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Escalated complement activation during hospitalization is associated with higher risk of 60-day mortality in SARS-CoV-2-infected patients

Andreas Barratt-Due[1], Kristin Pettersen[2], Tuva Børresdatter-Dahl[3], Jan Cato Holter[4,5], Renathe H. Grønli[2], Anne Ma Dyrhol-Riise[4,6], Tøri Vigeland Lerum[4,7], Aleksander Rygh Holten[4,8], Kristian Tonby[4,6], Marius Trøseid[3,4,9], Ole H. Skjønsberg[4,7], Beathe Kiland Granerud[4,5], Lars Heggelund[10,11], Anders Benjamin Kildal[12,13], Camilla Schjalm[4,14], Trond Mogens Aaløkken[4,15], Pål Aukrust[3,4,9], Thor Ueland[3,4], Tom Eirik Mollnes[2,14], Bente Halvorsen[3,4] & NOR-Solidarity study groupThe Norwegian SARS-CoV-2 study group

From the [1]Department of Anesthesia and Intensive Care Medicine, Oslo University Hospital, Oslo, Norway; [2]Research Laboratory, Nordland Hospital Trust, Bodø, Norway; [3]Research Institute for Internal Medicine, Oslo University Hospital, Oslo, Norway; [4]Insitute of Clinical Medicine, University of Oslo, Oslo, Norway; [5]Department of Microbiology, Oslo University Hospital, Oslo, Norway; [6]Department of Infectious Diseases, Oslo University Hospital, Oslo, Norway; [7]Department of Pulmonary Medicine, Oslo University Hospital, Oslo, Norway; [8]Department of Acute Medicine, Oslo University Hospital, Oslo, Norway; [9]Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital, University of Oslo, Oslo, Norway; [10]Department of Internal Medicine, Drammen Hospital, Vestre Viken Hospital Trust, Drammen, Norway; [11]Department of Clinical Science, Faculty of Medicine, University of Bergen, Bergen, Norway; [12]Department of Anesthesiology and Intensive Care, University Hospital of North Norway, Tromsø, Norway; [13]Department of Clinical Medicine, Faculty of Health Sciences, UIT–The Arctic University of Norway, Tromsø, Norway; [14]Department of Immunology, Oslo University Hospital, Oslo, Norway; and [15]Department of Radiology and Nuclear Medicine, Oslo University Hospital, Oslo, Norway

Abstract. Barratt-Due A, Pettersen K, Børresdatter-Dahl T, Holter JC, Grønli RH, Dyrhol-Riise AM, et al. Escalated complement activation during hospitalization is associated with higher risk of 60-day mortality in SARS-CoV-2-infected patients. *J Intern Med.* 2024;**296**:80–92.

Background. The complement system, an upstream recognition system of innate immunity, is activated upon SARS-CoV-2 infection. To gain a deeper understanding of the extent and duration of this activation, we investigated complement activation profiles during the acute phase of COVID-19, its persistence post-recovery and dynamic changes in relation to disease severity.

Methods. Serial blood samples were obtained from two cohorts of hospitalized COVID-19 patients (n = 457). Systemic complement activation products reflecting classical/lectin (C4d), alternative (C3bBbP), common (C3bc) and terminal pathway (TCC and C5a) were measured during hospitalization (admission, days 3–5 and days 7–10), at 3 months and after 1 year. Levels of activation and temporal profiles during hospitalization were related to disease severity defined as respiratory failure (PO_2/FiO_2 ratio <26.6 kPa) and/or admission to intensive care unit, 60-day total mortality and pulmonary pathology after 3 months.

Findings. During hospitalization, TCC, C4d, C3bc, C3bBbP and C5a were significantly elevated compared to healthy controls. Severely ill patients had significantly higher levels of TCC and C4d ($p < 0.001$), compared to patients with moderate COVID-19. Escalated levels of TCC and C4d during hospitalization were associated with a higher risk of 60-day mortality ($p < 0.001$), and C4d levels were additionally associated with chest CT changes at 3 months ($p < 0.001$). At 3 months and 1 year, we observed consistently elevated levels of most complement activation products compared to controls.

Conclusion. Hospitalized COVID-19 patients display prominent and long-lasting systemic complement activation. Optimal targeting of the system may be achieved through enhanced risk stratification and closer monitoring of in-hospital changes of complement activation products.

Keywords: C4d, complement, COVID, COVID-19, respiratory failure, TCC, SARS-CoV-2

Introduction

Hyperactivation of the complement system has been suggested to be a central player in the pathogenesis of COVID-19. Early in the pandemic, we demonstrated that systemic complement activation was prevalent among the majority of 39 hospitalized COVID-19 patients, and that the soluble terminal complement complex, TCC (sC5b-9), was associated with respiratory failure (RF) [1]. Moreover, increased complement activation measured as the consumption of C3 and concurrent increased levels of C3a and higher ratio of C3a/C3 have been identified as predictors of in-hospital mortality [2]. The C5a–C5aR1 axis was launched early as a potential therapeutic target and ultimately tested in a randomized clinical trial, demonstrating that administration of anti-C5a monoclonal antibody (mAb) to mechanically ventilated COVID-19 patients led to a significant reduction in all-cause mortality [3, 4]. However, the role of the complement system in COVID-19 is still not fully elucidated, and data on complement in relation to mortality and treatment options, as well as long-term data on complement activation following the acute phase, are still scarce or even lacking.

