner S, Müller-Deile J, Büttner-Herold M, Amann K, et al. Multiplex Gene Analysis Reveals T-Cell and Antibody-Mediated Rejection-Specific Upregulation of Complement in Renal Transplants. *Sci Rep* (2021) 11(1):15464. doi: 10.1038/s41598-021-94954-3
30. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-Based Map of the Human Proteome. *Science* (2015) 347(6220):1260419. doi: 10.1126/science.1260419
31. Malaquias MAS, Gadotti AC, Motta-Junior JDS, Martins APC, Azevedo MLV, Benevides APK, et al. The Role of the Lectin Pathway of the Complement System in SARS-CoV-2 Lung Injury. *Transl Res* (2020) 231:55–63. doi: 10.1016/j.trsl.2020.11.008
32. Noris M, Benigni A, Remuzzi G. The Case of Complement Activation in COVID-19 Multiorgan Impact. *Kidney Int* (2020) 98(2):314–22. doi: 10.1016/j.kint.2020.05.013
33. Conway EM, Pryzdial ELG. Is the COVID-19 Thrombotic Catastrophe Complement-Connected? *J Thromb Haemost* (2020) 18(11):2812–22. doi: 10.1111/jth.15050
34. Tomo S, Kumar KP, Roy D, Sankanagoudar S, Purohit P, Yadav D, et al. Complement Activation and Coagulopathy - An Ominous Duo in COVID19. *Expert Rev Hematol* (2021) 14(2):155–73. doi: 10.1080/17474086.2021.1875813
35. Krarup A, Wallis R, Presanis JS, Gál P, Sim RB. Simultaneous Activation of Complement and Coagulation by MBL-Associated Serine Protease 2. *PloS One* (2007) 2(7):e623. doi: 10.1371/journal.pone.0000623
36. Krarup A, Gulla KC, Gál P, Hajela K, Sim RB. The Action of MBL-Associated Serine Protease 1 (MASP1) on Factor XIII and Fibrinogen. *Biochim Biophys Acta* (2008) 1784(9):1294–300. doi: 10.1016/j.bbapap.2008.03.020
37. Ackermann M, Anders HJ, Bilyy R, Bowlin GL, Daniel C, De Lorenzo R, et al. Patients With COVID-19: In the Dark-NETs of Neutrophils. *Cell Death Differ* (2021) 28(11):3125–39. doi: 10.1038/s41418-021-00805-z
38. Skendros P, Mitsios A, Chrysanthopoulou A, Mastellos DC, Metallidis S, Rafailidis P, et al. Complement and Tissue Factor-Enriched Neutrophil Extracellular Traps Are Key Drivers in COVID-19 Immunothrombosis. *J Clin Invest* (2020) 130(11):6151–7. doi: 10.1172/jci141374
39. Carvelli J, Demaria O, Vély F, Batista L, Chouaki Benmansour N, Fares J, et al. Association of COVID-19 Inflammation With Activation of the C5a-C5aR1 Axis. *Nature* (2020) 588(7836):146–50. doi: 10.1038/s41586-020-2600-6
40. Defendi F, Leroy C, Epaulard O, Clavarino G, Vilotitch A, Le Marechal M, et al. Complement Alternative and Mannose-Binding Lectin Pathway Activation Is Associated With COVID-19 Mortality. *Front Immunol* (2021) 12:742446. doi: 10.3389/fimmu.2021.742446
41. Asgari E, Farrar CA, Lynch N, Ali YM, Roscher S, Stover C, et al. Mannan-Binding Lectin-Associated Serine Protease 2 Is Critical for the Development of Renal Ischemia Reperfusion Injury and Mediates Tissue Injury in the Absence of Complement C4. *FASEB J* (2014) 28(9):3996–4003. doi: 10.1096/fj.13-246306
42. Danobeitia JS, Djamali A, Fernandez LA. The Role of Complement in the Pathogenesis of Renal Ischemia-Reperfusion Injury and Fibrosis. *Fibrogene Tissue Repair* (2014) 7:16. doi: 10.1186/1755-1536-7-16
43. Bobka S, Ebert N, Koertvely E, Jacobi J, Wiesener M, Buttner-Herold M, et al. Is Early Complement Activation in Renal Transplantation Associated With Later Graft Outcome? *Kidney Blood Press Res* (2018) 43(5):1488–504. doi: 10.1159/000494014
44. Rambaldi A, Gritti G, Micò MC, Frigeni M, Borleri G, Salvi A, et al. Endothelial Injury and Thrombotic Microangiopathy in COVID-19: Treatment With the Lectin-Pathway Inhibitor Narsoplimab. *Immunobiology* (2020) 225(6):152001. doi: 10.1016/j.imbio.2020.152001
45. Diurno F, Numis FG, Porta G, Cirillo F, Maddaluno S, Ragozzino A, et al. Eculizumab Treatment in Patients With COVID-19: Preliminary Results From Real Life ASL Napoli 2 Nord Experience. *Eur Rev Med Pharmacol Sci* (2020) 24(7):4040–7. doi: 10.26355/eurrev_202004_20875
46. Laurence J, Mulvey JJ, Seshadri M, Racanelli A, Harp J, Schenck EJ, et al. Anti-Complement C5 Therapy With Eculizumab in Three Cases of Critical COVID-19. *Clin Immunol* (2020) 219:108555. doi: 10.1016/j.clim.2020.108555
47. Mentese IB, Velioglu A, Aykent B, Gunay DS, Arikan H, Tuglular S. Course of COVID-19 Pneumonia in a Kidney Transplant Patient Receiving Eculizumab. *Prog Transplant* (2021) 31(3):279–80. doi: 10.1177/15269248211024629
48. Mastaglio S, Ruggeri A, Risitano AM, Angelillo P, Yancopoulou D, Mastellos DC, et al. The First Case of COVID-19 Treated With the Complement C3 Inhibitor AMY-101. *Clin Immunol* (2020) 215:108450. doi: 10.1016/j.clim.2020.108450

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.

this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Immunology

REVIEW
published: 08 March 2022
doi: 10.3389/fimmu.2022.840137

How the Innate Immune System of the Blood Contributes to Systemic Pathology in COVID-19-Induced ARDS and Provides Potential Targets for Treatment

Bo Nilsson[1], Barbro Persson[1], Oskar Eriksson[1], Karin Fromell[1], Michael Hultström[2,3], Robert Frithiof[2], Miklos Lipcsey[2,4], Markus Huber-Lang[5] and Kristina N. Ekdahl[1,6]*

[1] Department of Immunology Genetics and Pathology, Uppsala University, Uppsala, Sweden, [2] Department of Surgical Sciences, Anesthesiology and Intensive Care, Uppsala University, Uppsala, Sweden, [3] Unit for Integrative Physiology, Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden, [4] Hedenstierna Laboratory, Anesthesiology and Intensive Care, Department of Surgical Sciences, Uppsala University, Uppsala, Sweden, [5] Institute for Clinical and Experimental Trauma-Immunology, University Hospital of Ulm, Ulm, Germany, [6] Linnaeus Centre for Biomaterials Chemistry, Linnaeus University, Kalmar, Sweden

OPEN ACCESS

Edited by:
Zoltán Prohászka, Semmelweis University, Hungary

Reviewed by:
Marina Noris, Mario Negri Pharmacological Research Institute (IRCCS), Italy
Daniel Ricklin, University of Basel, Switzerland
Christian Drouet, U1016 Institut Cochin (INSERM), France

***Correspondence:**
Bo Nilsson
bo.nilsson@igp.uu.se

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *20 December 2021*
Accepted: *14 February 2022*
Published: *08 March 2022*

Citation:
Nilsson B, Persson B, Eriksson O, Fromell K, Hultström M, Frithiof R, Lipcsey M, Huber-Lang M and Ekdahl KN (2022) How the Innate Immune System of the Blood Contributes to Systemic Pathology in COVID-19-Induced ARDS and Provides Potential Targets for Treatment. Front. Immunol. 13:840137. doi: 10.3389/fimmu.2022.840137

Most SARS-CoV-2 infected patients experience influenza-like symptoms of low or moderate severity. But, already in 2020 early during the pandemic it became obvious that many patients had a high incidence of thrombotic complications, which prompted treatment with high doses of low-molecular-weight heparin (LMWH; typically 150-300IU/kg) to prevent thrombosis. In some patients, the disease aggravated after approximately 10 days and turned into a full-blown acute respiratory distress syndrome (ARDS)-like pulmonary inflammation with endothelialitis, thrombosis and vascular angiogenesis, which often lead to intensive care treatment with ventilator support. This stage of the disease is characterized by dysregulation of cytokines and chemokines, in particular with high IL-6 levels, and also by reduced oxygen saturation, high risk of thrombosis, and signs of severe pulmonary damage with ground glass opacities. The direct link between SARS-CoV-2 and the COVID-19-associated lung injury is not clear. Indirect evidence speaks in favor of a thromboinflammatory reaction, which may be initiated by the virus itself and by infected damaged and/or apoptotic cells. We and others have demonstrated that life-threatening COVID-19 ARDS is associated with a strong activation of the intravascular innate immune system (IIIS). In support of this notion is that activation of the complement and kallikrein/kinin (KK) systems predict survival, the necessity for usage of mechanical ventilation, acute kidney injury and, in the case of MBL, also coagulation system activation with thromboembolism. The general properties of the IIIS can easily be translated into mechanisms of COVID-19 pathophysiology. The prognostic value of complement and KKsystem biomarkers demonstrate that pharmaceuticals, which are licensed or have passed the phase I trial stage are promising candidate drugs for treatment of COVID-19. Examples of such compounds include complement inhibitors AMY-101 and eculizumab

(targeting C3 and C5, respectively) as well as kallikrein inhibitors ecallantide and lanadelumab and the bradykinin receptor (BKR) 2 antagonist icatibant. In this conceptual review we discuss the activation, crosstalk and the therapeutic options that are available for regulation of the IIIS.

Keywords: cascade system, leukocytes, platelets, plasma proteins, COVID-19

INTRODUCTION

COVID-19 has been shown to have a multifaceted effect on the immune system. In a recently published article, we reported that the innate immune system of the blood, here designated the intravascular innate immune system (IIIS), is strongly activated in severe COVID-19 with ARDS (1), which is the major explanation for the serious course of the disease. In this article we review the IIIS and how its physiological function contributes to tissue injury and to the proinflammatory state during severe COVID-19. Finally, we review potential treatment targets within the IIIS with existing drugs that could be expected to modulate the disease course in COVID-19.

THE INTRAVASCULAR INNATE IMMUNE SYSTEM (IIIS)

The blood contains a large number of plasma proteins and cells that constitute our innate barrier both in terms of recognition and elimination of microorganisms. Here, we define the IIIS and focus on the network of proteins and cells that forms the innate immune system in blood leading to thromboinflammation (2). The IIIS consists of the cascade system of the blood: the complement system, the coagulation system, the kallikrein/kinin (KK; or contact system), and the fibrinolytic system. Also, individual proteins related to the cascade systems such as collectins, pentraxins, etc. belong to the IIIS, as do blood cells, e.g., granulocytes, monocytes, platelets and endothelial cells. These proteins are also available in the mucous membranes of the body, especially during inflammation either by passive diffusion or by active synthesis in the lining epithelial cells, while cells, such as granulocytes and monocytes, are recruited by chemotaxis mediated by the anaphylatoxins (C3a, C5a), by bradykinin (BK) and by chemokines.

Over the years, IIIS research has been very separated, not only in the laboratory but also in the collaboration between researchers and in the literature. For obvious reasons, human blood samples have been used for the studies in the various disciplines of the IIIS, and since the blood needs to be anticoagulated to be separated into a fluid phase and blood cells, different anticoagulants have been used. Divalent cations are necessary for IIIS function: the coagulation cascade requires Ca^{2+} and the complement cascade both Ca^{2+} and Mg^{2+} ions, which affects the usage of anticoagulant for the different cascade system. As a consequence, EDTA (chelating both Ca^{2+} and Mg^{2+}) is used for assessment of complement activation fragments/products in plasma, while the corresponding anticoagulant for the coagulation, contact and fibrinolytic systems is citrate (chelating Ca^{2+} ions, but not Mg^{2+}). Studies of the complement system function is performed using serum (i.e., the remaining fluid-phase after blood is coagulated), whereas for the coagulation, KK, and fibrinolysis systems, citrate reconstituted with Ca^{2+} ions is the plasma preparation of choice. These pre-analytic procedures have totally separated the research disciplines. Further aggravating the problem, cell studies have in many cases been performed on heparin blood, where activation of the coagulation and complement systems are considerably dampened by the high concentrations of heparin. Consequently, there is no generally used holistic assay format available for simultaneous assessment of all IIIS functions. However, for specialized *in vitro* studies heparin-coated tubes or tubing can be used, leaving the blood fresh and unaffected (3). In this conceptual review the intension is to consider these components of the blood as an intact network that gives rise to an integrated innate immune response in late-stage COVID-19. The findings by us and others support this approach.

ORGANIZATION OF THE IIIS

The schematic structure of the IIIS is described in **Figure 1**, which focuses on the interaction of the cascade systems of the blood, i.e., the complement, coagulation, KK and fibrinolytic systems. The reason for highlighting the cascade systems is that they contain most of the recognition molecules that specifically target DAMPs and PAMPs and trigger activation of the entire IIIS. In the figure, they are marked as components of the activation pathways that they initiate. The complement system has three defined activation pathways: the classical (CP), the lectin pathways (LP) and the alternative (AP) that are triggered by different stimuli (5).

Abbreviations: ACE, *angiotensin converting enzyme;* aHUS, atypical hemolytic uremic syndrome; AP, alternative pathway of complement; ARDS, acute respiratory distress syndrome; BK, bradykinin; BKR1/2, bradykinin receptor 1/2; C1-INH, C1 inhibitor; CAC, COVID-19-associated coagulopathy; CP, classical pathway of complement; CT, computed tomography; DAMPs, damage-associated molecular patterns; DIC, disseminated intravascular coagulation; DOAC, direct-acting oral anticoagulants; DVT, deep vein thrombosis; FXII, Factor XII; ICU, intensive care unit; HMWK, high molecular weight kininogen; IIIS, intravascular innate immune system; KK, kallikrein/kinin system; LMWH, low-molecular-weight heparin; LP, lectin pathway of complement; MERS, middle East respiratory syndrome; NETs, neutrophil extracellular traps; PAMPs, pathogen-associated molecular patterns; PMNs, polymorphonuclear leukocytes; PNH, paroxysmal noctural hemoglobinuria; RAS, renin-angiotensin system; SARS-1, severe acute respiratory ryndrome-1; TF, tissue factor; tPA, tissue plasminogen activator; vWF, von Willebrand factor.

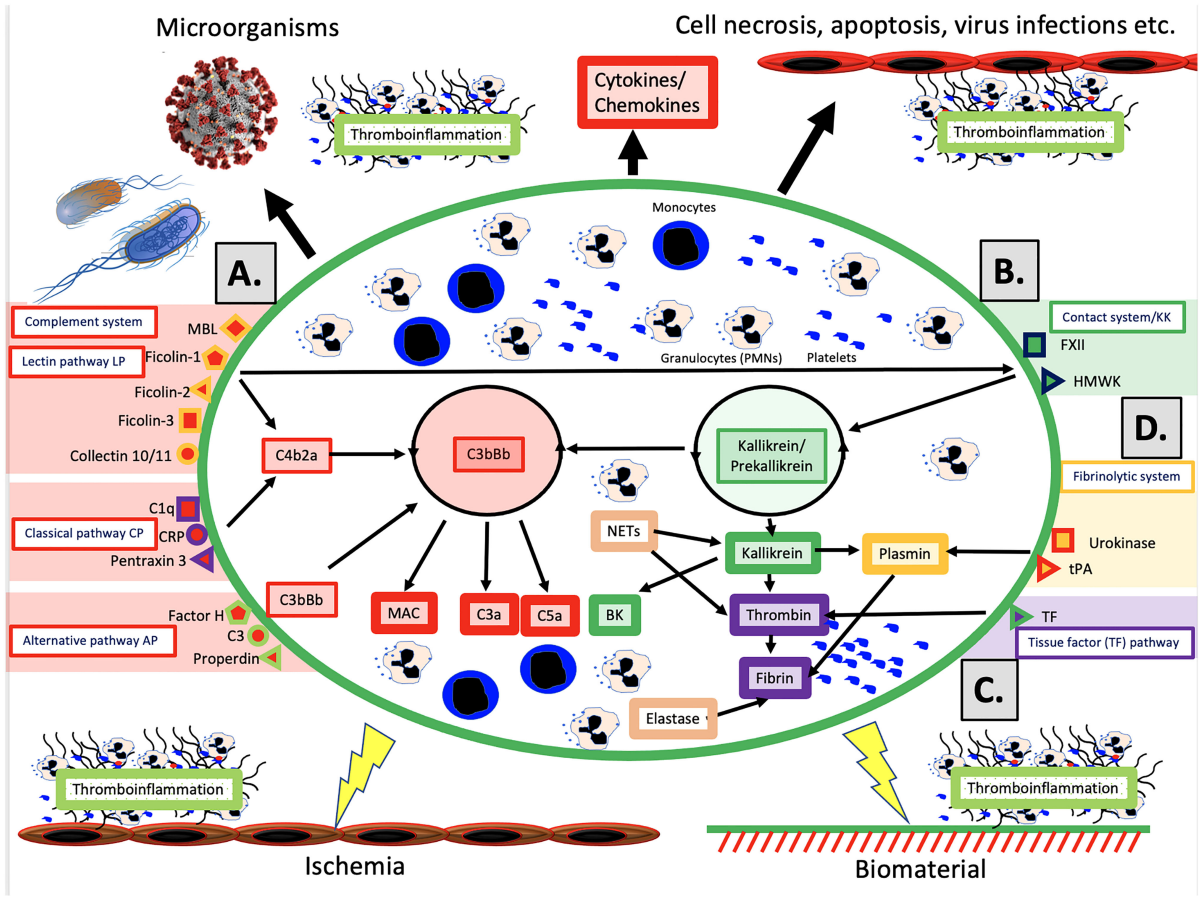


FIGURE 1 | Physiological and pathological conditions and treatments involving IIIS activation. The IIIS consists of the cascade systems of the blood: the complement system **(A)**, the contact or kallikrein/kinin system (KKS) **(B)**, the tissue factor (TF) pathway of the coagulation system **(C)**, and the fibrinolytic system **(D)**. Activation of IIIS occur in response to physiological stimuli such as microorganisms or necrotic, apoptotic or virus infected cells (top in figure), which leads to thromboinflammation. But also during pathological or therapeutic conditions such as ischemia during transplantation, or treatment with biomaterials e.g. stents, intravascular devices, and extracorporeal treatment (bottom in figure) a similar reaction occur. **(A)** The complement system has three activation pathways, which are triggered by different recognition molecules. The classical pathway (CP) is initiated by C1q, which binds to antigen bound IgG and IgM, but also to negatively charged surfaces and target-bound pentraxins e.g. CRP and pentraxin 3. The lectin pathway (LP) is activated by a number of carbohydrate binding proteins (lectins) such as mannan binding lectin (MBL), Ficolin-1, -2, -3 and Collectin 10/11 (4). The alternative pathway (AP) is be activated/regulated by surface-specific binding of factor H, C3 or properdin to a target, and has its main role as an amplifier. Complement activation leads to assembly of two enzyme complexes C4bC2a and C3bBb, which cleave C3 into the anaphylatoxin C3a and surface bound C3b (opsonization) and cleaves C5, which initiates the formation of the membrane attack complex (MAC) and the more potent anaphylatoxin C5a. **(B)** The primary function of the kallikrein/kinin and coagulation systems is in hemostasis but both systems are also engaged in inflammation. The recognition molecule in the contact/KK system is factor XII (FXII), which is activated, e.g., by negatively charged molecules such as LPS, glycosaminoglycans or extracellular matrix molecules exposed to the blood stream. The KK system also initiates an amplification loop, which involves prekallikrein that cleaves high molecular-weight kininogen (HMWK) leading to the generation of the proinflammatory mediator bradykinin (BK). **(C)** The main physiological trigger of coagulation, tissue factor (TF), is exposed in a functionally active form only after damage to vessels and activation of blood cells including platelets. It thereby initiates the extrinsic part of the coagulation cascade, which leads to formation of high amounts of thrombin. **(D)** The fibrinolytic system is initiated when urokinase or tissue plasminogen activator (tPA) activate plasminogen to plasmin, which degrades a formed fibrin network to soluble fibrin fragments. Please see the text for information on the roles of monocytes, PMNs and platelets.