Undoubtedly, the complement system serves a pivotal role within the realm of innate immunity, functioning as an upstream recognition alarm system that discerns threats originating from exogenous and endogenous sources [5]. Activation occurs through three distinct routes: the classical, the lectin and the alternative pathway, and it is highly likely that all of them are involved in the context of COVID-19. SARS-CoV-2-specific antibodies may bind to C1q and activate through the classical pathway; the virus itself may interact directly with mannose-binding lectin (MBL) or MBL-associated serine protease-2 and activate through the lectin pathway, whereas the alternative pathway always will play a dominant role amplifying an ongoing activation [6–10]. However, it is the cleavage-mediated activation of the crucial components C3 and C5, leading to the generation of the potent anaphylatoxins C3a and C5a, that primarily underlies the extensive inflammatory response. In particular, C5a manifests a wide array of inflammatory effects, encompassing the facilitation of phagocytosis and oxidative burst in neutrophils and monocytes, the upregulation of adhesion molecules, the induction of granular enzyme release and its pivotal role as a crucial chemoattractant for neutrophils [11–13]. Additionally, C5a increases thrombogenicity by upregulation of tissue factor on various cells [14]. Finally, TCC, reflecting the final step in the complement cascade and assessed in this study as soluble TCC, also exists as membrane-bound TCC, known as MAC. When MAC accumulates on cell surfaces, it may exert cytolytic effects, penetrating the membrane, or alternatively activate inflammatory pathways in a 'sub-lytic' manner [15].

The activation of the complement system in SARS-CoV-2 infection must be regarded as an integral part of the host inflammatory response, trying to preserve host integrity and homeostasis. Conversely, uncontrolled activation is undeniably a concern as this may unleash inappropriate inflammatory responses with the potential to be harmful rather than host-protective. In this situation, the inhibition of complement represents an attractive intervention strategy, although it is unlikely that all hospitalized COVID-19 patients will require or benefit from this approach. Moreover, the stratification of patients who may benefit from complement inhibitory strategy and optimal timing for such intervention remain unresolved questions, underscoring the need for additional and longitudinal data on complement activation during the hospitalization of COVID-19 patients.

We herein present the results of two prospective cohort studies, including 457 hospitalized COVID-19 patients, analysing different soluble complement activation products reflecting classical/lectin (C4d), alternative (C3bBbP), common (C3bc) and terminal pathways (TCC and C5a). The study aims to assess the dynamic changes of complement activation products during hospitalization in relation to disease severity, 60-day all-cause mortality and pulmonary pathology observed at 3 months following hospital admission. In addition, we evaluated the effect of therapeutic intervention (i.e. remdesivir and dexamethasone) on complement activation during hospitalization, as well as complement activation at 3 months and after 1 year.

Methods

Study design, population and COVID waves

Data from two prospective cohort studies were pooled and assessed in the present study. Cohort 1 (n = 163), known as the NOR-Solidarity trial

Last authorship shared by Thor Ueland, Tom Eirik Mollnes and Bente Halvorsen.

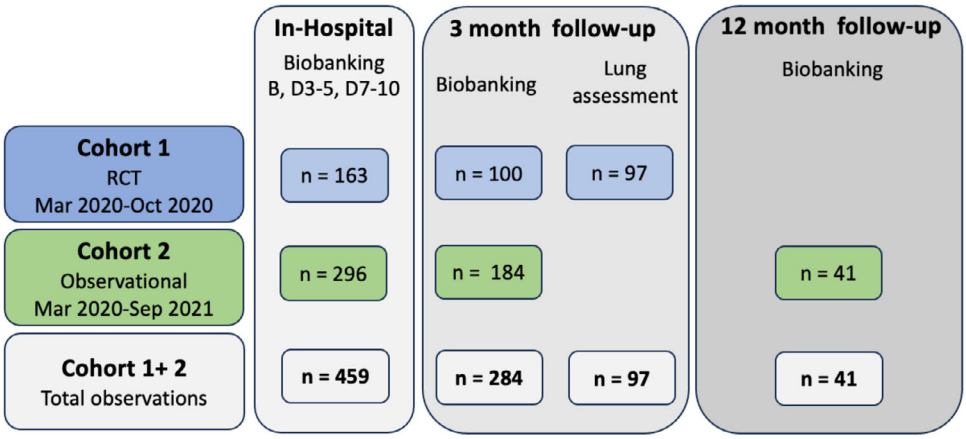

Fig. 1 *Flow chart depicting the assessment of participants from both cohorts, during their hospitalization and at 3 months and 1 year follow-up. In both studies, only patients ≥18 years who were admitted to the hospital with polymerase chain reaction (PCR)-confirmed SARS-CoV-2 infection were eligible for inclusion. Lung assessment included pulmonary function tests (DL_{CO}) and thin-section chest computed tomography (CT) images. B, baseline; D, day.*

(NCT04321616), was a multicentre open-label, adaptive randomized controlled trial conducted in 23 Norwegian hospitals, evaluating the efficacy of hydroxychloroquine (HCQ) and remdesivir in hospitalized SARS-CoV-2-infected patients. The study was part of, and an add-on trial, to the global WHO's Solidarity trial, and the participants were randomly assigned to one of three groups: (1) local standard of care (SoC); (2) SoC plus oral HCQ; or (3) SoC plus intravenous remdesivir. Cohort 2 ($n = 294$) was the Norwegian SARS-CoV-2 study (NCT04381819), an observational study on hospitalized COVID-19 patients admitted to five Norwegian hospitals. This study was conducted as part of the International Severe Acute Respiratory and Emerging Infection Consortium (ISARIC) WHO Clinical Characterization Protocol study [16].

In both studies, patients ≥18 years who were admitted to the hospital with polymerase chain reaction–confirmed SARS-CoV-2 infection in the oro/nasopharynx were eligible for inclusion. Three serial blood samples were collected throughout the first 10 days of hospitalization: (a) within 48 h of admission, (b) on days 3–5 and (c) on days 7–10. Additionally, blood samples were taken at 3 months and at 1 year (only cohort 2). In a subset of the patients in cohort 1, chest computed tomography (CT) images and lung function tests were performed at 3 months. A flow chart of the different cohorts and assessments is given in Fig. 1.

Cohort 1 patients were enrolled from March 28 to October 5, 2020, whereas cohort 2 patients were enrolled from March 8, 2020 to September 1, 2021. Thus, our data encompassed the initial three waves of the COVID-19 pandemic in Norway; wave one, March 8, 2020 to July 31, 2020; wave two, August 1, 2020 to February 17, 2021; and wave three, February 18, 2021 to July 31, 2021. By February 2021, the alpha variant was predominant, later replaced by the delta variant in July 2021.

Outcomes

In cohort 1 (NOR-Solidarity), the interventions did not affect clinical outcome, viral clearance or systemic inflammation [17, 18]. Thus, data from this cohort were pooled together with samples from cohort 2 (the Norwegian SARS-CoV-2 study) to examine levels of and dynamic changes of complement activation during hospitalization in relation to disease severity: (1) acute RF defined as PaO_2/FiO_2 ratio <26.6 kPa (<200 mmHg) and/or the requirement for admission to intensive care unit (ICU) during hospitalization and (2) 60-day post-admission all-cause mortality. In addition, we evaluated (3) complement activation in relation to pulmonary pathology at 3 months (performed in a subset of patients in cohort 1), (4) the persistence of complement activation at 3 months and after 1 year and (5) the effects of remdesivir (cohort 1) and dexamethasone (cohort 2) on complement dynamics during hospitalization.

Blood sampling protocol and biochemical analyses

Peripheral venous blood was drawn into pyrogen-free blood collection tubes with ethylenediamine tetraacetic acid (EDTA) as an anticoagulant, immediately immersed in melting ice and centrifuged at 4°C 2500 × *g* for 20 min within 60 min to obtain platelet-poor plasma. The biobank procedure was similar for both cohorts 1 and 2. For reference, complement activation products were analysed in 24 age- and sex-matched healthy controls, median age 54 (IQR 49,63), 58% men. All samples were stored at −80°C and thawed less than three times.

Routine laboratory variables, including C-reactive protein (CRP), ferritin and total leukocyte, neutrophil, lymphocyte and monocyte counts as well as creatinine and estimated glomerulus filtration rate (eGFR), were measured at the biochemical laboratories located within the participating hospitals.

Complement activation products and enzyme immunoassays

All samples were assayed in one batch in the same laboratory. The soluble terminal complement complex, TCC, was quantified in an enzyme-linked immunosorbent assay (ELISA) previously described in detail [19, 20]. Briefly, the mAb aE11, which reacts with a C9 neoepitope exposed after incorporation of C9 in the C5b-9 complex, was used as the capture Ab, and a biotinylated anti-C6 mAb (clone 9C4) was used as the detection Ab. The C3bc concentration was assessed by an ELISA based on the mouse anti-human C3bc mAb clone bH6, reacting with a neoepitope exposed in iC3b, C3b and C3c after C3 activation [21]. The alternative pathway activation was detected by quantifying the alternative pathway C3-convertase, C3bBbP, based on the monoclonal anti-factor P, clone number 2 (Quidel), binding the C3bBbP complex and detected by anti-C3c antibody reacting with all fragments [20]. The units of TCC, C3bc and C3bBbP, well-established in-house assays, are given according to an international standard defined as complement activation units per millilitre [20]. Commercially available assays were used to measure plasma C4d (SVAR Life Science) and C5a (Hycult Biotech). The reference values for C4d were given by the supplier, testing EDTA plasma from healthy donors, and for C5a, the reference range was based on previously published data [22].

Pulmonary function tests and CT imaging

In Cohort 1, lung function tests ($n = 90$), including spirometry and measurements of the diffusion capacity of the lungs for carbon monoxide (DL_{CO}), were conducted following established protocols as previously described [23]. We selected DL_{CO} as a measure of pulmonary function because it has been consistently identified as the parameter most frequently affected following hospitalization for COVID-19. DL_{CO} percentage of predicted value and the lower limit of normal (LLN) were calculated using Global Lung Function Initiative Network. Persistent respiratory dysfunction was defined as DL_{CO} below LLN. Thin-section chest CT scan was performed ($n = 91$) as previously described [23]. We evaluated the prevalence of ground-glass opacities exceeding 10% in at least one of the four lung zones, along with any mosaic pattern, and categorized these findings together as 'potentially reversible changes'. Reversible changes were seen as signs of ongoing inflammation in this study. Consolidations, reticular patterns, parenchymal bands, interlobular septal thickening or any bronchiectasis were considered 'potentially irreversible changes' and grouped together, indicating pulmonary fibrosis.