- The CP is initiated by C1q, which binds to negatively charged surfaces, to IgG and IgM in immune complexes, and to target-bound pentraxins such as CRP and pentraxin 3.
- The LP is activated by a number of lectins (i.e., carbohydrate-binding proteins) such as MBL, Ficolin-1, -2, and -3, and by the Collectins 10/11 (4).
- The AP functions primarily as an amplification loop but can be specifically regulated by properdin in concert with C3 and by factor H as an important recognition molecule controlling the AP convertase.

In vivo, the coagulation system is mainly activated by the extrinsic pathway elicited by tissue factor (TF), which is exposed in the vessel wall after endothelial cell damage. TF is also expressed by multiple cells in response to inflammatory signals (6) and when cell-bound TF is exposed to blood it leads to a strong coagulation activation. Active TF can also be expressed by activated monocytes in response to, e.g., C5a and on the endothelium during inflammation. The KK system is an alternative route for coagulation activation, initiated on contact between blood and negatively charged foreign surfaces. Activation of the contact system is the reason why blood collected without an anticoagulant, coagulates in a test tube. KK is also regulating the vascular permeability on endothelial cells (7).

Factor XII (FXII) has a dual role in that it is the starting point of both the intrinsic pathway activation of the coagulation system and KK system. FXII probably has a limited role in physiological hemostasis, which is illustrated by the fact that FXII deficiency (Hageman disease) does not lead to an increased tendency for bleeding (8). In contrast, inhibition of FXII is considered as a new possible strategy as an antithrombotic drug with minor risk for bleeding (9). The KK system consists of FXII, prekallikrein and high molecular weight kininogen (HMWK) (8). FXIIa cleaves prekallikrein generating kallikrein. Kallikrein can cleave FXII and prekallikrein thereby providing a positive feedback loop. The KK system elicits inflammation *via* kallikrein, which cleaves HMWK generating BK.

The fibrinolytic system is initiated by urokinase, tissue plasminogen activator (tPA) and FXIIa by activating plasminogen to plasmin (10). Activated plasmin in turn breaks down the fibrin network formed in the final stages of the coagulation reaction, and thus acts as a physiological restriction mechanism that limits the propagation of the clot (10).

THE FUNCTION OF THE IIIS

Significant cross-activation can take place directly or indirectly *via* leukocytes and platelets, which means that activation of one of the cascade systems can spread to the entire IIIS. The physiological end result of IIIS activation is thromboinflammation, which stops bleeding by sealing blood vessel leakage through fibrin formation and platelet aggregation, and supports the clearance of damaged cells by attracting leukocytes that remove the damaged tissue. The IIIS is the start of wound healing after an injury, where the "waste management function", i.e., removal of foreign substances, particles and apoptotic or necrotic cells is an important task. This process is combined with the release of growth factors from, e.g., activated platelets, which ultimately leads to tissue healing and scar formation (11).

In a similar way, the IIIS reacts to infections caused by different types of microorganisms. In these reactions, the IIIS helps to kill and remove the microorganisms or the infected cells, after which the tissue is cleansed and healed as a result of IIIS functions. Sometimes, however, the reaction can shoot over the target and become too strong, which leads to severe inflammation and tissue damage. The end result is an excessive and pathological thromboinflammation with activation of all IIIS components such as in sepsis (**Figure 1**).

COVID-19 AND IIIS ACTIVATION LEADING TO THROMBINFLAMMATION

The COVID-19 pandemic, which is caused by the corona virus SARS-CoV-2 was initially described in Wuhan, China at the end of 2019 and has since had an immense effect on human society worldwide. Most SARS-CoV-2 infected patients experience influenza-like symptoms of low or moderate severity that are characterized by sore throat, fever, a dry cough, intestinal problems, and a loss of taste and smell. Early in the pandemic, it was reported that most patients suffered from an acute-phase reaction, with high levels of certain plasma proteins, e.g., fibrinogen, C3, and ferritin that were sometimes multiple times higher than normal (12). It also became clear that there was an increased risk of severe thromboembolism (13, 14). In some patients, the disease aggravated after approximately 9 to 10 days and turned into a full-blown ARDS-like pulmonary inflammation with endothelialitis, thrombosis and vascular angiogenesis, which often required intensive care treatment with ventilator support (15). The lung injury in COVID-19 resembles the pulmonary complications that have been observed in earlier SARS-1 and MERS epidemics (16). In the beginning of the COVID pandemic, up to 30% of the intensive care unit (ICU) patients died due to pulmonary and other organ dysfunctions. Those patients who reached the late-stage syndrome developed a severe pulmonary inflammation with general cyto-/chemokine expression (17), reduced oxygen saturation, increased risk of pulmonary thrombosis, and signs of severe pulmonary damage revealed by CT, particularly in the periphery of the lungs (18, 19).

In 2020, we studied the first 65 patients with intensive care-requiring COVID-19 admitted to the ICU of the university hospital in Uppsala, Sweden (1). These patients were admitted to the ICU before the therapeutic procedures now used, such as corticosteroids, were introduced. These patients were comprehensively investigated and were found to have a pronounced activation of the IIIS, with activation of all cascade systems and especially the KK system with prekallikrein, FXII and HMWK consumption (i.e. activated) and kallikrein/C1-INH complex formation (1).

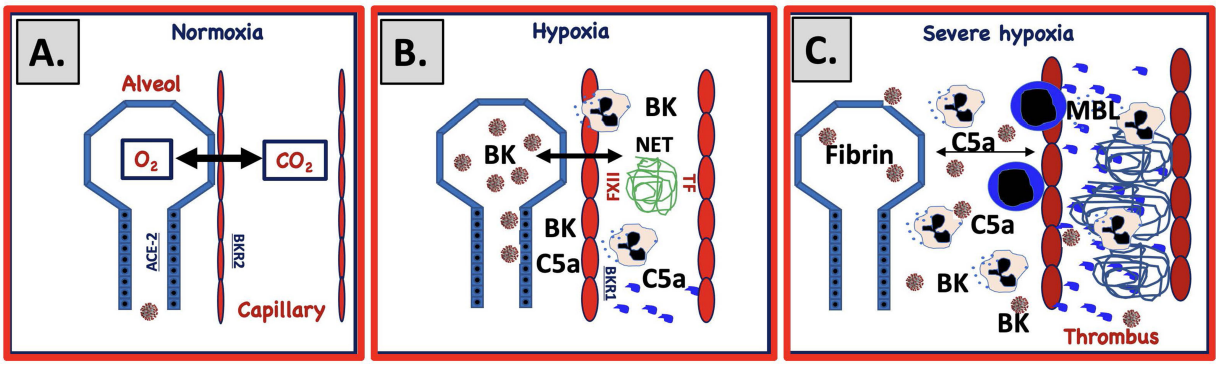


FIGURE 2 | Proposed mechanism for IIIS involvement in SARS-CoV-2-induced ARDS. **(A)** Under normal circumstances gas is exchanged over a narrow gap between alveolar epithelial cells (blue) and capillary endothelial cells (red). SARS-CoV-2 infects the alveolar/bronchial epithelial cells *via* ACE-2. Bronchial epithelial cells express ACE-2 and endothelial cells bradykinin receptor 2 (BKR2). Infection of alveolar epithelial cells activates the complement and KK systems and generates C5a and bradykinin (BK). **(B)** C5a and BK activate endothelial cells and elicit increased vascular permeability, which widens the gap between the cell linings. Activated endothelial cells also trigger complement and KK systems activation that upregulates BKR1, further increasing vascular permeability, damaging cells and inducing necrosis and apoptosis. BK and C5a elicit chemotaxis of PMNs that release neutrophil extracellular traps (NETs, depicted as a blue mesh). **(C)** Activated endothelial cells (TF) and NETs (TF and FXIIa) trigger coagulation activation and thrombus formation and further amplifie KK and complement activation. Plasma proteins leak into the alveolae causing fibrin precipitation. Invasion of PMNs and monocytes further increases the gap between the cell linings, ultimately leading to a collapsed exchange of gases over the epithelial and endothelial border.

THROMBOINFLAMMATORY CHANGES IN VARIOUS TISSUES OF COVID-19 PATIENTS

In COVID-19 patients, all parts of the IIIS are strongly activated, especially in those who are admitted to the ICU with ARDS-like symptoms. Most morphological studies have been performed on lung tissue from autopsies of patients that had succumbed at the ICU, e.g. (20, 21). Macroscopic observations show that large sections of the pulmonary lobes were clogged with cells and fluid, explaining the poor oxygenation of the blood in severely ill patients. From these cases of seriously ill COVD-19 patients, it was observed that the pulmonary tissue was infiltrated with immune cells (20). Initially, lymphocytes and macrophages were reported to be the dominating cell types (22), but later studies also showed that PMNs were important players (23). This discrepancy was probably due to that the latter observation were obtained in patients with earlier disease. Another important finding is that the alveolar space is filled with plasma proteins, e.g., fibrinogen giving rise to fibrin clots and that the interalveolar capillaries are activated and obstructed with platelet-containing thrombi (**Figure 2**). A few cases with immunohistochemical studies of complement components have been performed (24, 25). They show deposition of C4d, C3d, factor B, sC5b-9 on the endothelium in various tissues, preferentially in the lung, which can be interpreted as if complement activation *via* the CP or/and the LP occurs with amplification *via* the AP. Taken together these findings are consistent with a widespread thromboinflammation in the lungs.

Late-stage severe COVID-19 primarily affects the lungs but extends to other organs causing, in the more serious cases, multiorgan impact including cardiac, central nervous system and kidney injury (26–28). Myocardial dysfunction, arrhythmia and acute coronary syndrome have been reported in association with COVID-19, but in a recently performed study on 30 patients with severe COVID-19 disease late cardiac pathology of biopsies showed no relevant cardiac histopathological alterations (29, 30). At present the contribution of SARS-CoV2 on cardiac lesions remains to be established (31). Activation of the complement system might be involved in the worsening of renal symptoms since deposition of C3 was significant in renal arteries and in glomerular capillaries of COVID-19 biopsies (32). This is supported by findings in our own study, which shows a correlation between C3a generation and glomerular filtration rate (1). Neurological disorders associated with COVID-19, such as outright encephalitis is rare but has been reported with direct SARS-CoV2 detection in brain tissue (33). However, severe COVID-19 with long ICU stay has been associated with critical illness involving neuro/myopathy (34) and post intensive care syndrome (35) where thromboinflammation may play a role.

THROMBOSIS IN COVID-19

Already early in the COVID-19 pandemic, it appeared that moderately to seriously ill patients showed a very strong involvement of the coagulation system, and a high incidence of thromboembolic complications was reported on both the arterial and the venous side. In order to prevent these complications, treatment of the patients with high doses (typically 150-300 IU/kg) of LMWH was used (13, 14). Initially it was thought that the cause of the thromboembolism was an effect directly targeting the coagulation system but there was no real evidence of a systemic activation. Morphological data from the lungs showed that defined sections of the tissue were rich in microcapillary

thrombi suggesting rather that the clotting process was taking place locally close to the activated endothelium (18). The coagulation abnormalities that occur during COVID -19 disease were given a special name, COVID -19-associated coagulopathy (CAC), to emphasize what has emerged over time, that it is a condition with certain characteristics of its own. It is clear that CAC differs from the picture seen in sepsis and the so-called DIC (disseminated intravascular coagulation) where low platelet counts are a typical sign and extensive consumption of platelets and coagulation factors leads to thrombosis and bleeding at the same time (12, 36). Severely ill COVID -19 patients instead have normal or high platelet levels and are characterized by greatly elevated plasma levels of the fibrin degradation product D-dimer, which is a sensitive marker of fibrinolysis and coagulation turnover and probably reflects widespread thrombosis in e.g. small vessels in the lungs. It has been possible to reproduce the CAC phenotype in a primate model infected with SARS-CoV-2, and where an extensive fibrin deposition in the lung tissue was observed, in line with findings from autopsy materials of deceased COVID -19 patients (15, 37).

A number of hypotheses regarding the mechanisms behind CAC have been proposed. We have studied the relationship between complement and CAC, and shown that thrombosis in intensive care COVID -19 patients is linked to high activity in the LP and high plasma levels of the LP protein MBL (38). Future studies may show whether there is an underlying causal relationship between the LP and thrombosis, something that is supported by preclinical results where LP components MASP-1 and MASP-2 have a coagulation factor-like activity and can directly activate fibrinogen and prothrombin in *in vitro* experiments (39). There are also reports that neutrophil extracellular traps (NETs) that are released in response to C5a may be an initiator of coagulation since NETs expose TF and bind FXII (40). Similarly, activated endothelial cells express TF, which may contribute to the thromboinflammation.

ACTIVATION INDIVIDUAL COMPONENTS OF IIIS IN COVID-19

In COVID-19 an acute phase reaction increases the concentration of a number of plasma proteins multi-fold and thereby facilitates IIIS activation (41). Multiple proinflammatory cytokines and chemokines such as TNF, IL-1β, IL-6, and MCP-1 are produced in response to C3a and C5a generation that further amplifies the production of acute-phase proteins (41, 42). The IIIS activation is multifactorial: The virus itself can activate the IIIS directly *via* the CP and LP of the complement system and *via* the KK system (43–45). Another likely activation mechanism is tissue damage in the lungs with apoptotic and necrotic cells that initiates the overactivation of IIIS in its function to eliminate cells or cell debris (46).

The Complement System

The complement system was early suspected to be involved in the pathophysiology of severe COVID-19 disease (26, 47–51) and has thereafter been thoroughly investigated and shown to be involved in the disease and correlated with the severity of the disease. The first indication was reported early in the pandemic where increased levels of sC5b-9 were detected and a bit later a more complete report was published; both demonstrating that increased levels of sC5b-9 were associated with severe disease (52, 53). Thereafter, several comprehensive studies have been published, which assess a number of different complement parameters. These studies show that individual complement components increase and decrease in concentration during the course of the disease without any clear patterns (1, 54). For instance, C3 and C4 and other components such as C1q and MBL, and complement function (CP and AP) are elevated in some patients and decreased in others, indicating that both overexpression and complement activation with consumption occur. Overexpressed and elevated levels of factor B tend to be associated with severe COVID-19. In addition to sC5b-9 generation, formation of other activation markers occurs: C4d, Bb, C3a, and C3d,g are generated and seem to be activated early in the disease. The early trigger of complement activation is still unclear; most likely several activators elicit this response. Activation has been reported to be mediated by the intact virus *via* intracellular complement activation (55) but also by virus proteins expressed on the surface of infected cells. The virus itself has been shown to bind MBL, Ficolin-2 and Collectin-11, *via* its S- and N-proteins, with subsequent LP-mediated C3b and C4b deposition (44, 45). Structural parts of the virus have also been shown to bind C1q and trigger complement activation (43). COVID-19 is strongly associated with ischemic cells, apoptosis, and cell death (15, 20, 40), initially occurring in the lung tissue. These conditions lead to binding of MBL, MASP-2 and C1q to the cells (see below), which may be the combined initiators of the accelerating complement activation that occurs particularly in severely ill patients. In our own study, the C1q concentration is low (consumed) in several patients at admission to the ICU approximately at day 10, possibly caused by damaged cells in the lungs (1).

The combined data indicate that the maximum activation occurs at approximately day 10 coinciding with when patients are most likely to be admitted to the ICU. C4 and C3 consumption combined with C4d, C3a and C3d,g generation supports complement activation *via* CP/LP activation. Daily monitoring of C3d,g has been reported to predict outcome in patients hospitalized with COVID-19 in combination with SARS-CoV-2 nucleocapsid antigen, RNA in blood, IL-6, and CRP (56).

The KK System

In several early reviews and *in vitro studies*, the KK system was suggested to participate in the thromboinflammation of COVID-19 (43, 57–59), without presenting any evidence of KK system activation *in vivo*. No direct evidence of such activation was published until reports from us, and Busch et al. showed strong KK system activation in severely ill ICU patients (1, 60). The KK system activation was assessed by consumption of prekallikrein, HMWK and FXII, and by increased levels of kallikrein/C1-INH complexes (1). The activation of prekallikrein and HMWK on

the admission to the ICU was shown to have prognostic significance for survival and the need for mechanical ventilation, but also for other systemic and specific organ parameters, such as, poor kidney function.

The Fibrinolytic System

A consequence of the ongoing coagulation activation is generation of fibrin from fibrinogen. In the clot that is formed, plasmin is generated, which cleaves fibrin into fragments including D-dimer. D-dimer is an important clinical biomarker for fibrinolysis and indirectly for clot formation. Due to its general availability, this marker was one of the first biomarkers of IIIS reported to increase in COVID-19 and the levels were found to be associated with disease severity (61).