Statistical analysis

Baseline characteristics were described by median and interquartile range (25th and 75th percentiles [IQR]) for continuous variables and percentages for categorical variables. Differences between outcome groups in levels and temporal responses were analysed by linear mixed models using untransformed complement levels and a log link function. Subject was used as random effect and time outcome measure as fixed effects (also as interaction). Several outcomes (i.e. ICU/RF, 60-day mortality) and other group comparisons (i.e. treatment modality, pulmonary pathology at 3 months) were performed with some comparisons performed only in sub-populations. Covariates used for adjustment therefore differed somewhat. Covariates included reflected general demography (i.e. age, sex, comorbidities), factors that could influence complement levels (i.e. treatment modalities and dexamethasone, COVID-wave, vaccination status) and kidney function. We made a composite score of all comorbidities (i.e. chronic cardiac disease, hypertension, chronic pulmonary disease, obesity and diabetes), which was graded as having or not having comorbid disease. Thus, for the RF/ICU and

mortality outcome, treatment, dexamethasone use, age, sex, eGFR, COVID-wave, comorbidities and vaccination status were included as covariates. Pulmonary pathology at 3 months and randomized treatment (i.e. the NORSOL study) was assessed only in cohort 1, so vaccination status, dexamethasone use and COVID-wave were not applicable, and age, sex, treatment, eGFR and comorbidity were included as covariates. For assessing dexamethasone effects in cohort 2, age, sex, eGFR, COVID-wave, comorbidities and vaccination status were included as covariates. Complement levels at each time point were compared with healthy controls by a multivariate general linear model using age and sex as covariates.

The association between admission levels of complement factors and 60-day all-cause mortality was first assessed by receiver-operating characteristic (ROC) analysis using baseline levels or delta values of changes at 3–5 or 7–10 days. The association among changes (i.e., increase or decrease) in complement factors during hospitalization with 60-day all-cause mortality was further assessed by the Kaplan–Meier analysis. As max change in the assessed complement factors was at 7–10 days, this time point was used and supplemented by change to 3–5 days in patients without blood samples at 7–10 days. For analysis of 60-day mortality in an adjusted Cox regression model, we evaluated the change in complement factors (i.e. increase or decrease from baseline to 3–5 or 7–10 days as described above) and performed several adjustments: Step 1 was general demographic data (age, sex, comorbidities); step 2 was covariates from step 1 + CRP, eGFR, PaO_2/FiO_2-ratio, as covariates one-by-one.

Ethics

Both cohort studies on which this study is based were approved by the Committee for Medical Research Ethics Region South-east Norway (Cohort 1: approval no. 118684, date 13.03.2020; Cohort 2: approval no. 106624, 13.02.2020). Cohort 1 was additionally approved by the Norwegian Medicines Agency (20/04950-23). Prior to inclusion, all participants either provided informed consent directly or through a legally authorized representative. Participants granted their consent for both engagement in the study and the subsequent publication of results in a medical journal.

Results

Baseline characteristics

Table 1 provides demographic data and clinical characteristics for the combined cohort ($n = 457$, comprising cohorts 1 and 2), stratified by patient admitted to the hospital ward only ($n = 265$, 57%), and those who developed RF and/or were admitted to ICU (RF/ICU, $n = 192$, 39%). The groups were comparable concerning age, gender and comorbidity. As expected, during the first 10 days, there was a statistically significant increased usage of dexamethasone, oxygen therapy, a higher number of obese patients (BMI > 30), elevated levels of CRP, ferritin, total white blood cell count, neutrophils; and a lower level of PaO_2/FiO_2-ratio, haemoglobin and lymphocytes in the RF/ICU group. The characteristics between cohorts 1 and 2 were highly comparable, with the notable distinction that patients in cohort 1 received interventional treatment (HCQ and remdesivir), whereas patients in cohort 2 were administered dexamethasone (Table S1), introduced as a standard treatment option for severe COVID-19 in early autumn 2020.

Temporal profile of complement activation products in relation to disease severity during hospitalization

During the first 10 days of hospitalization, complement activation products were assessed in serial samples for each patient: TCC, reflecting activation of the terminal pathway implying release of C5a, C4d, reflecting activation of the classical and lectin pathway, C3bBbP, reflecting alternative pathway activation and C3bc, reflecting the common pathway from all three initial pathways. Hospitalized patients maintained markedly higher levels of four of the five activation products but with a considerable overlap for C5a, compared to healthy controls ($n = 24$) in age- and sex-adjusted analyses (Fig. 2). In a linear mixed model, evaluation of temporal profiles revealed that patients with RF/ICU outcome maintained higher levels of TCC and C4d, compared to other patients, adjusting for treatment, dexamethasone use, age, sex, eGFR, COVID-wave, comorbidity and vaccination status ($p < 0.001$ for both) (Fig. 2A,B). A similar distinction in disease severity was not discernible in the levels of C3bBbP, C3bc and C5a as no significant differences were observed at any time point (Fig. 2C–E). With respect to treatment effects, remdesivir had a modest impact on complement activation products during hospitalization,

Table 1. *Demographic, clinical and biochemical characteristics in 457 patients hospitalized for COVID-19, stratified according to ICU admission or respiratory failure (ICU/RF) during the first 10 days of hospitalization.*

Parameter	Ward ($n = 265$)	ICU/RF ($n = 192$)
Age, years	55.6 [47.0, 65.6]	59.7 [49.0, 71.9]*
Male gender (%)	169 (64%)	120 (63%)
Body mass index, kg/m^2	27.4 [25.0, 30.7]	28.3 [25.3, 32.0]
Treatment group		
Standard of care (SoC) (%)	212 (80%)	163 (85%)
SoC + hydroxychloroquine (%)	29 (11%)	16 (8%)
SoC + remdesivir (%)	24 (9%)	13 (7%)
Vaccinated ≥1 dose	4 (2%)	4 (2%)
Dexametasone (%)	49 (19%)	90 (47%)***
Oxygen therapy (%)	149 (56%)	170 (89%)***
Current smoker (%)	15(6%)	9 (5%)
Comorbidities		
Chronic cardiac disease (%)	46 (18%)	34 (18%)
Hypertension (%)	87 (34%)	66 (36%)
Chronic pulmonary disease (%)	60 (22%)	47 (25%)
Obesity, BMI ≥ 30 (%)	60 (24%)	62 (33%)*
Diabetes (%)	54 (21%)	42 (34%)
Any comorbidity	170 (65%)	138 (72%)
Outcomes	12 (5%)	8 (5%)
ICU admission (%)	0 (0%)	121 (63%)
Respiratory failure (%)	0 (0%)	140 (75%)
ICU/RF (%)	0 (0%)	192 (100%)
60-day death (%)	4 (2%)	34 (18%)***
P/F-ratio at admission, kPa	45.2 [37.8, 51.9]	29.7 [21.0, 43.2]***
Haemoglobin, g/dL	13.4 [12.6, 14.3]	12.8 [11.7, 13.6]***
C-reactive protein, mg/L	48 [24, 102]	92 [45, 160]***
Ferritin, μg/L	512 [265, 877]	897 [486, 1447]*
White blood cell count, $\times 10^9$/L	5.8 [4.5, 7.2]	7.7 [5.0, 9.8]***
Neutrophils, $\times 10^9$/L	3.8 [2.8, 5.3]	6.1 [3.7, 1.2]***
Lymphocytes, $\times 10^9$/L	1.2 [0.8, 1.5]	0.9 [0.6, 1.2]***
eGFR	94 [77, 108]	90 [71, 105]*

Note: Data are shown as median (25th and 75th percentile [IQR]) for continuous variables and n = (percentages) for categorical variables and were compared with the Mann–Whitney U test or chi-square test, respectively.
Abbreviations: eGFR, estimated glomerular filtration rate; ICU, intensive care unit; P/F-ratio, PaO_2/FiO_2-ratio; SoC, standard of care.
*$p < 0.05$, ***$p < 0.001$ between cohorts.

resulting in reduced levels of TCC (cohort 1), whereas the administration of dexamethasone to patients in cohort 2 was associated with increased levels of C4d, reduced levels of C3bc and no effect on TCC (Table S2).

Increased 60-day mortality in patients with escalated levels of TCC and C4d during hospitalization

Among the entire population, a total of 37 patients died within 60 days, accounting for 18% of those with RF/ICU outcomes and 2% of those admitted