Granulocytes (PMN)

The neutrophilic granulocytes, which are found in the lungs early in the course of the disease (23),have a profound effect on the outcome of COVID-19 (23). Several drivers of cell infiltration exist and the anaphylatoxins of the IIIS (C3a and C5a) as well as BK are likely to be important contributors. Chemotactic effects are mediated by the specific receptors C3aR (C3a), C5aR1 and 2 (C5a) and by BKR1 and 2 (BK). These receptors also trigger activation of the PMNs, which release proteases and other granular proteins, e.g., elastase, myeloperoxidase and protein-arginine deiminase type 4. One specific effect related to PMNs is the generation of NETs, which are large, extracellular, web-like structures consisting of cytosolic and granule proteins with a scaffold of decondensed chromatin in which the majority of the DNA originates from the nucleus (62). NETs protect against infection but have also been associated with a number of other immunoregulatory events such as binding antimicrobial peptides and priming immune cells (62). In addition to this, NETs promote thrombotic events, since they bind both TF (40) and FXIIa (63) thereby being able to trigger both the extrinsic pathway of coagulation as well as the contact system and consequently the intrinsic pathway of coagulation. NET formation has been suggested to be an important promotor of thrombosis in the lungs of COVID-19 patients where C5a has been proposed to elicit the release of these morphological structures (60, 62).

Platelets and Endothelial Cells

Increased platelet activation and platelet-monocyte aggregates were observed in COVID-19 patients but not in patients presenting a mild syndrome (64). Platelet counts tend to increase in COVID-19 patients and not be consumed and decrease in number as in patients with thromboembolism. This observed difference, which speaks against that the thrombi found in the lungs in COVID-19 patients are the result of embolism with a major thrombus formed elsewhere such as in deep vein thrombosis (DVT). Instead, it has been observed that COVID-19 patients exhibit reduced procoagulant platelet responses (65), but platelets are found in the thrombi formed in the lung capillaries either as a result of activated endothelial cells or NETs formation (40). Endothelial cells are poorly infected by Cov-Sars-2 since these cells express a low number of ACE-2 molecules (66), but generation of both C5a and BK by the nearby pneumocytes can initiate expression of TF on both endothelial cells (67)and neutrophils (40, 68) thereby promoting local thrombus formation.

ISCHEMIC INJURY

Hypoxia is anticipated to occur, particularly in the lungs during COVID-19, where major parts of the small airways may be totally clogged with fibrin, platelet-rich thrombi, and cells [**Figure 2**; (46)]. Ischemia is expected to be a mechanism that is involved in the injury mediated by the IIIS in COVID-19, considering the low oxygen saturation in these patients. Ischemia is a major stress to the cell, which can lead to changes in the composition and protein expression of the cell membrane (62). Consequently, IIIS in contact with a cell subjected to hypoxia, can recognize the cell surface as foreign, causing a thromboinflammatory reaction and, by extension, ischemia/reperfusion injury-like damage and cell death. The ischemic cell has a distinctive phenotype compared to the native one, which leads to that IIIS recognition molecules of the complement and the KK systems target the cell as foreign (non-self). In ischemia/reperfusion injury, MBL (69) and MASP-2 (70) of the LP and innate IgM antibodies (71, 72) of the CP have been suspected to be involved in the IIIS activation that occurs.

THE RENIN-ANGIOTENSIN SYSTEM (RAS)

Hypertension is linked to the renin-angiotensin system (RAS) and an increased risk for severe COVID-19 infection. The docking protein for SARS-CoV-2 on human cells is angiotensin converting enzyme (ACE)-2 of the angiotensinogen cascade system, which may destroy the function of this protein. ACE-1 inhibitors (common hypertensive drugs) block the cleavage of angiotensin I to angiotensin II. ACE-1 is a regulator of BK, making it feasible that inhibition of ACE-1 could aggravate the ARDS condition in COVID-19 patients by increasing the levels of active BK. Although in a meta study focusing on the effects of renin-angiotensin system (RAS) inhibitors on the RAS and the outcome of COVID-19, no support for this concept was found. However, since this study is based on several clinical trials that treat RAS inhibitors as a common group further studies are needed to elucidate this issue (58, 73).

CONCEPTUAL MECHANISMS INDUCING COVID-19-TRIGGERED ARDS

SARS-CoV-2-infected and ischemic, damaged epithelial and endothelial cells can activate all the IIIS cascade systems in a joint thromboinflammatory reaction in the lungs (**Figure 2**). During the development of ARDS, SARS CoV-2 and damaged cells (SARS CoV-2-infected, apoptotic, necrotic cells) are potential targets for the recognition molecules of the blood cascade systems. C1q, MBL, and FXII are known to recognize apoptotic and necrotic cells that

can trigger the CP and LP of complement ultimately leading to cleavage of C3/C5 into C3a/C5a and C3b/C5b. BK generated by the KK system activation can cause dry cough (74) and pulmonary inflammation with edema (75) by binding to BKR2. C3a and C5a bind to the anaphylatoxin receptors: C3aR, C5aR1, and C5aR2 and C5a is also able to activate endothelial cells in COVID-19 patients leading to von Willebrand Factor (vWF) and p-selectin exposure on the endothelial lining thereby contributing to the thrombotic phenotype (76). Supporting the importance of the C5a/C5aR1 axis is that COVID-19 patients generate C5a as detected in pulmonary lavage in proportion to the severity of the disease and high expression of C5aR1 was found in blood and on pulmonary myeloid cells (77). C5a also induces expression of BKR1, (which contrary to BKR2 is not constitutively expressed) and BK can then act *via* both BKR2 (expressed on both the pulmonary epithelium and endothelium) and after cleavage to desArg9-BK *via* BKR1 (78–80), which also elicits increased vascular permeability, PMN chemotaxis, and nerve end stimulation, amplifying all of the above-mentioned reactions. Thus, C3a, C5a, and BK combined have the potential to cause a local edema and pulmonary leukocyte infiltration/inflammation that increases the distance between epithelial and endothelial cells in the alveolae, thereby hindering oxygenation of the blood (**Figure 2**). The poor oxygenation can lead to further ischemia (as described above) that in turn leads to even more IIIS activation thereby creating a vicious circle. Local thrombosis in the vasculature of the lung induced by activated endothelial cells that bind MBL, and expose selectins, TF and vWF on their surfaces, further aggravates the hypoxia. Chemotaxis and activation of neutrophlic granulocytes also cause NET formation and FXII activation (40, 81). Taken together these phenomena are likely drivers of the endothelialitis, the thrombus formation and the increased vascular angiogenesis linked to COVID-19 patients (15).

Activation of the IIIS in COVID-19-induced ARDS have many similarities to ARDS of other etiologies, e.g., as in sepsis but there are also large differences both regarding activation mechanisms and the focus of the inflammation. We have previously reported that the activation of the IIIS in COVID-19 mainly occurs *via* the KK system and the LP and CP of the complement system (1). By contrast, in septic shock, it has been reported by others that the key steps of complement activation consist of first the AP followed by the CP (36). One explanation of these discrepancies may be the specific pathophysiologic process in SARS-CoV-2 infection as compared with the diverse and heterogeneous microbiology in sepsis of different origin. Another important dissimilarity between COVID-19 and sepsis-induced ARDS is the focus and origin of inflammation. In COVID-19, ARDS originates from the lung, which is in contrast to sepsis-induced ARDS that is caused by a secondary systemic inflammatory response in a distant organ, unless the infection focus already is in the lung (82, 83). In the latter case, usually termed pulmonary ARDS, the lung is the driver of IIIS activation with the highest IIIS activation locally, while in the former, usually termed extrapulmonary ARDS, the lung is only secondarily affected and IIIS activation measured as activation products in the plasma is less dependent on the severity of ARDS.

TABLE 1 | All trials currently (2022-01-28) registered in ClinicalTrials.gov testing targeting IIIS components in COVID-19.

Drug (target)	Identifier	Participants	Study design	Last update
Kallikrein				
lanadelumab (kallikrein)	NCT04422509	43	Randomized vs SOC	Nov 16, 2021
lanadelumab (kallikrein)	NCT04460105	0	Randomized vs placebo	Oct 20, 2020
ISIS 721744 (kallikrein antisense)	NCT04549922	111	Randomized vs placebo	April 19, 2021
Icatibant				
C1-INH ± icatibant	NCT05010876	44	Randomized vs SOC	Aug 18, 2021
Icatibant	NCT04978051	120	Randomized vs SOC	July 27, 2021
C1-INH				
Conestat alfa (recomb C1-INH)	NCT04414631	80	Randomized vs SOC	Nov 9, 2021
Ruconest (recomb C1-INH)	NCT04705831	40	Randomized vs SOC, crossover	Jan 12, 2021
Ruconest (recomb C1-INH)	NCT04530136	120	Randomized vs SOC	Dec 10, 2020
C5 cleavage inhibitors				
Eculizumab	NCT04346797	120	Randomized vs SOC	April 20, 2020
Eculizumab	NCT04288713	no info	no info found	March 20, 2020
Ravulizumab	NCT04390464	1167 (3 arms)	Randomized vs SOC	May 18, 2020
Ravulizumab	NCT04570397	32	Randomized vs SOC	Jan 14, 2021
Ravulizumab	NCT04369469	120	Randomized vs SOC	Sept 22, 2021
Zilucoplan (C5 cleavage inhibiting peptide)	NCT04382755	81	Randomized vs SOC + antibiotics	July 2, 2021
Zilucoplan (C5 cleavage inhibiting peptide)	NCT04590586	516 (7 arms)	Randomized vs SOC + placebo	Nov 23, 2021
C3 cleavage inhibitors				
AMY-101	NCT04395456	144	Randomized vs placebo	Feb 20, 2021
APL-9	NCT04402060	65	Randomized vs placebo	Sept 1, 2021
Lectin pathway inhibitor Narsoplimab (anti MASP-2) + Remdesivir (anti CD14)	NCT04488081	1500 (8 arms)	Randomized	July 21, 2021
C5aR antagonists				
avdoralimab (anti C5aR mAb)	NCT04371367	208	Randomized vs placebo	May 27, 2021
avdoralimab (anti C5aR mAb)	NCT04333914	219	Randomized vs SOC	Aug 5, 2021
vilobelimab (anti C5a mAb)	NCT04333420	390	Randomized vs SOC + placebo	Dec 31, 2021

SOC, standard of care.

In summary, we hypothesize that activation of the IIIS, either caused directly by SARS-CoV-2, or more likely by large quantities of activated and damaged cells resulting from viral infection and ischemia, is the pathophysiological mechanism of the thromboinflammatory reaction that triggers the ARDS linked to COVID-19.

THERAPEUTICS

A number of investigators have suggested the complement and KK systems as targets for treating COVID-19 (84). In addition to previously known treatment of IIIS activation *via* inhibition of the coagulation system (heparin, warfarin, direct-acting oral anticoagulants [DOAC], etc.), a large number of inhibitors have recently been developed to regulate the complement system. There are several licensed drugs that affect the components of the IIIS. Treatment of paroxysmal noctural hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS) and myasthenia gravis has been performed for a long time by inhibiting complement factor C5 (using the anti-C5 antibodies eculizumab and ravulizimab) with excellent results (85–87). Also, several other anti-complement drugs are being developed for use in, for example, treatment of COVID-19. Two of these AMY-101 and pegcatacoplan (both C3 inhibitors of the compstatin family) were recently tested and AMY-101 shown promising results with faster clinical recovery and a reduction in a number of inflammatory parameters such as plasma levels of IL-6 (88). In addition, the LP inhibitor narsoplimab (anti-MASP-2) has been tested in a small number of patients (89).

Inhibition of the KK system appears to be a possible step in the search for therapeutic alternatives for treatment of COVID-19. In support of this hypothesis are several publications including the successful treatment of ARDS with icatibant in hantavirus infection (90). The latter indicates that this may be a route of success, since it confirms that a BKR2 antagonist alleviated other types of virus-induced ARDS. Several currently registered drugs in clinical use for the treatment of angioedema target the KK system, which include icantibant, the kallikrein inhibitors lanadelumab (monoclonal antibody) and ecallantide (small molecular inhibitor), which is licensed in the USA. Also, C1-INH (both recombinant and in purified form), which inhibits both the complement and the KK systems, are examples of pharmaceuticals with a potential use to control IIIS activation in, e.g., COVID-19 patients. In a case control study C1-INH was investigated and shown to be safe and have some clinical effect (91). In a case study without controls (92) with recombinant C1-INH (conestat alfa) the drug was well taken but the study was indecisive. Considering reports of very high concentrations of C1-INH in COVID-19 patients, this finding may explain this result (60, 92).

In summary, all IIIS drugs were promising and had the expected effect on the targeted IIIS components and provided evidence that they affected thromboinflammation induced by IIIS to a varying degree. However, most of the studies included few patients without controls, which made evaluation of the results difficult. Further randomized and controlled studies will give us a deeper insight into the effect of these drugs alone. However, reflecting on the content of this review, combinations of IIIS inhibitors are most likely to be needed to get an optimal effect on COVID-19 ARDS. All trials currently registered in ClinicalTrials.gov testing IIIS-targeted components in COVID-19 are summarized in **Table 1**.

AUTHOR CONTRIBUTIONS

BN and KE have written and edited the major part of the review. OE, KF, and MH-L have contributed to the writing and editing of the article. All authors contributed to the article and approved the submitted version.

FUNDING

The study was funded by the Swedish Research Council grants 2016-01060, 2016-04519, 2020-05762, 2021-02252, the Swedish Heart-Lung Foundation grant HLF 2020-0398 and by faculty grants from the Linnaeus University.

REFERENCES

1. Lipcsey M, Persson B, Eriksson O, Blom AM, Fromell K, Hultström M, et al. The Outcome of Critically Ill COVID-19 Patients Is Linked to Thromboinflammation Dominated by the Kallikrein/Kinin System. *Front Immunol* (2021) 12:627579. doi: 10.3389/fimmu.2021.627579
2. Ekdahl KN, Teramura Y, Hamad OA, Asif S, Duehrkop C, Fromell K, et al. Dangerous Liaisons: Complement, Coagulation, and Kallikrein/Kinin Cross-Talk Act as a Linchpin in the Events Leading to Thromboinflammation. *Immunol Rev* (2016) 274(1):245–69. doi: 10.1111/imr.12471
3. Ekdahl KN, Hong J, Hamad OA, Larsson R, Nilsson B. Evaluation of the Blood Compatibility of Materials, Cells, and Tissues: Basic Concepts, Test Models, and Practival Guidelines. *Adv Exp Med Biol* (2013) 735:257–70. doi: 10.1007/978-1-4614-4118-2_18
4. Howard M, Farrar CA, Sacks SH. Structural and Functional Diversity of Collectins and Ficolins and Their Relationship to Disease. *Semin Immunopathol* (2018) 40:75–85. doi: 10.1007/s00281-017-0642-0
5. Ricklin D, Mastellos DC, Lambris JD. Therapeutic Targeting of the Complement System. *Nat Rev Drug Discovery* (2019) 9:1–1. doi: 10.1038/s41573-019-0055-y
6. *Tissue Factor* (2022). Available at: https://www.proteinatlas.org/ENSG00000117525-F3/tissue (Accessed January 27, 2022).
7. Kaplan AP, Ghebrehiwet B. The Plasma Bradykinin-Forming Pathways and its Interrelationships With Complement. *Mol Immunol* (2010) 47:2161–9. doi: 10.1016/j.molimm.2010.05.010
8. Hess R, Wujak L, Hesse C, Sewald K, Jonigk D, Warnecke G, et al. Coagulation Factor XII Regulates Inflammatory Responses in Human Lungs. *Thromb Haemostasis* (2017) 117:1896–907. doi: 10.1160/th16-12-0904
9. Wilbs J, Kong X-D, Middendorp SJ, Prince R, Cooke A, Demarest CT, et al. Cyclic Peptide FXII Inhibitor Provides Safe Anticoagulation in a Thrombosis Model and in Artificial Lungs. *Nat Commun* (2020) 11:3890. doi: 10.1038/s41467-020-17648-w
10. Maas C. Plasminflammation-An Emerging Pathway to Bradykinin Production. *Front Immunol* (2019) 10:2046. doi: 10.3389/fimmu.2019.02046