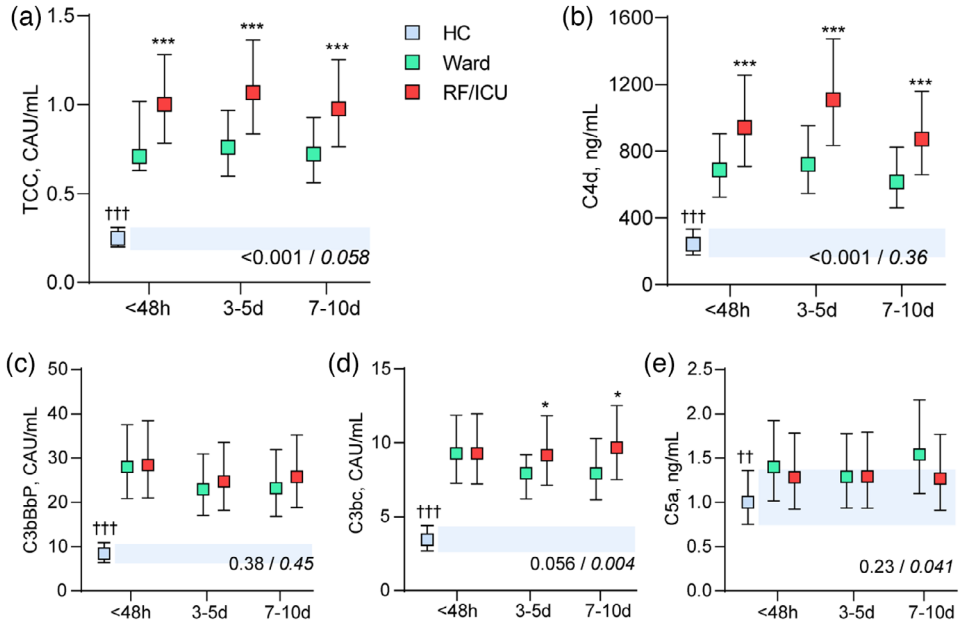

Fig. 2 *Temporal profiles of complement activation products during the acute phase in 459 hospitalized COVID-19 patients in relation to a combined outcome of respiratory failure (RF) and intensive care unit (ICU) admission (n = 192) or ward (n = 265). (A) TCC, terminal complement complex (B) C4d, degradation product of the activated complement factor C4 (C) C3bBbP, alternative pathway activation product (D) C3bc, complement activation product (E) C5a, complement activation product. Data are shown as estimated marginal means and 95% CI from linear mixed model analysis. The p-values reflect the group (RF/ICU) effect, and group × time (italic) from the linear mixed models adjusted for treatment, dexamethasone use, age, sex, estimated glomerulus filtration rate (eGFR), COVID-wave, comorbidity and vaccination status. *p < 0.05, **p < 0.01, ***p < 0.001 between groups are sequential Sidak-adjusted p-values from the mixed model analysis. HC, healthy controls (n = 24). ††p < 0.01, †††p < 0.001 versus patients at all time points as assessed by ANCOVA using age and sex as covariates. HC levels are also shown as blue-shaded area. Number of observations in Ward versus ICU/RF at baseline (<48 h) (n = 271/142), 3–5 days (n = 185/137) and 7–10 days (n = 88/133).*

to the ward only. ROC analysis of admission levels of complement activation products did not reveal any significant association with increased mortality at day 60 (Fig. 3A). However, when assessing the temporal profiles of complement activation products during hospitalization in relation to 60-day mortality, we observed a significant escalation of TCC and C4d among those patients that did not survive ($p < 0.001$ for both time × death interactions) (Fig. 3B). A similar trend was not observed for C3bBbP, C3bc and C5a (Fig. 3C). ROC analysis of the changes in TCC and C4d during hospitalization in relation to 60-day mortality showed descent discriminatory power in identifying individuals who did not survive to day 60 (Fig. 3D). Kaplan–Meyer analyses based on in-hospital increase in TCC and C4d confirmed a higher risk of death within day 60 (Fig. 3E). In a Cox regression analysis, after sequential adjustment for CRP, PaO_2/FiO_2-ratio, comorbid pulmonary disease and comorbid cardiac disease, the hazard ratios exhibited highly significant values within a substantial range (2.71–3.94) for increased risk of death at day 60, for both TCC and C4d (Fig. 3F).

Complement activation in relation to pulmonary pathology after 3 months

In cohort 1, lung function tests and chest CT were assessed in 97 patients at 3 months (90 with DL_{CO} and 91 with CT assessment). (Admission demographic, clinical and biochemical characteristics for these patients are described in Table S3.) Diffusion capacity (DL_{CO}) was identified below the LLN in 28 of the patients, whereas 57 patients had reversible changes on chest CT (Table S3). We evaluated temporal profiles of the complement activation products during the first 10 days of admission in relation to reversible CT changes measured at 3 months. Linear mixed model analysis revealed