11. Broughton G, Janis JE, Attinger CE. The Basic Science of Wound Healing. *Plast Reconstr Surg* (2006) 117:12S–34S. doi: 10.1097/01.prs.0000225430.42531.c2
12. van Dam LF, Kroft LJM, van der Wal LI, Cannegieter SC, Eikenboom J, de Jonge E, et al. Clinical and Computed Tomography Characteristics of COVID-19 Associated Acute Pulmonary Embolism: A Different Phenotype of Thrombotic Disease? *Thromb Res* (2020) 193:86–9. doi: 10.1016/j.thromres.2020.06.010
13. Marietta M, Coluccio V, Luppi M. COVID-19, Coagulopathy and Venous Thromboembolism: More Questions Than Answers. *Intern Emerg Med* (2020) 15(8):1375–87. doi: 10.1007/s11739-020-02432-x
14. Stattin K, Lipcsey M, Andersson H, Pontén E, Anderberg SB, Gradin A, et al. Inadequate Prophylactic Effect of Low-Molecular Weight Heparin in Critically Ill COVID-19 Patients. *J Crit Care* (2020) 60:249–52. doi: 10.1016/j.jcrc.2020.08.026
15. Ackermann M, Verleden SE, Kuehnel M, Haverich A, Welte T, Laenger F, et al. Pulmonary Vascular Endothelialitis, Thrombosis, and Angiogenesis in Covid-19. *New Engl J Med* (2020) 383:120–8. doi: 10.1056/nejmoa2015432
16. Zeidler A, Karpinski TM. SARS-CoV, MERS-CoV, SARS-CoV-2 Comparison of Three Emerging Coronaviruses. *Jundishapur J Microb* (2020) 13(6): e103744. doi: 10.5812/jjm.103744
17. Skevaki C, Fragkou PC, Cheng C, Xie M, Renz H. Laboratory Characteristics of Patients Infected With the Novel SARS-CoV-2 Virus. *J Infection* (2020) 81:205–12. doi: 10.1016/j.jinf.2020.06.039
18. Cavagna E, Muratore F, Ferrari F. Pulmonary Thromboembolism in COVID-19: Venous Thromboembolism or Arterial Thrombosis? *Radiol Cardiothorac Imaging* (2020) 2:e200289. doi: 10.1148/ryct.2020200289
19. Mackman N, Antoniak S, Wolberg AS, Kasthuri R, Key NS. Coagulation Abnormalities and Thrombosis in Patients Infected With SARS-CoV-2 and Other Pandemic Viruses. *Arterioscler Thromb Vasc Biol* (2020) 40(9):2033–44. doi: 10.1161/atvbaha.120.314514
20. Szekely L, Bozoky B, Bendek M, Ostad M, Lavignasse P, Haag L, et al. Pulmonary Stromal Expansion and Intra-Alveolar Coagulation are Primary Causes of COVID-19 Death. *Heliyon* (2021) 7:e07134. doi: 10.1016/j.heliyon.2021.e07134
21. Azkur AK, Akdis M, Azkur D, Sokolowska M, Veen W, Brüggen M, et al. Immune Response to SARS-CoV-2 and Mechanisms of Immunopathological Changes in COVID-19. *Allergy* (2020) 75:1564–81. doi: 10.1111/all.14364
22. Stassi C, Mondello C, Baldino G, Cardia L, Asmundo A, Spagnolo EV. An Insight Into the Role of Postmortem Immunohistochemistry in the Comprehension of the Inflammatory Pathophysiology of COVID-19 Disease and Vaccine-Related Thrombotic Adverse Events: A Narrative Review. *Int J Mol Sci* (2021) 22:12024. doi: 10.3390/ijms222112024
23. Reusch N, Domenico ED, Bonaguro L, Schulte-Schrepping J, Baßler K, Schultze JL, et al. Neutrophils in COVID-19. *Front Immunol* (2021) 12:652470. doi: 10.3389/fimmu.2021.652470
24. Macor P, Durigutto P, Mangogna A, Bussani R, D'Errico S, Zanon M, et al. Multi-Organ Complement Deposition in COVID-19 Patients. *Biomedicines* (2021) 9:1003. doi: 10.3390/biomedicines9081003
25. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement Associated Microvascular Injury and Thrombosis in the Pathogenesis of Severe COVID-19 Infection: A Report of Five Cases. *Transl Res J Lab Clin Med* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
26. Noris M, Benigni A, Remuzzi G. The Case of Complement Activation in COVID-19 Multiorgan Impact. *Kidney Int* (2020) 98:314–22. doi: 10.1016/j.kint.2020.05.013
27. Luther T, Bülow-Anderberg S, Larsson A, Rubertsson S, Lipcsey M, Frithiof R, et al. COVID-19 Patients in Intensive Care Develop Predominantly Oliguric Acute Kidney Injury. *Acta Anaesth Scand* (2021) 65:364–72. doi: 10.1111/aas.13746
28. Virhammar J, Nääs A, Fällmar D, Cunningham JL, Klang A, Ashton NJ, et al. Biomarkers for Central Nervous System Injury in Cerebrospinal Fluid are Elevated in COVID-19 and Associated With Neurological Symptoms and Disease Severity. *Eur J Neurol* (2021) 28:3324–31. doi: 10.1111/ene.14703
29. Gupta A, Madhavan MV, Sehgal K, Nair N, Mahajan S, Sehrawat TS, et al. Extrapulmonary Manifestations of COVID-19. *Nat Med* (2020) 26:1017–32. doi: 10.1038/s41591-020-0968-3
30. Rosén J, Noreland M, Stattin K, Lipcsey M, Frithiof R, Malinovschi A, et al. Group UICC-19 R. ECG Pathology and its Association With Death in Critically Ill COVID-19 Patients, a Cohort Study. *PloS One* (2021) 16: e0261315. doi: 10.1371/journal.pone.0261315
31. Ferrer-Gómez A, Pian-Arias H, Carretero-Barrio I, Navarro-Cantero A, Pestaña D, de Pablo R, et al. Late Cardiac Pathology in Severe Covid-19. A Postmortem Series of 30 Patients. *Front Cardiovasc Med* (2021) 8:748396. doi: 10.3389/fcvm.2021.748396
32. Pfister F, Vonbrunn E, Ries T, Jäck H-M, Überla K, Lochnit G, et al. Complement Activation in Kidneys of Patients With COVID-19. *Front Immunol* (2021) 11:594849. doi: 10.3389/fimmu.2020.594849
33. Virhammar J, Kumlien E, Fällmar D, Frithiof R, Jackmann S, Sköld MK, et al. Acute Necrotizing Encephalopathy With SARS-CoV-2 RNA Confirmed in Cerebrospinal Fluid. *Neurology* (2020) 95:445–9. doi: 10.1212/wnl.0000000000010250
34. Frithiof R, Rostami E, Kumlien E, Virhammar J, Fällmar D, Hultström M, et al. Critical Illness Polyneuropathy, Myopathy and Neuronal Biomarkers in COVID-19 Patients: A Prospective Study. *Clin Neurophysiol Off J Int Fed Clin Neurophysiol* (2021) 132:1733–40. doi: 10.1016/j.clinph.2021.03.016
35. Wallin E, Hultström M, Lipcsey M, Frithiof R, Rubertsson S, Larsson I-M. Intensive Care-Treated COVID-19 Patients' Perception of Their Illness and Remaining Symptoms. *Acta Anaesth Scand* (2022) 66:240–7. doi: 10.1111/aas.13992
36. Charchaflieh J, Wei J, Labaze G, Hou YJ, Babarsh B, Stutz H, et al. The Role of Complement System in Septic Shock. *Clin Dev Immunol* (2012) 2012:407324. doi: 10.1155/2012/407324
37. Aid M, Busman-Sahay K, Vidal SJ, Maliga Z, Bondoc S, Starke C, et al. Vascular Disease and Thrombosis in SARS-CoV-2-Infected Rhesus Macaques. *Cell* (2020) 183:1354–1366.e13. doi: 10.1016/j.cell.2020.10.005
38. Eriksson O, Persson B, Lipcsey M, Ekdahl K, Nilsson B. Mannose-Binding Lectin is Associated With Thrombosis and Coagulopathy in Critically Ill COVID-19 Patients. *Thromb Haemostasis* (2020) 120(12):1720–4. doi: 10.1055/s-0040-1715835
39. Ip WKE, Chan KH, Law HKW, Tso GHW, Kong EKP, Wong WHS, et al. Mannose-Binding Lectin in Severe Acute Respiratory Syndrome Coronavirus Infection. *J Infect Dis* (2005) 191:1697–704. doi: 10.1086/429631
40. Skendros P, Mitsios A, Chrysanthopoulou A, Mastellos DC, Metallidis S, Rafailidis P, et al. Complement and Tissue Factor-Enriched Neutrophil Extracellular Traps are Key Drivers in COVID-19 Immunothrombosis. *J Clin Invest* (2020) 130(11):6151–7. doi: 10.1172/jci141374
41. Epstein FH, Gabay C, Kushner I. Acute-Phase Proteins and Other Systemic Responses to Inflammation. *New Engl J Med* (1999) 340:448–54. doi: 10.1056/nejm199902113400607
42. Wang R, Xiao H, Guo R, Li Y, Shen B. The Role of C5a in Acute Lung Injury Induced by Highly Pathogenic Viral Infections. *Emerg Microbes Infect* (2019) 4:1–7. doi: 10.1038/emi.2015.28
43. Savitt AG, Manimala S, White T, Fandaros M, Yin W, Duan H, et al. SARS-CoV-2 Exacerbates COVID-19 Pathology Through Activation of the Complement and Kinin Systems. *Front Immunol* (2021) 12:767347. doi: 10.3389/fimmu.2021.767347
44. Ali YM, Ferrari M, Lynch NJ, Yaseen S, Dudler T, Gragerov S, et al. Lectin Pathway Mediates Complement Activation by SARS-CoV-2 Proteins. *Front Immunol* (2021) 12:714511. doi: 10.3389/fimmu.2021.714511
45. Stravalaci M, Pagani I, Paraboschi E, Pedotti M, Doni A, Scavello F, et al. Recognition and Inhibition of SARS-CoV-2 by Humoral Innate Immunity Pattern Recognition Molecules. *Nat Immunol* (2022) 23(2):275–86. doi: 10.1038/s41590-021-01114-w.
46. Brosnahan SB, Jonkman AH, Kugler MC, Munger JS, Kaufman DA. COVID-19 and Respiratory System Disorders: Current Knowledge, Future Clinical and Translational Research Questions. *Arterioscler Thromb Vasc Biol* (2020) 40:2586–97. doi: 10.1161/atvbaha.120.314515
47. Cugno M, Meroni PL, Gualtierotti R, Griffini S, Grovetti E, Torri A, et al. Complement Activation and Endothelial Perturbation Parallel COVID-19 Severity and Activity. *J Autoimmun* (2021) 116:102560. doi: 10.1016/j.jaut.2020.102560
48. Java A, Apicelli AJ, Liszewski MK, Coler-Reilly A, Atkinson JP, Kim AHJ, et al. The Complement System in COVID-19: Friend and Foe? *JCI Insight* (2020) 5(15):e140711. doi: 10.1172/jci.insight.140711
49. Lo MW, Kemper C, Woodruff TM. COVID-19: Complement, Coagulation, and Collateral Damage. *J Immunol* (2022) 205(6):1488–90. doi: 10.4049/jimmunol.2000644
50. Risitano AM, Mastellos DC, Huber-Lang M, Yancopoulou D, Garlanda C, Ciceri F, et al. Complement as a Target in COVID-19? *Nat Rev Immunol* (2020) 20:343–4. doi: 10.1038/s41577-020-0320-7

51. Tomo S, Kumar KP, Roy D, Sankanagoudar S, Purohit P, Yadav D, et al. Complement Activation and Coagulopathy - an Ominous Duo in COVID19. *Expert Rev Hematol* (2021) 14:1–19. doi: 10.1080/17474086.2021.1875813
52. Holter JC, Pischke SE, de BE, Lind A, Jenum S, Holten AR, et al. Systemic Complement Activation is Associated With Respiratory Failure in COVID-19 Hospitalized Patients. *Proc Natl Acad Sci* (2020) 117:25018–25. doi: 10.1073/pnas.2010540117
53. Cugno M, Meroni PL, Gualtierotti R, Griffini S, Grovetti E, Torri A, et al. COMPLEMENT ACTIVATION IN PATIENTS WITH COVID-19: A NOVEL THERAPEUTIC TARGET. *J Allergy Clin Immunol* (2020) 146:215–7. doi: 10.1016/j.jaci.2020.05.006
54. Ma L, Sahu SK, Cano M, Kuppuswamy V, Bajwa J, McPhatter J, et al. Increased Complement Activation is a Distinctive Feature of Severe SARS-CoV-2 Infection. *Sci Immunol* (2021) 6:eabh2259. doi: 10.1126/sciimmunol.abh2259
55. Yan B, Freiwald T, Chauss D, Wang L, West E, Mirabelli C, et al. SARS-CoV-2 Drives JAK1/2-Dependent Local Complement Hyperactivation. *Sci Immunol* (2021) 6:eabg0833. doi: 10.1126/sciimmunol.abg0833
56. Brasen CL, Christensen H, Olsen DA, Kahns S, Andersen RF, Madsen JB, et al. Daily Monitoring of Viral Load Measured as SARS-CoV-2 Antigen and RNA in Blood, IL-6, CRP and Complement C3d Predicts Outcome in Patients Hospitalized With COVID-19. *Clin Chem Lab Med Cclm* (2021) 0:000010151520210694. doi: 10.1515/cclm-2021-0694
57. Colarusso C, Terlizzi M, Pinto A, Sorrentino R. A Lesson From a Saboteur: High Molecular Weight Kininogen (HMWK) Impact in COVID-19. *Brit J Pharmacol* (2020) 177(21):4866–72. doi: 10.1111/bph.15154
58. Garvin MR, Alvarez C, Miller JI, Prates ET, Walker AM, Amos BK, et al. A Mechanistic Model and Therapeutic Interventions for COVID-19 Involving a RAS-Mediated Bradykinin Storm. *Elife* (2020) 9:e59177. doi: 10.7554/elife.59177
59. Maglakelidze N, Manto KM, Craig TJ. A Review: Does Complement or the Contact System Have a Role in Protection or Pathogenesis of COVID-19? *Pulm Ther* (2020) 6(2):169–76. doi: 10.1007/s41030-020-00118-5
60. Busch MH, Timmermans SAMEG, Nagy M, Visser M, Huckriede J, Aendekerk JP, et al. Neutrophils and Contact Activation of Coagulation as Potential Drivers of COVID-19. *Circulation* (2020) 142:1787–90. doi: 10.1161/circulationaha.120.050656
61. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical Course and Risk Factors for Mortality of Adult Inpatients With COVID-19 in Wuhan, China: A Retrospective Cohort Study. *Lancet* (2020) 395:1054–62. doi: 10.1016/s0140-6736(20)30566-3
62. Papayannopoulos V. Neutrophil Extracellular Traps in Immunity and Disease. *Nat Rev Immunol* (2018) 18:134–47. doi: 10.1038/nri.2017.105
63. Oehmcke S, Mörgelin M, Herwald H. Activation of the Human Contact System on Neutrophil Extracellular Traps. *J Innate Immun* (2009) 1:225–30. doi: 10.1159/000203700
64. Hottz ED, Azevedo-Quintanilha IG, Palhinha L, Teixeira L, Barreto EA, Pão CRR, et al. Platelet Activation and Platelet-Monocyte Aggregate Formation Trigger Tissue Factor Expression in Patients With Severe COVID-19. *Blood* (2020) 136:1330–41. doi: 10.1182/blood.2020007252
65. Denorme F, Manne BK, Portier I, Petrey AC, Middleton E, Kile BT, et al. COVID-19 Patients Exhibit Reduced Procoagulant Platelet Responses. *J Thromb Haemost* (2020) 18(11):3067–73. doi: 10.1111/jth.15107
66. Liu F, Han K, Blair R, Kenst K, Qin Z, Upcin B, et al. SARS-CoV-2 Infects Endothelial Cells *In Vivo* and *In Vitro*. *Front Cell Infect Mi* (2021) 11:701278. doi: 10.3389/fcimb.2021.701278
67. Ikeda K, Nagasawa K, Horiuchi T, Tsuru T, Nishizaka H, Niho Y. C5a Induces Tissue Factor Activity on Endothelial Cells. *Thromb Haemostasis* (1997) 77:394 398. doi: 10.1055/s-0038-1655974
68. Ritis K, Doumas M, Mastellos D, Micheli A, Giaglis S, Magotti P, et al. Lambris JD. A Novel C5a Receptor-Tissue Factor Cross-Talk in Neutrophils Links Innate Immunity to Coagulation Pathways. *J Immunol* (2006) 177:4794–802. doi: 10.4049/jimmunol.177.7.4794
69. van der Pol P, Schlagwein N, van Gijlswijk DJ, Berger SP, Roos A, Bajema IM, et al. Mannan-Binding Lectin Mediates Renal Ischemia/Reperfusion Injury Independent of Complement Activation: MBL Is Cytotoxic to Tubular Epithelial Cells. *Am J Transplant* (2012) 12:877–87. doi: 10.1111/j.1600-6143.2011.03887.x
70. Zhang M, Hou YJ, Cavusoglu E, Lee DC, Steffensen R, Yang L, et al. MASP-2 Activation is Involved in Ischemia-Related Necrotic Myocardial Injury in Humans. *Int J Cardiol* (2013) 166:499 504. doi: 10.1016/j.ijcard.2011.11.032
71. Zhang M, Alicot EM, Carroll MC. Human Natural IgM can Induce Ischemia/Reperfusion Injury in a Murine Intestinal Model. *Mol Immunol* (2008) 45:4036–9. doi: 10.1016/j.molimm.2008.06.013
72. van der Pol P, Roos A, Berger SP, Daha MR, van Kooten C. Natural IgM Antibodies are Involved in the Activation of Complement by Hypoxic Human Tubular Cells. *Am J Physiol-renal* (2011) 300:F932 40. doi: 10.1152/ajprenal.00509.2010
73. Chung MK, Karnik S, Saef J, Bergmann C, Barnard J, Lederman MM, et al. SARS-CoV-2 and ACE2: The Biology and Clinical Data Settling the ARB and ACEI Controversy. *Ebiomedicine* (2020) 58:102907. doi: 10.1016/j.ebiom.2020.102907
74. Hewitt MM, Adams G, Mazzone SB, Mori N, Yu L, Canning BJ. Pharmacology of Bradykinin-Evoked Coughing in Guinea Pigs. *J Pharmacol Exp Ther* (2016) 357:620–8. doi: 10.1124/jpet.115.230383
75. Ostenfeld S, Bygum A, Rasmussen ER. Life-Threatening ACE Inhibitor-Induced Angio-Oedema Successfully Treated With Icatibant: A Bradykinin Receptor Antagonist. *BMJ Case Rep* (2015) 2015):bcr2015212891. doi: 10.1136/bcr-2015-212891
76. Aiello S, Gastoldi S, Galbusera M, Ruggenenti PL, Portalupi V, Rota S, et al. C5a and C5aR1 are Key Drivers of Microvascular Platelet Aggregation in Clinical Entities Spanning From aHUS to COVID-19. *Blood Adv* (2022) 6 (3):866–81. doi: 10.1182/bloodadvances.2021005246
77. Carvelli J, Demaria O, Vély F, Batista L, Benmansour NC, Fares J, et al. Association of COVID-19 Inflammation With Activation of the C5a-C5aR1 Axis. *Nature* (2020) 588(7836):146–50. doi: 10.1038/s41586-020-2600-6
78. Kaplan AP, Joseph K. Pathogenic Mechanisms of Bradykinin Mediated Diseases: Dysregulation of an Innate Inflammatory Pathway. *Adv Immunol* (2014) 121:41 89. doi: 10.1016/b978-0-12-800100-4.00002-7
79. Mugisho OO, Robilliard LD, Nicholson LFB, Graham ES, O'Carroll SJ. Bradykinin Receptor-1 Activation Induces Inflammation and Increases the Permeability of Human Brain Microvascular Endothelial Cells. *Cell Biol Int* (2020) 44:343–51. doi: 10.1002/cbin.11232
80. Terzuoli E, Corti F, Nannelli G, Giachetti A, Donnini S, Ziche M. Bradykinin B2 Receptor Contributes to Inflammatory Responses in Human Endothelial Cells by the Transactivation of the Fibroblast Growth Factor Receptor FGFR-1. *Int J Mol Sci* (2018) 19:2638. doi: 10.3390/ijms19092638
81. Renné T, Stavrou EX. Roles of Factor XII in Innate Immunity. *Front Immunol* (2019) 10:2011. doi: 10.3389/fimmu.2019.02011
82. Pelosi P, D'Onofrio D, Chiumello D, Paolo S, Chiara G, Capelozzi VL, et al. Pulmonary and Extrapulmonary Acute Respiratory Distress Syndrome are Different. *Eur Respir J* (2003) 22:48s–56s. doi: 10.1183/09031936.03.00420803
83. Gibson PG, Qin L, Puah SH. COVID-19 Acute Respiratory Distress Syndrome (ARDS): Clinical Features and Differences From Typical Pre-COVID-19 ARDS. *Med J Aust* (2020) 213:54. doi: 10.5694/mja2.50674
84. Campbell CM, Kahwash R. Will Complement Inhibition Be the New Target in Treating COVID-19-Related Systemic Thrombosis? *Circulation* (2020) 141:1739–41. doi: 10.1161/circulationaha.120.047419
85. Diurno F, Numis FG, Porta G, Cirillo F, Maddaluno S, Ragozzino A, et al. Eculizumab Treatment in Patients With COVID-19: Preliminary Results From Real Life ASL Napoli 2 Nord Experience. *Eur Rev Med Pharmaco* (2020) 24:4040–7. doi: 10.26355/eurrev_202004_20875
86. Giudice V, Pagliano P, Vatrella A, Masullo A, Poto S, Polverino BM, et al. Combination of Ruxolitinib and Eculizumab for Treatment of Severe SARS-CoV-2-Related Acute Respiratory Distress Syndrome: A Controlled Study. *Front Pharmacol* (2020) 11:857. doi: 10.3389/fphar.2020.00857
87. Laurence J, Mulvey JJ, Seshadri M, Racanelli A, Harp J, Schenck EJ, et al. Anti-Complement C5 Therapy With Eculizumab in Three Cases of Critical COVID-19. *Clin Immunol Orlando Fla* (2020) 219:108555. doi: 10.1016/j.clim.2020.108555
88. Mastaglio S, Ruggeri A, Risitano AM, Angelillo P, Yancopoulou D, Mastellos DC, et al. The First Case of COVID-19 Treated With the Complement C3 Inhibitor AMY-101. *Clin Immunol* (2020) 215:108450. doi: 10.1016/j.clim.2020.108450
89. Rambaldi A, Gritti G, Micò MC, Frigeni M, Borleri G, Salvi A, et al. Endothelial Injury and Thrombotic Microangiopathy in COVID-19: Treatment With the Lectin-Pathway Inhibitor Narsoplimab. *Immunobiology* (2020) 225:152001. doi: 10.1016/j.imbio.2020.152001
90. Laine O, Leppänen I, Koskela S, Antonen J, Mäkelä S, Sinisalo M, et al. Severe Puumala Virus Infection in a Patient With a Lymphoproliferative Disease Treated With Icatibant. *Infect Dis* (2014) 47:107–11. doi: 10.3109/00365548.2014.969304

91. Mansour E, Palma AC, Ulaf RG, Ribeiro LC, Bernardes AF, Nunes TA, et al. Safety and Outcomes Associated With the Pharmacological Inhibition of the Kinin–Kallikrein System in Severe COVID-19. *Viruses* (2021) 13:309. doi: 10.3390/v13020309
92. Urwyler P, Moser S, Charitos P, Heijnen IAFM, Rudin M, Sommer G, et al. Treatment of COVID-19 With Conestat Alfa, a Regulator of the Complement, Contact Activation and Kallikrein-Kinin System. *Front Immunol* (2020) 11:2072. doi: 10.3389/fimmu.2020.02072

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 27 April 2022
doi: 10.3389/fimmu.2022.841759

Secondary Complement Deficiency Impairs Anti-Microbial Immunity to *Klebsiella pneumoniae* and *Staphylococcus aureus* During Severe Acute COVID-19

Youssif M. Ali[1,2], Nicholas J. Lynch[1], Priyanka Khatri[1], Ifeoluwa E. Bamigbola[1], Andrew C. Y. Chan[1], Munehisa Yabuki[3], Gregory A. Demopulos[3], Jonathan L. Heeney[1], Sumita Pai[4], Helen Baxendale[4] and Wilhelm J. Schwaeble[1*]*

[1] Department of Veterinary Medicine, School of Biological Sciences, University of Cambridge, Cambridge, United Kingdom, [2] Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt, [3] Omeros Corporation, Seattle, WA, United States, [4] Royal Papworth Hospital NHS Foundation Trust, Cambridge, United Kingdom

A high incidence of secondary *Klebsiella pneumoniae* and *Staphylococcus aureus* infection were observed in patients with severe COVID-19. The cause of this predisposition to infection is unclear. Our data demonstrate consumption of complement in acute COVID-19 patients reflected by low levels of C3, C4, and loss of haemolytic activity. Given that the elimination of Gram-negative bacteria depends in part on complement-mediated lysis, we hypothesised that secondary hypocomplementaemia is rendering the antibody-dependent classical pathway activation inactive and compromises serum bactericidal activity (SBA). 217 patients with severe COVID-19 were studied. 142 patients suffered secondary bacterial infections. Klebsiella species were the most common Gram-negative organism, found in 58 patients, while *S. aureus* was the dominant Gram-positive organism found in 22 patients. Hypocomplementaemia was observed in patients with acute severe COVID-19 but not in convalescent survivors three months after discharge. Sera from patients with acute COVID-19 were unable to opsonise either *K. pneumoniae* or *S. aureus* and had impaired complement-mediated killing of Klebsiella. We conclude that hyperactivation of complement during acute COVID-19 leads to secondary hypocomplementaemia and predisposes to opportunistic infections.

Keywords: *K. pneumoniae*, COVID-19, SARS-CoV-2, complement system, bacterial infection, *S. aureus*

OPEN ACCESS

Edited by:
Zoltán Prohászka, Semmelweis University, Hungary

Reviewed by:
Pál Gyombolai, Semmelweis University, Hungary
Uday Kishore, Brunel University London, United Kingdom

***Correspondence:**
Youssif M. Ali
myima2@cam.ac.uk
Wilhelm J. Schwaeble
hws24@cam.ac.uk

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *22 December 2021*
Accepted: *21 March 2022*
Published: *27 April 2022*

Citation:
Ali YM, Lynch NJ, Khatri P, Bamigbola IE, Chan ACY, Yabuki M, Demopulos GA, Heeney JL, Pai S, Baxendale H and Schwaeble WJ (2022) Secondary Complement Deficiency Impairs Anti-Microbial Immunity to Klebsiella pneumoniae and Staphylococcus aureus During Severe Acute COVID-19. Front. Immunol. 13:841759. doi: 10.3389/fimmu.2022.841759

INTRODUCTION

Coronavirus disease 2019 (COVID-19), a predominantly respiratory disease caused by Severe Acute Respiratory Syndrome-Coronavirus type 2 (SARS-CoV-2), is responsible for the current global health pandemic, with a high rate of mortality, especially among the elderly and patients with underlying medical conditions (1). Secondary bacterial infections with different microbial

pathogens such as *Streptococcus pneumoniae, Klebsiella pneumoniae, Haemophilus influenzae, Escherichia coli*, *Staphylococcus aureus*, and Aspergillus have been reported during COVID-19 (2). The mechanisms involved in the increased risk of secondary infection are likely multifactorial including known underlying risk factors for infection such as immune deficiency, gastric reflux/aspiration and gut ischaemia associated with severe disease, and the use of catheters and lines to support critical care management provides a portal for infection and sustained colonisation. Lung tissue injury through SARS-CoV-2 infection may facilitate bacterial colonisation, resulting in airway dysfunction, cytopathology, tissue destruction and damage to the protective mucosa in the lung, exacerbating disease severity and increasing the risk of septicaemia and admission to the intensive care unit (ICU) (3). In the acute inflammatory phase of severe COVID-19, a secondary innate and adaptive immune incompetence is likely to increase the risk of secondary infections. It is well established that sepsis can impair many aspects of immune functions (4) *K. pneumoniae* is a Gram-negative opportunistic pathogen that causes serious pathology such as pneumonia, septicaemia, urinary tract infection (UTI) and pyogenic liver abscesses (5). The incidence of Klebsiella infection is increasing, with the highest incidence in older age groups, as has recently been reported in England (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/615375/hpr1817_klbsll.pdf).

The clinical management of secondary *K. pneumoniae* infections became a serious issue during the COVID-19 pandemic because *Klebsiella* strains (and possibly other opportunistic pathogens) have developed mechanisms to resist a wide range of antimicrobial agents, such as β-lactams, aminoglycosides, quinolones, and polymyxins (6). Although antibiotic treatment of patients infected with *K. pneumoniae* may reduce bacterial load, most antibiotics offer insufficient protection from organ damage resulting from an exaggerated immune response. *K. pneumoniae* produces a wide range of virulence factors, such as capsular polysaccharides and lipopolysaccharide (endotoxins), and leads to biofilm formation (mucoid layer), all of which increase the pathogenicity of the bacteria (7). The contribution of the mucoid layer to the pathogenicity of *K. pneumoniae* strains has been reported to increase the resistance to phagocytosis and serum killing activity by preventing direct complement activation on the bacterial surface (7). As such, an anti-capsular antibody is required to enable complement fixation and optimal bacterial clearance of mucoid strains. Mucoid strains of *K. pneumoniae* are normally responsible for invasive disease and community-acquired pneumonia, whereas non-mucoid strains of Klebsiella are less virulent (8).

Staphylococcus aureus is a Gram-positive opportunistic bacterium causing infections that vary from superficial skin infection to life-threatening invasive disease including pneumonia and sepsis (9). The transition from an opportunistic commensal to an invasive pathogen requires evasion from the immune defence and the ability of the bacterium to exploit different niches within the host. Secondary infections caused by *S. aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) are commonly feared, especially among immunocompromised and severely ill patients as they contribute to further morbidity and mortality (10). The increased risk for *S. aureus* infections during COVID-19 was reported in previous studies showing an association between secondary infections with *S. aureus* and MRSA and mortality (10, 11).

The complement system is a major component of innate immunity and plays a pivotal role in the prevention of invasive microbial infections (12). The complement activation cascade is initiated *via* three different pathways: the classical (CP), the lectin (LP), and the alternative (AP) pathways (13, 14). Initiation of complement activation converges in the generation of enzyme complexes that cleave the most abundant complement component C3, generating the activation fragments C3b and C3a. While C3a is an anaphylatoxin, C3b binds covalently to activating surfaces, like the surface of bacteria, to enhance their uptake and removal by phagocytic cells. C3b binds in close proximity of the C3 convertase complexes C3bBb and C4bC2a, switching their substrate specificity from C3 to C5. C5 is split into the anaphylatoxin C5a and the larger fragment C5b, which initiates the formation of the terminal pathway and results in the formation of C5b-9, ultimately leading to the insertion of this complex – the membrane attack complex (MAC) – in the cell wall, forming a channel-like pore composed of polymers of C9. Membrane penetrating C5b-C9 complexes cause osmotic leakage and lyse bacteria (15).

The first indication that complement is likely to be involved in the inflammatory pathology of severe acute respiratory syndrome coronavirus (SARS-CoV) infection was published before the emergence of SARS-CoV-2 in a mouse model of SARS-CoV-1 where gene-targeted C3-deficient mice were protected from the significant weight loss and respiratory dysfunction seen in C56BL/6J wildtype control mice infected with an equivalent viral load (16).

Following the emergence of SARS-CoV-2 in 2019, it became clear that some infected individuals can develop moderate, to severe, to life-threatening forms of COVID-19 presenting as an acute respiratory distress syndrome (ARDS), and an early histopathology study in the tissue of patients that succumbed to COVID-19 provided strong evidence of an intrinsic involvement of complement activation and microangiopathies in the pathophysiology of COVID-19 (17). Many articles have been published since reporting the involvement of complement in the pathophysiology of COVID-19, but how and through which initiation pathway a substantial activation of complement occurs is still elusive (18, 19).

Several previous studies reported the essential role of complement activation during *K. pneumoniae* and *S. aureus* (20–23). This report demonstrates that hyperactivation of complement seen in every COVID-19 patient serum assessed in the acute phase of severe disease leads to a secondary loss of complement-dependent opsonisation of *K. pneumoniae* and *S. aureus* and complement–mediated lysis of *K. pneumoniae*. The findings of this study provide an explanation for the frequent occurrence of opportunistic secondary infections with *K. pneumoniae and S. aureus* in patients with severe COVID-19.

MATERIALS AND METHODS

Clinical Data and Serum Samples

SARS-CoV-2 infected patients referred to the Royal Papworth Hospital, Cambridge, UK for critical care were recruited to the study. Clinical assessment and WHO criteria scoring of severity was conducted following the 'COVID-19 Clinical Management: living guidance'. (*COVID-19 Clinical Management: Living Guidance*. Available at: https://www.who.int/publications/i/item/WHO-2019-nCoV-clinical-2021-1. 217 patients included in our study were classified as severely ill (scoring between 3 and 7 in the WHO severity score, see above). Blood and sputum cultures were collected and processed using standard microbiological techniques as part of routine clinical care. Bacteria were identified according to UK standards for Microbiological investigations https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/800451/B_57i3.5.pdf. Serum samples were taken at defined time intervals from hospital admission up to convalescence from 25 severely ill patients (scoring between 4-7). In this 25-patient group, there are 10 females and 15 males with an average age of 51 years (ranging from 30-73 years). All critically ill patients are at risk of venous thromboembolism and dissemminated intravascular coagulation (DIC). A high incidence of venous and arterial embolism (25-30%) has recently been reported in COVID-19 patients (24, 25). Patients with the more severe form of COVID-19 pneumonia display high D-dimers, low antithrombin, high fibrinogen and sometimes abnormal prothrombin time and activated thromboplastin time consistent with DIC. To manage the risk of thrombosis, all patients in our cohort received anticoagulant treatment throughout ICU stay and hospitalisation (either infusion with heparin [4500 IU/day] for all patients on extracorporeal membrane oxygenation (ECMO) or with low-molecular-weight heparin [LMWH] at intermediate doses of 50-60mg/day. To assess the impact of therapeutic doses of heparin or LMWH in the patients' sera as a limiting confounding factor that might affect complement functional activity, we compared normal human serum (NHS), heparinised plasma and EDTA plasma in a C3b or C4b deposition assay on *K. pneumoniae*-coated ELISA plates. In addition, the CH_{50} of NHS, heparinised plasma (10 IU heparin/mL) or EDTA plasma (10μM EDTA) was determined. No difference in complement functional activity was observed (**Figure S1**), an observation underlined by recent literature reporting that SBA against *K. pneumoniae* was not diminished in heparinised (5 IU/mL) blood (26).

The NHS control group is composed of sera from 6 females and 8 males with an average age of 47 years (ranging from 32 to 54 years). All NHS blood donors were tested negative for SARS-CoV-2 prior to blood collection.

The study was approved by Research Ethics Committee Wales, IRAS: 96194 12/WA/0148. Amendment 5. All participants or legal consent representatives provided written, informed consent prior to enrolment in the study. Sera from non-infected healthy volunteers were used as a control (NHS).

Bacterial Strains

Mucoid (ATCC 43816) and non-mucoid (Ecl8) strains were kindly provided by Dr. Sebastian Bruchmann, Department of Veterinary Medicine, University of Cambridge, UK. ***Staphylococcus aureus*** (*S. aureus*) Newman strain D2C (ATCC® 25904™) was purchased from ATCC.

Measurement of Serum Levels of Complement Proteins and Complement Activation Products

Circulating C5a and sC5b-9 levels were measured using a sandwich ELISA kit supplied by R&D systems (Cat. No. DY2037) and (BD OptEIA Human C5b-9 ELISA set). Complement C3 and C4 levels were measured using Abcam C3 and C4 ELISA kits.

Haemolytic Assay

2mL of packed sheep erythrocytes were washed 3 times using GVB buffer (10mM barbital, 145mM NaCl, 0.1%w/v bovine gelatine) containing 10mM EDTA. The final concentration of RBCs was adjusted to $1x10^9$/mL in the same buffer. RBCs were sensitised by incubation with 10μg/mL anti-sheep RBCs at 37°C with gentle shaking for 30 minutes. Finally, RBCs were washed with GVB buffer containing 2mM Ca^{2+} and 1mM Mg^{2+} (GVB^{++}). Serum samples were serially diluted in GVB^{++} buffer in 96 well plates and 10^7 RBCs were added to each well. Wells receiving water were used as a positive control to achieve 100% lysis of RBCs. Wells containing buffer only were used as a negative control. After 1 h incubation at 37°C, plates were centrifuged and released haemoglobin was measured at 405 nm. % RBCs haemolysis was calculated as previously described (27). In some experiments the haemolytic assay was performed using sera from acute COVID-19 patients reconstituted with purified human C4 (10μg/mL). C4 was purified from plasma given by healthy donors as previously described (28).

Complement Activation Assay

Maxisorp polystyrene microtiter ELISA plates were coated with 10μg/mL mannan or formalin-fixed *K. pneumoniae* or *S. aureus* (OD600 = 0.6) in carbonate buffer (15mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). The next day, wells were blocked with 1% BSA in TBS buffer (10mM Tris-HCl, 140mM NaCl, pH7.4) for 2 hours then washed with TBS buffer containing 0.05% (v/v) Tween 20 and 5 mM $CaCl_2$. NHS were diluted in BBS^{++} buffer (4mM barbital, 145mM NaCl, 2mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) (starting from 1:100), added to the plate and incubated for 1 hour at 37°C then washed. Deposition of C3b, C4b and C5b-9 was detected using either rabbit anti-C3c (Dako), rabbit anti-C4c (Dako) or rabbit anti-C5b-9 (Abcam), respectively, followed by peroxidase-conjugated goat anti-rabbit IgG. After 1 hour, wells were washed and 100 μL of 1-Step Ultra TMB Solution (Thermo Fisher Scientific) was then added to each well and incubated for 5 minutes at room temperature. The reaction was stopped by the addition of 2M H_2SO_4 and the optical density at 450 nm was immediately measured. To assess complement deposition *via* the alternative pathway, ELISA plates coated with *K. pneumoniae* and *S. aureus* were incubated with serial dilutions of NHS in EGTA buffer (4mM barbital, 145mM NaCl, 5mM $MgCl_2$, 20mM EGTA, pH 7.4) starting from 1:5. The plate was incubated at 37°C for 1 hour then washed. C3b deposition was detected as described above.

LP and CP Specific Complement Deposition Assay

To assess LP-mediated C4b deposition on the surface of *K. pneumoniae* and *S. aureus*, sera were diluted in MBL binding buffer (20mM Tris-HCl, 1 M NaCl, 10mM CaCl2, 0.05% (v/v) Triton X-100 pH 7.4) then incubated with ELISA plates coated with the bacteria for 1 hour at 37°C. After three washing steps, 100µL of 1µg/mL purified human C4 (Comp Tech, USA) in BBS^{++} was added to each well then incubated (29). After 1-hour incubation, plates were washed and bound C4b was detected as previously mentioned. To assess for CP activation, C1q-depleted serum (Comp Tech, USA) was serially diluted in BBS^{++} then incubated for 1 h at 37°C with ELISA plates coated with *K. pneumoniae*. As a control, C1q-depleted serum was reconstituted with 10µg/mL of purified human C1q. Complement C3b deposition was detected as described before. To inhibit the CP-mediated C3b deposition we used a potent monospecific anti-human C1s antibody (TNT003), which is a potent CP inhibitor (30). In this experiment, 2% NHS was incubated with different concentrations of monospecific anti-human C1s antibody (TNT003) at room temperature for 15 minutes then incubated with an ELISA plate coated with the bacteria for 15 minutes at 37°C. After several washing steps, complement C3b deposition was detected as described before.

Complement Deposition From Acute and Convalescent COVID-19 Patients' Sera on *K. pneumoniae* and *S. aureus*

ELISA plates coated with *K. pneumoniae* and *S. aureus* were incubated at 37°C with sera from acutely ill and convalescent COVID-19 patients (diluted 1:100) in BBS^{++}. NHS was used as a control. After 1 hour, plates were washed and C3b, C4b or C5b-9 were detected using either rabbit anti-C3c, rabbit anti-C4c or rabbit anti-C5b-9, respectively, followed by peroxidase-conjugated goat anti-rabbit IgG as described before (31).