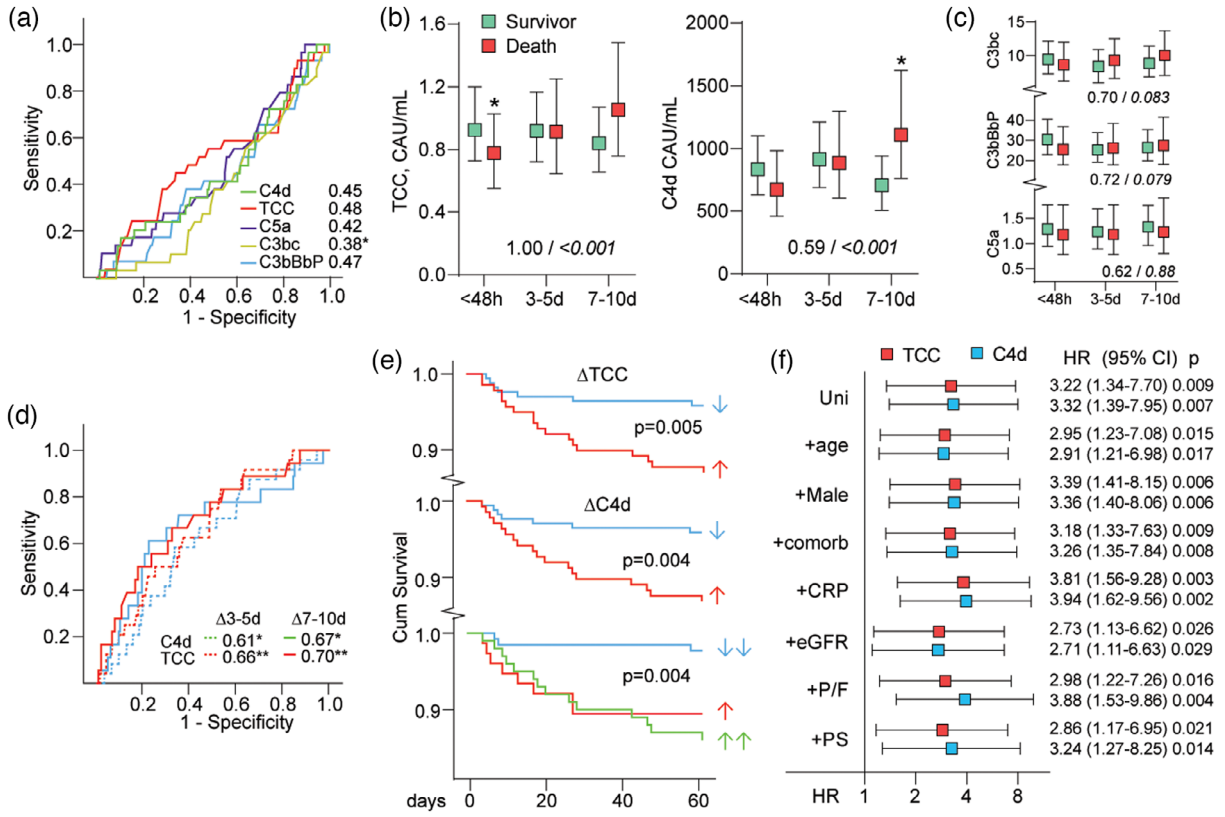

Fig. 3 *Increased 60-day mortality in patients with an increase in TCC and C4d during hospitalization. (A) Receiver operating characteristic (ROC) analysis of admission levels of complement markers in relation to 60-day mortality. *p < 0.05. Temporal profiles of (B)TCC and C4d and (C) C3bc, C3bBbP and C5a during the first 10 days after admission according to 60-day mortality shown as estimated marginal means and 95% CI from linear mixed model analysis. The p-values reflect the group (60-day mortality) effect, and group × time (italic) from the linear mixed models adjusted for treatment, dexamethasone use, age, sex, estimated glomerulus filtration rate (eGFR), COVID-wave, comorbidity and vaccination status. *p < 0.05 between groups are sequential Sidak-adjusted p-values from the mixed model analysis. (D) ROC analysis of change in TCC and C4d during hospitalization in relation to 60-day mortality. *p < 0.05, **p < 0.01. (E)Kaplan–Meier analysis of 60-day mortality according to if TCC or C4d increase or decrease from baseline to 3–5 or 7–10 days. The bottom panel shows a combination of both factors, where red reflects an increase in either TCC or C4d and green reflects an increase in both. p-Values are from the log-rank test. (F) Cox regression of change (i.e. increase vs. decrease) in TCC and C4d during hospitalization in relation to 60-day mortality in univariate analysis or following one-by-one adjustment with covariates as well as a propensity score (PS) combing all covariates. CRP, C-reactive protein; P/F ratio, PO_2/FiO_2 ratio; C. Pulm, comorbid pulmonary disease; C. Card, comorbid cardiac disease. Data are shown as hazard ratios (HR) and 95% CI's with p-values from the Cox regression. Number of observations in survivors versus non-survivors at baseline (<48 h) (n = 383/30), 3–5 days (n = 293/29) and 7–10 days (n = 194/27). For the composite change score, survivors n = 283 versus non-survivors n = 25.*

consistently higher C4d levels at all time points in patients with reversible CT changes thought to reflect persistent pulmonary inflammation compared to those patients who did not exhibit CT changes (p < 0.001–0.05) (Fig. 4A), following adjustment for randomized treatment, age and sex. None of the complement activation products were associated with impaired pulmonary function (DCLO < LLN) or irreversible CT changes at 3 months (Table S4).

Long-term activation of complement in hospitalized COVID-19 patients

Among the 457 included patients in this study, biobanking and measurement of complement

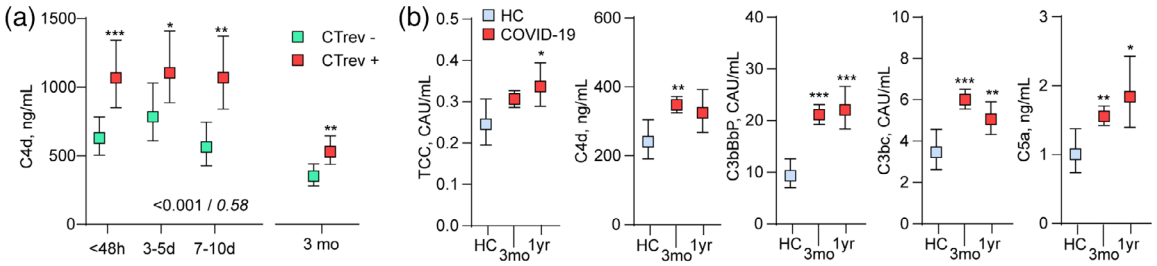

Fig. 4 *Levels of complement activation products at 3-month and 1-year follow-up and C4d in relation to pulmonary pathology at 3 months. (A) Temporal profile of C4d during the first 10 days after admission according to reversible CT changes at 3-month follow-up shown as estimated marginal means and 95% CI from linear mixed model analysis. The p-values reflect the group (reversible CT changes) effect and group × time interaction (italic) from the linear mixed models adjusting for randomized treatment, age, sex, estimated glomerulus filtration rate (eGFR) and comorbidity. *p < 0.05, **p < 0.01, ***p < 0.001 between groups are sequential Sidak-adjusted p-values from the mixed model analysis. (B) Complement factor levels at 3-month and 1-year follow-up in COVID-19 patients who were hospitalized in relation to levels in healthy controls (HC). Data were analysed at each time point using multivariate GLM with complement factors as dependent, patient/control as fixed and age and sex as covariates. The HCs were assessed only once and used for comparison at all time points. *p < 0.05, **p < 0.01, ***p < 0.001 versus HC. Number of observations: HC n = 24, 3-month post-COVID patients n = 284, 1-year post-COVID patients n = 41. For reversible CT changes at 3-month follow-up: no changes versus reversible CT changes at baseline (<48 h) (n = 36/54), 3–5 days (n = 34/55), 7–10 days (n = 19/37) and 3 months (n = 34/57).*

activation products were performed for 284 and 41 patients at 3 and 12 months, respectively (Fig. 1) and compared with healthy controls. In the SARS-CoV-2 infected patients, TCC and C4d levels decreased substantially from in-hospital values but remained elevated compared to healthy controls at 3 months and 1 year. TCC was significantly higher at 1 year ($p < 0.05$), whereas C4d was significantly higher at 3 months ($p < 0.01$) (Fig. 4B). Notably, also the other measured complement activation products C3bBbP, C3bc and C5a were significantly higher compared to healthy controls at both 3 months and 1 year ($p < 0.001$–0.05) (Fig. 4B).