Serum Bactericidal Assay (SBA)

K. pneumoniae isolates were grown in nutrient broth at 37°C for overnight with gentle shaking. The next day, 10 mL offresh nutrient broth were seeded with 100 µL of overnight bacterial culture and incubated at 37°C with gentle shaking until mid-logarithmic phase. Bacterial cultures were collected, washed twice using BBS^{++} and then adjusted to a final concentration of 1×10^7 CFU mL–1. 1×10^4 CFU were incubated with 75% serum NHS or sera from acute/convalescent in BBS at 37°C with gentle shaking. After 2 hours, samples were taken and plated out on nutrient agar plates then incubated overnight at 37°C. Serum bactericidal activity was calculated by measuring the decrease in the viable bacterial count after 2 h incubation with each serum compared to heat-inactivated normal human serum (HI-NHS) (32).

Determination of Antibody Titer Against *K. pneumoniae* in Patients' Sera

Nunc Maxisorp microplates coated with *K. pneumoniae* and blocked using 1% BSA in TBS buffer as described above were used in this experiment. Wells were incubated at room temperature with different serum dilutions from SARS-CoV-2 or convalescent patients. Sera from non-COVID-19 volunteers were used as controls. After 1 h incubation, wells were washed with wash buffer and 100µL of peroxidase-conjugated goat anti-human IgG were added to each well. After 1 h, wells were washed and 100µL of 1-Step Ultra TMB Solution (Thermo Fisher Scientific) was then added to each well and incubated for 5 minutes at room temperature. The reaction was stopped by the addition of 2 M H_2SO_4 and the optical density at 450 nm was immediately measured. Antibody titre was calculated as the highest serum dilution that gave positive results (33).

RESULTS

K. pneumoniae and *S. aureus* Secondary Bacterial Infections Are High Among COVID-19 Patients

An unusually high rate of secondary bacterial infection was observed in patients admitted with severe symptoms of COVID-19 infection. Our analysis showed that, in our cohort of 217 severely ill COVID-19 patients, 142 presented with a secondary bacterial infection. A wide range of pathogens was isolated from sputum, BAL or blood, and the most common Gram-negative bacterial isolates were *Klebsiella* species (**Figures 1A, B**). Gram-positive bacteria were also identified in blood and respiratory cultures where *Staphylococcus* species were the dominant bacteria (**Figures 1C, D**).

K. pneumoniae and *S. aureus* Activate Complement *via* the LP, CP, and the AP

In a set of preliminary experiments, we assessed complement deposition on the surface of mucoid and non-mucoid strains of *K. pneumoniae* using pooled sera from healthy volunteers. High levels of C3b, C4b and C5b-9 deposition were observed on the surface of the bacteria under conditions that allow activation of both the LP and CP, i.e., where serum was diluted in BBS with Ca^{+2} and Mg^{+2} (**Figures 2A–C**). Activation of the complement system was also observed on the surface of *S. aureus* where high levels of complement C3b, C4b and C5b-9 deposition were detected (**Figures 2E–G**).

Involvement of the AP in complement-mediated opsonisation was assessed by measuring C3b deposition under conditions that allow only activation of the AP, i.e., where serum samples were diluted in EGTA buffer with Mg^{+2}. High levels of C3b deposition *via* the AP were detected on both *K. pneumoniae* and *S. aureus* (**Figures 2D, H**).

To evaluate complement activation on the surface of the bacteria by either the LP or the CP we used pathway-specific assay conditions. Sera diluted in MBL-binding buffer were incubated on ELISA plates coated with *K. pneumoniae* or *S. aureus* to measure the LP-dependent deposition of C4b (**Figures 3A, D**). The high salt content of the MBL-binding buffer dissociates the CP initiation complex C1 while it leaves the LP-initiation complexes intact (29). In an additional series of experiments, the contribution of the LP towards the deposition

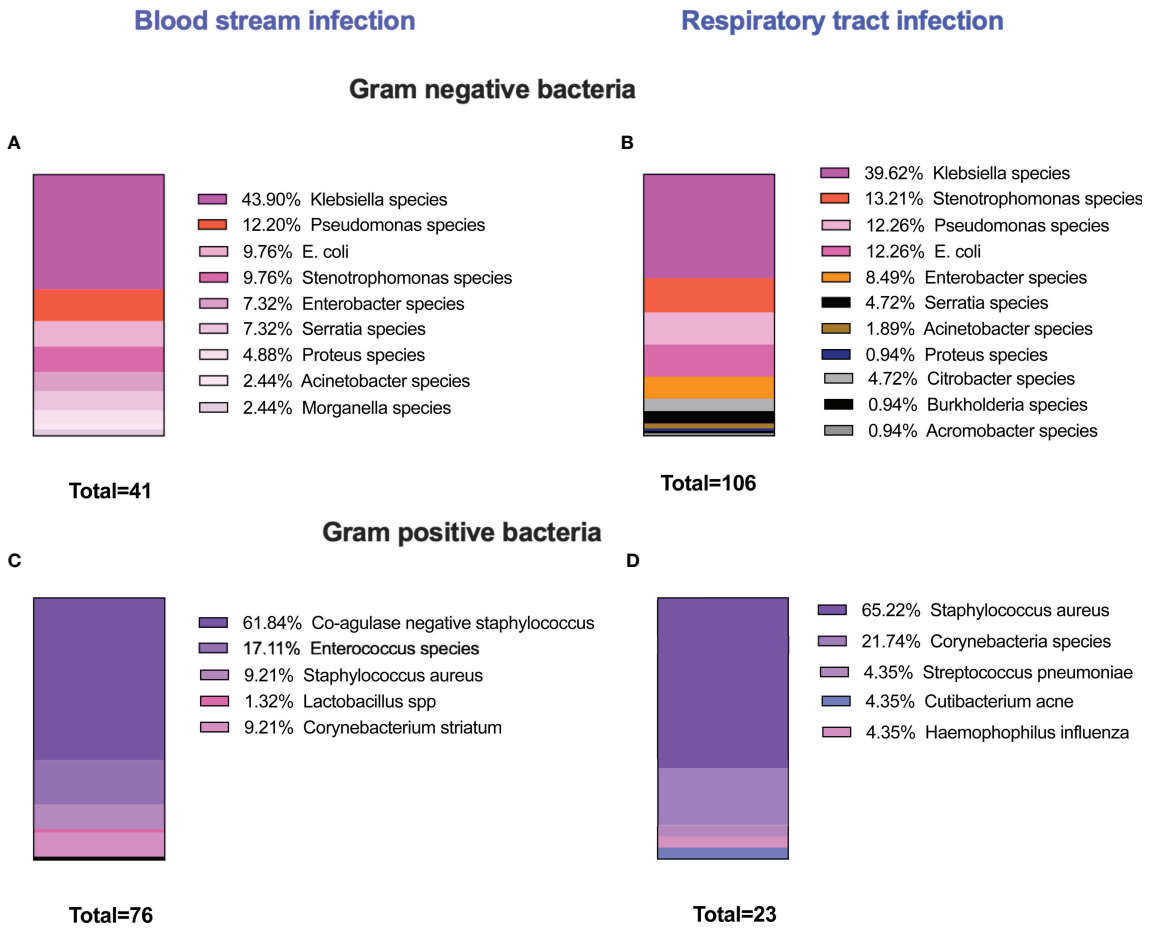


FIGURE 1 | Prevalence of secondary bacterial infection among severely ill COVID-19 patients. 217 patients were recruited in this study. 142 patients developed secondary bacterial infections. The microbiology analysis showed that different microbial species were isolated either from blood or respiratory cultures. The majority of infections were caused by Gram-negative bacteria, and *Klebsiella* species were the most common organism detected either in blood or respiratory cultures **(A, B)**. High incidences of Gram-positive bacteria were also identified and coagulase-negative staphylococcus species were the dominant Gram-positive bacteria in blood cultures, and *S. aureus* was the predominant organism in respiratory cultures **(C, D)**. In several occasions, multiple microorganisms were isolated from one patient.

of C3b on the surface of bacteria was shown by rendering the CP inactive either by using C1q-depleted serum or by using an inhibitory antibody directed against the CP effector enzyme C1s. Reconstituting C1q-depleted serum with purified human C1q (10 µg/mL) restored CP-mediated C3b deposition (**Figures 3B, E**). In addition, inhibition of the CP using the anti-C1s inhibitory antibody TNT003 significantly reduced C3b deposition on the surface of *K. pneumoniae* and *S. aureus*. Under the chosen conditions, the residual C3b deposition is most likely the result of LP functional activity (**Figures 3C, F**).

Secondary Loss of Complement Functional Activity Was Observed In Acute Severe COVID-19

We investigated the activity of the complement system in sera from acute and convalescent COVID-19 patients using a haemolytic assay with antibody-sensitised sheep erythrocytes. This assay provides an end-to-end measurement of complement activation *via* the CP and is sensitive to the reduction, absence and/or inactivity of any component of the CP and components involved in the formation of the lytic membrane attack complex.

Sera were taken from 25 survivors of severe COVID-19 on admission to the ICU (acute sera) and 3 months after discharge (convalescent sera). All sera of patients with acute severe COVID-19 (on admission to ICU) showed little or no complement-mediated lysis, while convalescent sera from the same patients showed normal complement-mediated lysis 3 months after release from hospital (**Figure 4A**). The serum levels of C3 and C4 were also significantly lower in acute-phase sera compared to the convalescent sera of the same patients and significantly lower than in the NHS controls, supporting the hypothesis that acute-phase sera are hypocomplementaemic due to complement consumption in the early phase of severe COVID-19 (**Figures 4B, C**). Reconstitution of acute-phase sera with purified complement C4 restored the defective haemolytic activity, indicating that low levels of C4 in

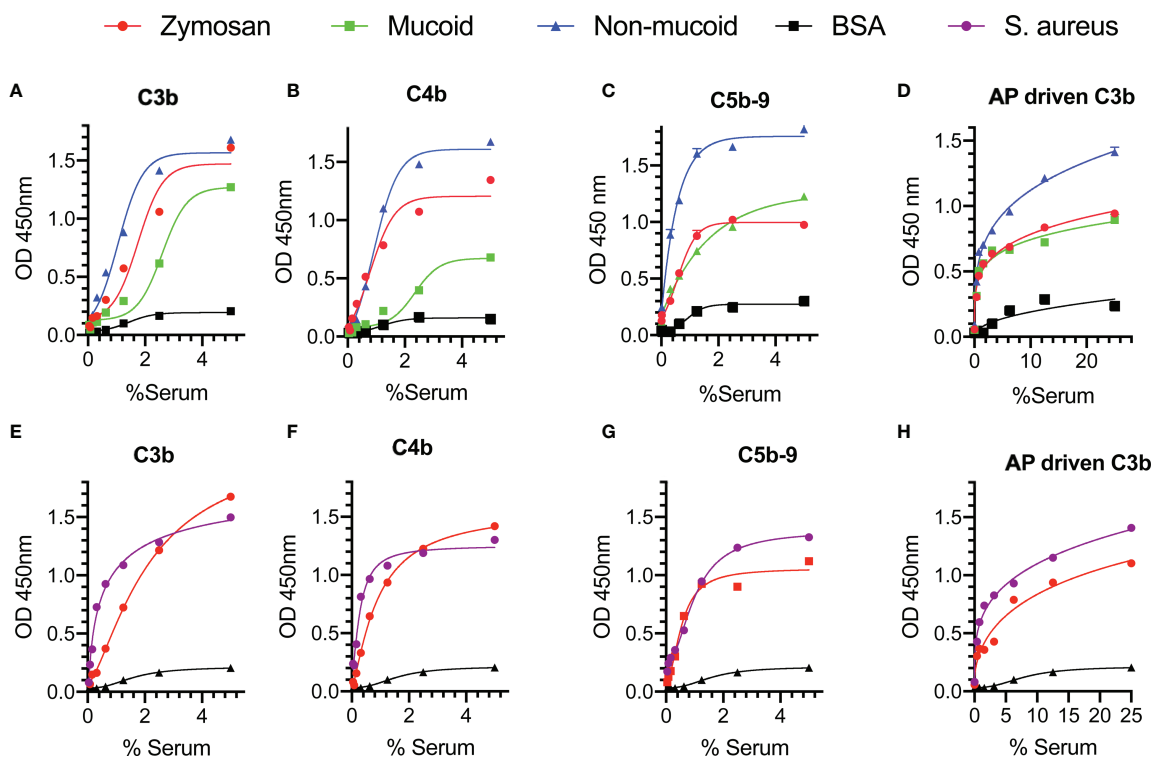


FIGURE 2 | Complement is activated on the surface of *Klebsiella pneumoniae* and *Staphylococcus aureus*. ELISA plates were coated with mucoid or non-mucoid strains of *K. pneumoniae* or with *S. aureus*. Wells coated with zymosan were used as a control. Plates were incubated with NHS in BBS with Ca^{+2} and Mg^{+2} **(A–C, E–G)** or EGTA buffer **(D, H)**. Complement C3b, C4b and C5b-9 deposition were detected using specific antibodies. C3b, C4b and C5b-9 deposition were observed on the surface of *K. pneumoniae* **(A–C)** and *S. aureus* **(E–G)** in conditions permissive for both the CP and the LP pathways. High levels of C3b *via* the AP were also detected on the surface of the bacteria **(D, H)**. Results are means of duplicates ± SD.

these sera at least contribute to defective CP and LP functional activity (**Figure 4D**). The hypothesis that hypocomplementaemia is resulting from hyperactivation of the complement system in the early phase of severe COVID-19 patients is supported by the detection of high levels of the complement activation markers C5a and sC5b-9 in acute patient sera compared to the levels seen in convalescent sera (**Figure 5**). To assess whether complement activation on the surface of *K. pneumoniae* is compromised during COVID-19 infection, we measured complement deposition on the surface of a mucoid strain (ATCC 43816) and a non-mucoid strain (Ecl8) using longitudinal serum samples of our study group of 25 patients with severe COVID-19. The levels of C3b, C4b and C5b-9 deposition were significantly lower in sera taken during the acute phase of the disease when compared to convalescent sera and NHS (**Figures 6A–C**). Similar results were obtained when the ELISA plates were coated with the non-mucoid strain Ecl8 (data not shown).

In addition, we assessed and compared complement deposition from acute and convalescent sera on the surface of *S. aureus* (the most common Gram-positive bacterium isolated in this study). As described for *K. pneumoniae*, all sera taken at the acute phase of severe COVID-19 were significantly compromised in their ability to deposit complement activation products, such as C3b, C4b and C5b-9, on the surface of *S. aureus* when compared to the degree of complement opsonisation seen in parallel when using NHS and convalescent sera of the same patients taken 3 months after release from hospital (**Figures 6D–F**).

Serum Bactericidal Activity Against *K. pneumoniae* Is Impaired During Acute Severe COVID-19

Having shown that serum from patients with acute severe COVID-19 is compromised in its ability to opsonise *K. pneumoniae* with complement C3b and C4b, we tested whether the bactericidal activity of the serum was similarly affected. Mucoid and non-mucoid strains of *K. pneumoniae* were incubated with sera from acute or convalescent patients and the number of viable bacteria after 2 hours was determined. Significantly lower levels of bacterial killing were observed when using sera from acute patients compared to sera from the same patients after recovery or to control NHS sera. Heat-inactivated serum (HI-NHS) was used as a negative control (**Figures 7A, B**). The presence of high antibody titres against mucoid and non-mucoid *K. pneumoniae* (**Figures 7C, D**) was also detected.

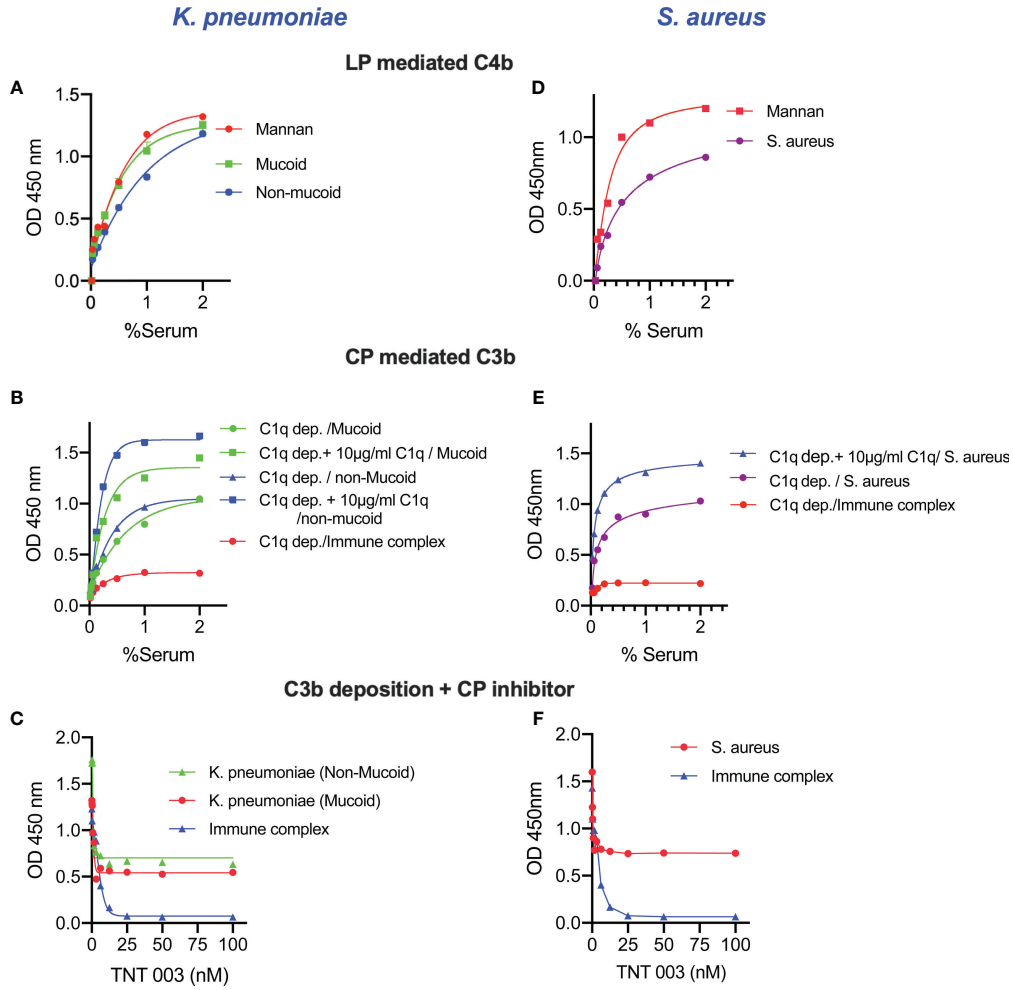


FIGURE 3 | Complement activation pathway-specific assay conditions demonstrate that both LP and CP are activated on the surface of *K. pneumoniae* and *S. aureus*. ELISA plates coated with zymosan (as control), mucoid, non-mucoid strains of *K. pneumoniae* or *S. aureus* were incubated with NHS diluted in MBL-binding buffer for 1 h at 37°C then washed. Purified human C4 was incubated with the ELISA plate for another 1 h at 37°C. After washing steps, C4b deposition was detected. High levels of LP-mediated C4b deposition were observed in the surface of *K. pneumoniae* and *S. aureus* **(A, D)**. Likewise, the use of C1q-depleted serum resulted in a significant reduction of C3b deposition. In absence of CP functional activity, only LP-dependent C3b deposition was measured on the surface of *K.pneumoniae* and *S. aureus*. Reconstitution of C1q-depleted serum with purified human C1q restored CP-mediated C3b deposition. No C3b deposition was observed when using C1q-depleted serum on the surface of immune complexes **(B, E)**. Using the C1s inhibitory antibody TNT003 blocked CP-mediated C3b deposition on the surface of immune complexes and all CP-mediated C3b opsonisation on *K. pneumoniae* and *S. aureus*. Under the chosen assay conditions, the residual deposition of C3b on *Klebsiella* and *S. aureus* is LP-mediated **(C, F)**. Results are means of duplicates ± SD.

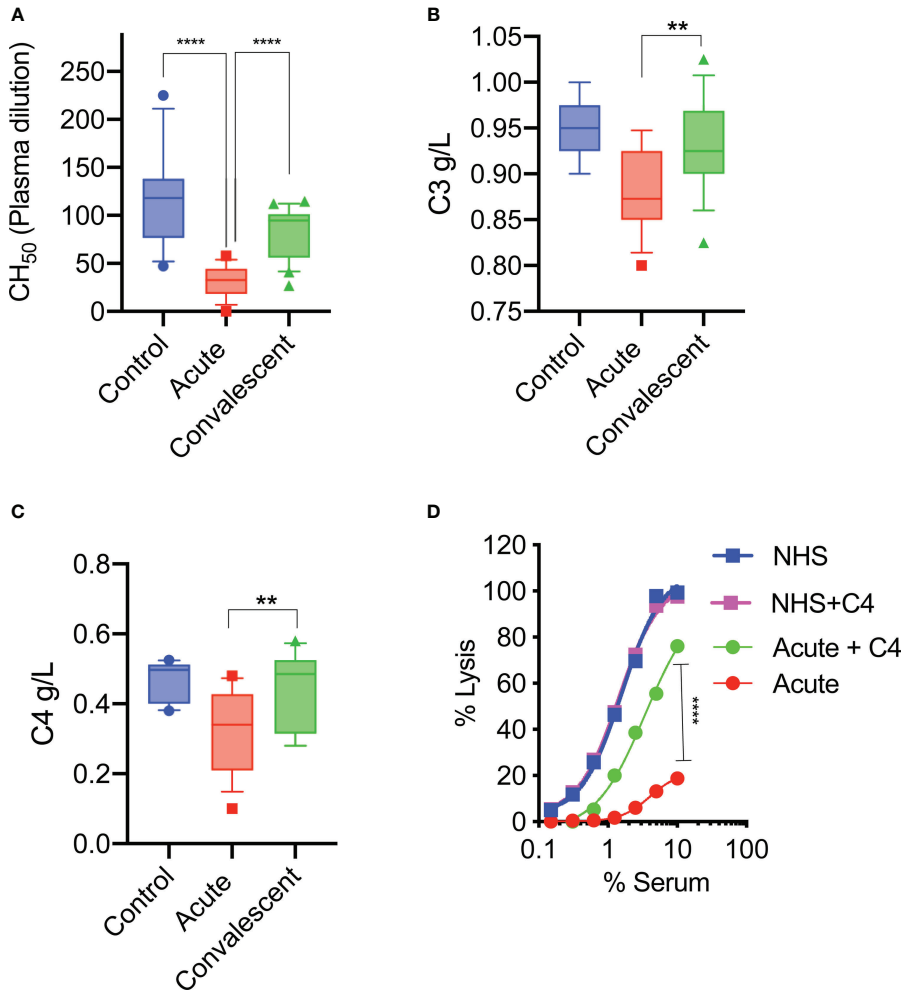


FIGURE 4 | At hospital admission, sera of severely ill COVID-19 patients lack complement functional activity. Sheep RBCs were coated with anti-sheep erythrocyte antibodies and incubated with different serum concentrations. The serum dilution required to lyse 50% of RBCs (CH_{50}) was calculated. The haemolytic activity of sera from acute COVID-19 patients (n= 25) is significantly impaired compared to sera from the same patients after recovery or to those of control NHS (n=14) **(A)**. Serum levels of complement C3 and C4 were also significantly lower in acute sera compared to convalescent sera and control sera (NHS) **(B, C)**. Results were analysed using 1-way ANOVA, with Dunnett's correction for multiple comparisons. $^{****}p < 0.0001$, $^{**}p < 0.01$. Reconstitution of sera taken during the acute phase of severe COVID-19 with 10 µg/mL of purified C4 restored the deficient haemolytic activity **(D)**. Results were analysed using 2-way ANOVA with Sidak's multiple comparison test.

DISCUSSION

High rates of bacterial co-infection during previous outbreaks of pandemic and epidemic respiratory viral infection caused by H1N1, SARS-CoV-1 and MERS were associated with a high morbidity and mortality among infected patients (34, 35). Secondary infections with respiratory pathogens in COVID-19 patients have been reported in several previous studies (36–38). The upper respiratory tract hosts a wide range of commensal microorganisms, some of which are potential opportunistic pathogens including *Legionella pneumophila*, *Streptococcus pyogenes, Neisseria meningitidis, Moraxella catarrhalis, S. pneumoniae*, *H. influenzae*, *S. aureus*, *Pseudomonas aeruginosa* and *K. pneumoniae* (39). Infection with opportunistic bacteria and fungi in patients with severe COVID-19 is not surprising since studies from previous pandemic viral infections reported co-infection with opportunistic bacteria, fungi, and even other viruses (40).

It has been established that secondary bacterial infections in acute COVID-19 patients are associated with greater severity of COVID-19 and poorer outcome (41, 42) with a high incidence of Gram-negative infections, especially *K. pneumoniae* (43, 44). A high incidence of Gram-positive bacterial infections was also reported in COVID-19, mostly with *Staphylococcus species* (34). Co-infections with Gram-negative pathogens such as *Pseudomonas aeruginosa, Escherichia coli*, and enterobacteriaceae were also observed, yet

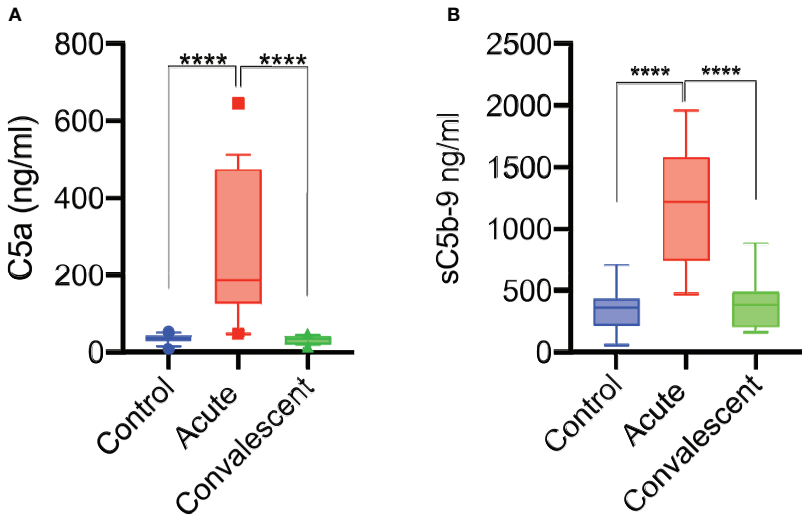


FIGURE 5 | Complement system is activated during acute COVID-19 infection. Anaphylatoxin C5a levels in patients sera increase during acute disease and return to normal on recovery (n = 25) **(A)**. sC5b-9 levels were also significantly increased during the acute phase (n = 25) of severe COVID-19 and returned to levels seen in NHS (n = 14) of healthy blood donors and sera taken from convalescent patients **(B)**. Results were analysed using 1-way ANOVA, with Dunnett's correction for multiple comparisons. ****$p < 0.0001$.

Klebsiella species dominated and were identified in approximately 27% of patients infected with Gram-negative bacteria.

The prevalance of *Klebsiella* species was recently attributed to the ability of these bacteria to release several virulence factors that can overcome host immune defences, as well as the emergence of multi-drug-resistant strains of *K. pneumoniae* (45). The critical role of complement in fighting bacterial infections has been established in numerous animal studies as well as in clinical studies of inherited or acquired complement deficiencies (46, 47). Immune complex-mediated activation of the CP is recognised as an important mechanism in the control of *Klebsiella* infection (48, 49). In the presence of antibodies, *Klebsiella species* are generally kept in check by antibody and complement-mediated lysis and/or opsonophagocytosis (8), which makes them non-pathogenic commensals for most individuals, but the loss of complement functional activity poses a significantly increased risk for invasive infection.

Our present report showed a loss of complement functional activity in all acute-phase sera of patients assessed with severe COVID-19. Sera from these severely ill patients showed low CH_{50} and low levels of C3 and C4 (**Figure 4**). At the same time, complement activation products C5a and sC5b-9 were detected in abundance, suggesting that complement activation is a hallmark of the early phase of severe COVID-19 as previously postulated (50). Reconstitution of these sera with purified human C4 restored the defective complement-mediated haemolytic activity to levels seen in NHS. This supports the hypothesis that consumption of complement components during the early phase of severe COVID-19 leads to secondary hypocomplementaemia and impairs complement functional activity. The molecular events driving substantial complement activation in the early phase of severe COVID-19 are presently unknown. The reconstitution of haemolytic activity in acute-phase sera through the addition of purified C4 indicates involvement of either the lectin or the classical activation pathway, or both. A deficient alternative pathway functional activity seen in all acute-phase sera suggests that all 3 complement activation pathways may be involved. The high consumption of complement leading to hypocomplementaemia in blood taken at ICU admission implies that substantial complement activation occurs at an early phase of severe COVID-19, a time point that needs to be further defined.

A connection between complement activation and disease severity of COVID-19 was recently established by showing that the ratio between high levels of the complement activation product C3a and serum levels of C3 correlates with disease severity and might serve as a predictive marker of disease outcome (51, 52). Sinkovits et al. additionally described a low CH_{50} in sera of patients with severe COVID-19 (51). A loss of complement functional activity in sera of patients with severe COVID-19 has also been reported by Charitos et al. (53), showing a correlation between COVID-19 severity and a loss of complement functional activity *via* the classical and the alternative pathway. A study by Defendi et al. reported similar results demonstrating a significant reduction of total haemolytic activity in sera of a cohort of patients with severe COVID-19 compared to sera in a cohort of patients with a milder course of disease (54).

Whilst all patient sera in our study were positive for antibodies against *K. pneumoniae*, both SBA and complement opsonisation of *K. pneumoniae* (including deposition of C3b, C4b, and C5b-9) were compromised in sera of acute COVID-19 patients due to a loss of complement functional activity. This phenomenon is likely to increase the susceptibility not only to

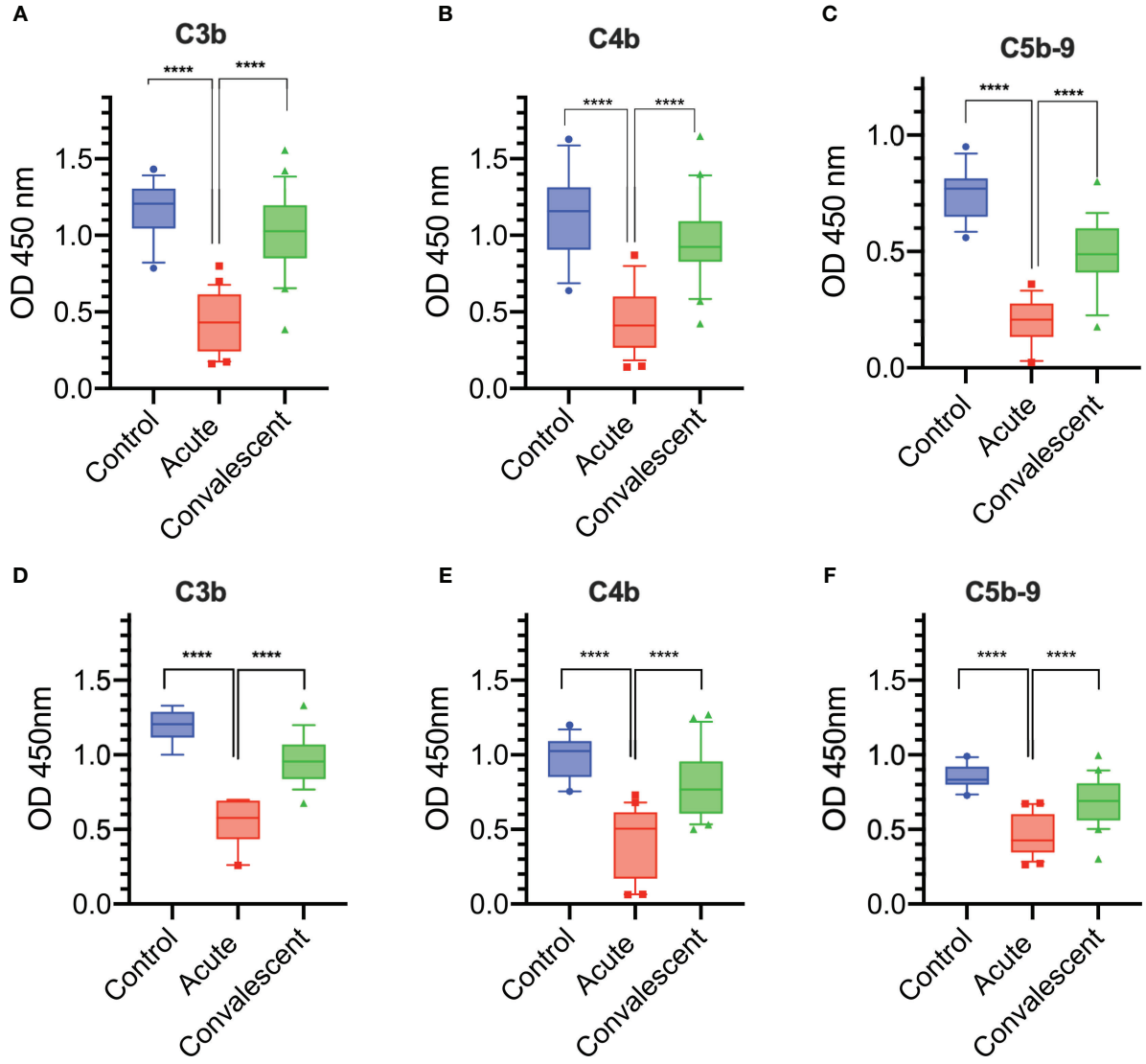


FIGURE 6 | Complement deposition on the surface of *K. pneumoniae* and *S. aureus* is impaired during acute severe COVID-19. A significant reduction in C3b, C4b, and C5b-9 deposition levels on the surface of mucoid *K. pneumoniae* **(A–C)** and on the surface of *S. aureus* **(D–F)** were detected when using sera from acutely ill patients compared to sera from the same patients taken 3 months after discharge (n = 25) or to control sera (n= 14). Results were analyzed using 1-way ANOVA, with Dunnett's correction for multiple comparisons. ****$p < 0.0001$.

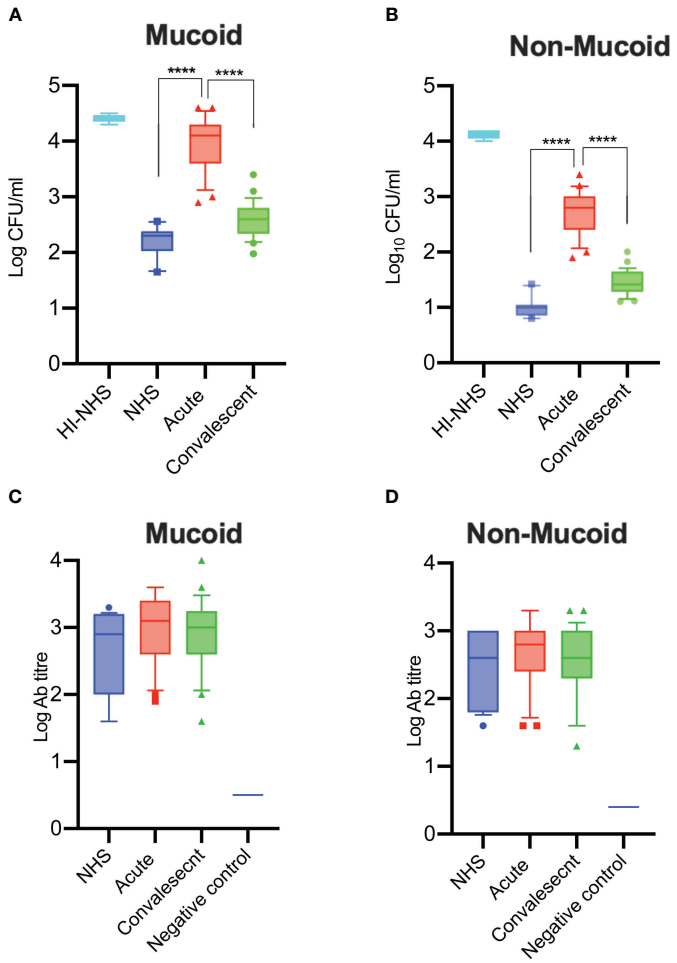


FIGURE 7 | Serum bactericidal activity of sera from COVID-19 patients in the acute phase of severe disease is impaired and has recovered in convalescent sera. Complement-mediated killing of mucoid **(A)** and non-mucoid **(B)** strains of *K. pneumoniae* is significantly impaired in sera from acute patients compared to recovered patients in convalescence (n = 25 paired acute/convalescent patients). NHS (n = 12) and HI-NHS were used as controls. High antibody titres against *K. pneumoniae* species were detected in all 25 sera of patients with severe COVID-19 (C-D). An individual NHS sample with no detectable antibody titres against *K. pneumoniae* was used as a negative control **(C, D)**. Results were analysed using 1-way ANOVA, with Dunnett's correction for multiple comparisons. ****$p < 0.0001$.

secondary infections with *K. pneumoniae* but also to other opportunistic pathogens that are usually held in check through complement-driven immune defense mechanisms.

In order to assess this, we measured complement deposition through each of the three complement activation pathways on a laboratory strain of *Staphylococcus aureus*, the most frequent Gram-positive opportunistic pathogen we detected in our group of severely ill COVID-19 patients. Again, complement deposition was highly impaired in sera of patients in the acute phase of severe COVID-19 and recovered in convalescent patient sera taken 3 months after hospital release. Acquired complement deficiency and low levels of C3, C4 and CH_{50} were previously reported to increase the risk of infection caused by *S. aureus* infection (55). A defect in complement opsonisation of *S. aureus* in sera of patients with acute severe COVID-19 may be a contributing factor to the previously reported high increase of nosocomial *Staphylococcus aureus* and *methicillin-resistant Staphylococcus aureus* (MRSA) infections in these patients (10).