Discussion

The present study demonstrates the activation of the complement system in hospitalized COVID-19 patients. The findings of this study revealed several key points: (i) A marked and significant elevation of various complement activation products, TCC, C4d, C3bc, C3bBbP and C5a, was observed among hospitalized patients compared to healthy controls; (ii) patients who developed severe disease, that is RF or required ICU admission, exhibited significantly higher in-hospital levels of TCC and C4d, when compared to patients admitted to the ward only; (iii) temporal profiles demonstrated that escalated levels of TCC and C4d during the first 10 days of hospitalization were associated with increased risk of mortality; (iv) high C4d levels during hospitalization were significantly associated with inflammatory CT pathology at 3 months; and (v) complement activation products remained persistently and significantly elevated 3 months and 1 year after admission with SARS-CoV-2, in contrast to healthy controls.

This study reaffirms prior observations indicating activation of the complement system upon SARS-CoV-2 infection, emphasizing the existence of a graded activation pattern that aligns with the severity of the disease [1, 24, 25]. However, in this study examining a much larger study population, we observed that the heightened risk of mortality exclusively was associated with patients who exhibited escalated complement activation revealed by increased levels of TCC and C4d during hospitalization, rather than being related to the degree of complement activation upon admission. In addition, increased levels of C4d during hospitalization were associated with persistent inflammatory changes on CT at 3 months. These observations are intriguing as they raise the question of whether a pronounced complement activation poses a threat to the host, provided that the activation is short-lived and rapidly diminishes. Conversely, a non-resolving complement activation, characterized by persistent and even increased activation, would indicate an inappropriate and dysregulated response, intrinsically signifying harm to the host [26].

This perspective is consistent with the research conducted by Xiao et al., concluding that it is the duration rather than the peak level of the inflammatory response that is crucial for the final outcome [27]. Given this consideration, and in light of the findings in the present study, it is tempting to speculate whether complement inhibitory intervention could be guided according to a stratification approach, targeting those patients exhibiting escalated complement activation following their admission to hospital. This risk stratification implies a delayed treatment intervention, but it also provides an opportunity or a window to assess the necessity of such intervention through a personalized therapeutic approach. Although early intervention has always been a priority when discussing complement intervention in relation to sepsis and poly-trauma [28], it is worth noting that delayed intervention with the C5a inhibitor vilobelimab, up to 14 days after symptom onset, given to mechanically ventilated SARS-CoV-2-infected patients, significantly reduced mortality [4]. Although the assessment of complement activation at a daily basis is still not feasible in a clinical context, there are strong reasons to investigate potential risk stratifications for patients in the context of complement intervention. This underscores the potential for precise, targeted therapies against complement activation in COVID-19 subgroups.

A striking finding in this study was the enduring and consistently elevated complement activation observed at both 3 months and even after 1 year when compared to healthy controls. To the best of our knowledge, we are unaware of any similar reports indicating such prolonged and noteworthy low-grade but consistent systemic complement activation following SARS-CoV-2 infection. We cannot exclude that this long-lasting activation may be part of what is described as a long COVID, a condition known to comprise a wide range of symptoms and affect various organs with varying pathologies [29, 30]. The elevated complement levels observed a year post-disease may stem from prolonged endogenous activation following SARS-CoV-2, but as we lack clinical information, we are not able to align this elevation to the long-term COVID condition. It is, however, questionable whether this pattern is solely attributed to COVID-19, as other community-acquired pneumonias (e.g. influenza and others) may induce a similar inflammatory fingerprint.

In this study, a broad mapping of complement activation products has been assessed, reflecting different branches of the system. C4d reflects classical and lectin pathway activation, C3bBbP reflects alternative pathway activation and C3bc expresses activation of all three initial pathways, whereas C5a and TCC (sC5b-9) are products of terminal pathway activation. Notably, the interpretation of each of these products and the comparison between them should be made with caution for two main reasons. Their in vivo half-lives differ substantially, and a number of in vitro issues post-sampling play a role for the final result obtained after the testing. Of particular interest is the difference in in vivo half-life of the two main products formed after C5 cleavage, C5a and TCC, which are released in equimolar amounts. C5a, which is the most potent complement inflammatory mediator, has a half-life of less than 1 min due to its rapid binding to the C5a receptors, whereas plasma TCC has a half-life of approximately 1 h [31, 32]. Furthermore, TCC displays superior in vitro stability, allowing plasma to endure up to 10 freeze–thaw cycles without altering TCC concentration [20]. Paradoxically, it is most likely that C5a is the main mediator in the pathogenesis since inhibition of C5a was found to improve prognosis in COVID patients [4]. However, plasma TCC stands out as the most important biomarker for complement activation, serving as a surrogate marker for C5a release.

There are certain limitations in this study, which analyses pooled data from two distinct cohort studies providing different treatment. Although adjustments for both treatment and dexamethasone are integrated in the linear mixed model analyses, there are still uncertainties related to when, during the disease course, dexamethasone was administrated. The timing of dexamethasone administration may