CONCLUSION

Our results support the hypothesis that secondary hypocomplementemia caused by complement consumption in the early phase of severe SARS-CoV-2 infection is a key risk factor for secondary microbial infections.

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 study was approved by Research Ethics Committee Wales, IRAS: 96194 12/WA/0148. Amendment 5. The patients/participants or their legal consent representatives provided written informed consent to participate in this study prior to enrolment.

AUTHOR CONTRIBUTIONS

YA, NL, SP, IEB, PK, AC, HB, and WS designed and performed the experiments. YA, NL, HB, and WS wrote and revised the manuscript. GD and MY provided essential reagents and revised the manuscript. YA and NL had full access to all data in the study, take responsibility for the integrity of the data, and affirm that the manuscript is an honest, accurate, and transparent account of the study being reported, and that no important aspects of the study have been omitted. All authors contributed to the article and approved the submitted version.

FUNDING

This study was undertaken by HICC (Human Immune Correlates to COVID-19) consortium supported by NIHR/UKRI grant COV0170 awarded to WS.

ACKNOWLEDGMENTS

We thank the RPH Foundation Trust COVID-19 research and clinical teams including intensive care clinicians, Catherine Denny, Maria Nizami, Erin Hopley for supporting recruitment to this study, HCWs and outpatients who participated in this study. Heidi Utsugi and Galina Gragerova (Omeros Corporation) provided excellent technical support for production of recombinant reagents.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.841759/full#supplementary-material

Supplementary Figure 1 | Heparin has no effect on complement activation at a concentration up to 10 IU/ml. NHS and heparinised plasma (10 IU/ml) or EDTA plasma (10 μM) were serially diluted in BBS^{++} and incubated in ELISA plates coated with *K. pneumoniae*. Complement C3b **(A)** and C4b **(B)** deposition were measured as described in materials and methods. No significant differences were observed in the degree of C3b and C4b deposition on *K. pneumoniae* when using NHS or plasma collected in heparin or EDTA tubes. The haemolytic activity of NHS and heparin or EDTA plasma was measured as described in materials and methods **(C)**. This assay provides an end-to-end measurement of complement activation *via* the CP, including components that are shared in all three pathways. No significant difference was observed in the haemolytic activity between serum and heparinised plasma used in this experiment.

REFERENCES

1. Mizrahi B, Shilo S, Rossman H, Kalkstein N, Marcus K, Barer Y, et al. Longitudinal Symptom Dynamics of COVID-19 Infection. *Nat Commun* (2020) 11:6208. doi: 10.1038/s41467-020-20053-y
2. Hughes S, Troise O, Donaldson H, Mughal N, Moore LSP. Bacterial and Fungal Coinfection Among Hospitalized Patients With COVID-19: A Retrospective Cohort Study in a UK Secondary-Care Setting. *Clin Microbiol Infect* (2020) 26:1395–9. doi: 10.1016/j.cmi.2020.06.025
3. Sharifipour E, Shams S, Esmkhani M, Khodadadi J, Fotouhi-Ardakani R, Koohpaei A, et al. Evaluation of Bacterial Co-Infections of the Respiratory Tract in COVID-19 Patients Admitted to ICU. *BMC Infect Dis* (2020) 20:646. doi: 10.1186/s12879-020-05374-z
4. Han S, Mallampalli RK. The Acute Respiratory Distress Syndrome: From Mechanism to Translation. *J Immunol* (2015) 194:855–60. doi: 10.4049/jimmunol.1402513
5. Martin RM, Bachman MA. Colonization, Infection, and the Accessory Genome of Klebsiella Pneumoniae. *Front Cell Infect Microbiol* (2018) 8:4. doi: 10.3389/fcimb.2018.00004
6. Navon-Venezia S, Kondratyeva K, Carattoli A. Klebsiella Pneumoniae: A Major Worldwide Source and Shuttle for Antibiotic Resistance. *FEMS Microbiol Rev* (2017) 41:252–75. doi: 10.1093/femsre/fux013
7. Lee HC, Chuang YC, Yu WL, Lee NY, Chang CM, Ko NY, et al. Clinical Implications of Hypermucoviscosity Phenotype in Klebsiella Pneumoniae Isolates: Association With Invasive Syndrome in Patients With Community-Acquired Bacteraemia. *J Intern Med* (2006) 259:606–14. doi: 10.1111/j.1365-2796.2006.01641.x
8. Yu VL, Hansen DS, Ko WC, Sagnimeni A, Klugman KP, von Gottberg A, et al. Virulence Characteristics of Klebsiella and Clinical Manifestations of K. Pneumoniae Bloodstream Infections. *Emerg Infect Dis* (2007) 13:986–93. doi: 10.3201/eid1307.070187
9. Pollitt EJG, Szkuta PT, Burns N, Foster SJ. Staphylococcus Aureus Infection Dynamics. *PloS Pathog* (2018) 14:e1007112. doi: 10.1371/journal.ppat.1007112
10. Adalbert JR, Varshney K, Tobin R, Pajaro R. Clinical Outcomes in Patients Co-Infected With COVID-19 and Staphylococcus Aureus: A Scoping Review. *BMC Infect Dis* (2021) 21:985. doi: 10.1186/s12879-021-06616-4
11. Cusumano JA, Dupper AC, Malik Y, Gavioli EM, Banga J, Berbel Caban A, et al. Staphylococcus Aureus Bacteremia in Patients Infected With COVID-19: A Case Series. *Open Forum Infect Dis* (2020) 7:ofaa518. doi: 10.1093/ofid/ofaa518
12. Heesterbeek DAC, Martin NI, Velthuizen A, Duijst M, Ruyken M, Wubbolts R, et al. Complement-Dependent Outer Membrane Perturbation Sensitizes Gram-Negative Bacteria to Gram-Positive Specific Antibiotics. *Sci Rep* (2019) 9:3074. doi: 10.1038/s41598-019-38577-9
13. Schwaeble W, Dahl MR, Thiel S, Stover C, Jensenius JC. The Mannan-Binding Lectin-Associated Serine Proteases (MASPs) and MAp19: Four Components of the Lectin Pathway Activation Complex Encoded by Two Genes. *Immunobiology* (2002) 205:455–66. doi: 10.1078/0171-2985-00146
14. Lachmann PJ. The Amplification Loop of the Complement Pathways. *Adv Immunol* (2009) 104:115–49. doi: 10.1016/S0065-2776(08)04004-2
15. Ali YM, Hayat A, Saeed BM, Haleem KS, Alshamrani S, Kenawy HI, et al. Low-Dose Recombinant Properdin Provides Substantial Protection Against Streptococcus Pneumoniae and Neisseria Meningitidis Infection. *Proc Natl Acad Sci USA* (2014) 111:5301–6. doi: 10.1073/pnas.1401011111
16. Gralinski LE, Sheahan TP, Morrison TE, Menachery VD, Jensen K, Leist SR, et al. Complement Activation Contributes to Severe Acute Respiratory Syndrome Coronavirus Pathogenesis. *mBio* (2018) 9. doi: 10.1128/mBio.01753-18
17. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement Associated Microvascular Injury and Thrombosis in the Pathogenesis of Severe COVID-19 Infection: A Report of Five Cases. *Transl Res* (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
18. Java A, Apicelli AJ, Liszewski MK, Coler-Reilly A, Atkinson JP, Kim AH, et al. The Complement System in COVID-19: Friend and Foe? *JCI Insight* (2020) 5. doi: 10.1172/jci.insight.140711
19. Agostinis C, Mangogna A, Balduit A, Aghamajidi A, Ricci G, Kishore U, et al. COVID-19, Pre-Eclampsia, and Complement System. *Front Immunol* (2021) 12:775168. doi: 10.3389/fimmu.2021.775168
20. Jensen TS, Opstrup KV, Christiansen G, Rasmussen PV, Thomsen ME, Justesen DL, et al. Complement Mediated Klebsiella Pneumoniae Capsule Changes. *Microbes Infect* (2020) 22:19–30. doi: 10.1016/j.micinf.2019.08.003

21. van der Maten E, de Jonge MI, de Groot R, van der Flier M, Langereis JD. A Versatile Assay to Determine Bacterial and Host Factors Contributing to Opsonophagocytotic Killing in Hirudin-Anticoagulated Whole Blood. *Sci Rep* (2017) 7:42137. doi: 10.1038/srep42137
22. Doorduijn DJ, Bardoel BW, Heesterbeek DAC, Ruyken M, Benn G, Parsons ES, et al. Bacterial Killing by Complement Requires Direct Anchoring of Membrane Attack Complex Precursor C5b-7. *PloS Pathog* (2020) 16: e1008606. doi: 10.1371/journal.ppat.1008606
23. Abu-Humaidan AH, Elven M, Sonesson A, Garred P, Sorensen OE. Persistent Intracellular Staphylococcus Aureus in Keratinocytes Lead to Activation of the Complement System With Subsequent Reduction in the Intracellular Bacterial Load. *Front Immunol* (2018) 9:396. doi: 10.3389/fimmu.2018.00396
24. Cui S, Chen S, Li X, Liu S, Wang F. Prevalence of Venous Thromboembolism in Patients With Severe Novel Coronavirus Pneumonia. *J Thromb Haemost* (2020) 18:1421–4. doi: 10.1111/jth.14830
25. Klok FA, Kruip MJHA, van der Meer NJM, Arbous MS, Gommers D A M P J, Kant KM, et al. Incidence of Thrombotic Complications in Critically Ill ICU Patients With COVID-19. *Thromb Res* (2020) 191:145–7. doi: 10.1016/j.thromres.2020.04.013
26. DeLeo FR, Kobayashi SD, Porter AR, Freedman B, Dorward DW, Chen L, et al. Survival of Carbapenem-Resistant Klebsiella Pneumoniae Sequence Type 258 in Human Blood. *Antimicrob Agents Chemother* (2017) 61. doi: 10.1128/AAC.02533-16
27. Sahu A, Morikis D, Lambris JD. Compstatin, a Peptide Inhibitor of Complement, Exhibits Species-Specific Binding to Complement Component C3. *Mol Immunol* (2003) 39:557–66. doi: 10.1016/S0161-5890(02)00212-2
28. Dodds AW. Small-Scale Preparation of Complement Components C3 and C4. *Methods Enzymol* (1993) 223:46–61. doi: 10.1016/0076-6879(93)23037-N
29. Petersen SV, Thiel S, Jensen L, Steffensen R, Jensenius JC. An Assay for the Mannan-Binding Lectin Pathway of Complement Activation. *J Immunol Methods* (2001) 257:107–16. doi: 10.1016/S0022-1759(01)00453-7
30. Peerschke EI, Panicker S, Bussel J. Classical Complement Pathway Activation in Immune Thrombocytopenia Purpura: Inhibition by a Novel C1s Inhibitor. *Br J Haematol* (2016) 173:942–5. doi: 10.1111/bjh.13648
31. Kenawy HI, Ali YM, Rajakumar K, Lynch NJ, Kadioglu A, Stover CM, et al. Absence of the Lectin Activation Pathway of Complement Does Not Increase Susceptibility to Pseudomonas Aeruginosa Infections. *Immunobiology* (2012) 217:272–80. doi: 10.1016/j.imbio.2011.10.001
32. Bidmos FA, Chan H, Praekelt U, Tauseef I, Ali YM, Kaczmarski EB, et al. Investigation Into the Antigenic Properties and Contributions to Growth in Blood of the Meningococcal Haemoglobin Receptors, HpuAB and HmbR. *PloS One* (2015) 10:e0133855. doi: 10.1371/journal.pone.0133855
33. Lundberg U, Senn BM, Schuler W, Meinke A, Hanner M. Identification and Characterization of Antigens as Vaccine Candidates Against Klebsiella Pneumoniae. *Hum Vaccin Immunother* (2013) 9:497–505. doi: 10.4161/hv.23225
34. Langford BJ, So M, Raybardhan S, Leung V, Westwood D, MacFadden DR, et al. Bacterial Co-Infection and Secondary Infection in Patients With COVID-19: A Living Rapid Review and Meta-Analysis. *Clin Microbiol Infect* (2020) 26:1622–9. doi: 10.1016/j.cmi.2020.07.016
35. Joseph C, Togawa Y, Shindo N. Bacterial and Viral Infections Associated With Influenza. *Influenza Other Respir Viruses* (2013) 7 Suppl 2:105–13. doi: 10.1111/irv.12089
36. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, et al. Clinical Course and Outcomes of Critically Ill Patients With SARS-CoV-2 Pneumonia in Wuhan, China: A Single-Centered, Retrospective, Observational Study. *Lancet Respir Med* (2020) 8:475–81. doi: 10.1016/S2213-2600(20)30079-5
37. Russell CD, Fairfield CJ, Drake TM, Turtle L, Seaton RA, Wootton DG, et al. Co-Infections, Secondary Infections, and Antimicrobial Use in Patients Hospitalised With COVID-19 During the First Pandemic Wave From the ISARIC WHO CCP-UK Study: A Multicentre, Prospective Cohort Study. *Lancet Microbe* (2021) 2:e354–65. doi: 10.1016/S2666-5247(21)00090-2
38. He S, Liu W, Jiang M, Huang P, Xiang Z, Deng D, et al. Clinical Characteristics of COVID-19 Patients With Clinically Diagnosed Bacterial Co-Infection: A Multi-Center Study. *PloS One* (2021) 16:e0249668. doi: 10.1371/journal.pone.0249668
39. Morris DE, Cleary DW, Clarke SC. Secondary Bacterial Infections Associated With Influenza Pandemics. *Front Microbiol* (2017) 8:1041. doi: 10.3389/fmicb.2017.01041
40. Frias-De-Leon MG, Pinto-Almazan R, Hernandez-Castro R, Garcia-Salazar E, Meza-Meneses P, Rodriguez-Cerdeira C, et al. Epidemiology of Systemic Mycoses in the COVID-19 Pandemic. *J Fungi (Basel)* (2021) 7. doi: 10.3390/jof7070556
41. Farrell JM, Zhao CY, Tarquinio KM, Brown SP. Causes and Consequences of COVID-19-Associated Bacterial Infections. *Front Microbiol* (2021) 12:682571. doi: 10.3389/fmicb.2021.682571
42. Feldman C, Anderson R. The Role of Co-Infections and Secondary Infections in Patients With COVID-19. *Pneumonia (Nathan)* (2021) 13:5–021. doi: 10.1186/s41479-021-00083-w
43. Emeraud C, Figueiredo S, Bonnin RA, Khecharem M, Ouzani S, Leblanc P, et al. Outbreak of CTX-M-15 Extended-Spectrum β-Lactamase-Producing *Klebsiella Pneumoniae* ST394 in a French Intensive Care Unit Dedicated to COVID-19. *Pathogens* (2021) 10:1426. doi: 10.3390/pathogens10111426
44. Mędrzycka-Dąbrowska W, Lange S, Zorena K, Dąbrowski S, Ozga D, Tomaszek L. Carbapenem-Resistant *Klebsiella Pneumoniae* Infections in ICU COVID-19 Patients—A Scoping Review. *J Clin Med* (2021) 10:2067. doi: 10.3390/jcm10102067
45. Kim D, Park BY, Choi MH, Yoon EJ, Lee H, Lee KJ, et al. Antimicrobial Resistance and Virulence Factors of Klebsiella Pneumoniae Affecting 30 Day Mortality in Patients With Bloodstream Infection. *J Antimicrob Chemother* (2019) 74:190–9. doi: 10.1093/jac/dky397
46. Brown EJ, Hosea SW, Hammer CH, Burch CG, Frank MM. A Quantitative Analysis of the Interactions of Antipneumococcal Antibody and Complement in Experimental Pneumococcal Bacteremia. *J Clin Invest* (1982) 69:85–98. doi: 10.1172/jci110444
47. Bousfiha A, Jeddane L, Picard C, Ailal F, Bobby Gaspar H, Al-Herz W, et al. The 2017 IUIS Phenotypic Classification for Primary Immunodeficiencies. *J Clin Immunol* (2018) 38:129–43. doi: 10.1007/s10875-017-0465-8
48. Bengoechea JA, Sa Pessoa J. Klebsiella Pneumoniae Infection Biology: Living to Counteract Host Defences. *FEMS Microbiol Rev* (2019) 43:123–44. doi: 10.1093/femsre/fuy043
49. Alberti S, Marques G, Hernandez-Alles S, Rubires X, Tomas JM, Vivanco F, et al. Interaction Between Complement Subcomponent C1q and the Klebsiella Pneumoniae Porin Ompk36. *Infect Immun* (1996) 64:4719–25. doi: 10.1128/iai.64.11.4719-4725.1996
50. Ma L, Sahu SK, Cano M, Kuppuswamy V, Bajwa J, McPhatter J, et al. Increased Complement Activation Is a Distinctive Feature of Severe SARS-CoV-2 Infection. *Sci Immunol* (2021) 6. doi: 10.1126/sciimmunol.abh2259
51. Sinkovits G, Mezo B, Reti M, Muller V, Ivanyi Z, Gal J, et al. Complement Overactivation and Consumption Predicts In-Hospital Mortality in SARS-CoV-2 Infection. *Front Immunol* (2021) 12:663187. doi: 10.3389/fimmu.2021.663187
52. de Nooijer AH, Grondman I, Janssen NAF, Netea MG, Willems L, van de Veerdonk FL, et al. Complement Activation in the Disease Course of Coronavirus Disease 2019 and Its Effects on Clinical Outcomes. *J Infect Dis* (2021) 223:214–24. doi: 10.1093/infdis/jiaa646
53. Charitos P, Heijnen IAFM, Egli A, Bassetti S, Trendelenburg M, Osthoff M. Functional Activity of the Complement System in Hospitalized COVID-19 Patients: A Prospective Cohort Study. *Front Immunol* (2021) 12:765330. doi: 10.3389/fimmu.2021.765330
54. Defendi F, Leroy C, Epaulard O, Clavarino G, Vilotitch A, Le Marechal M, et al. Complement Alternative and Mannose-Binding Lectin Pathway Activation Is Associated With COVID-19 Mortality. *Front Immunol* (2021) 12:742446. doi: 10.3389/fimmu.2021.742446
55. Homann C, Varming K, Hogasen K, Mollnes TE, Graudal N, Thomsen AC, et al. Acquired C3 Deficiency in Patients With Alcoholic Cirrhosis Predisposes to Infection and Increased Mortality. *Gut* (1997) 40:544–9. doi: 10.1136/gut.40.4.544

Conflict of Interest: WS, NL, YA are consultants to Omeros Corporation, which is developing inhibitors of the lectin pathway. GD and MY are employed by Omeros Corporation.

The remaining 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.

this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership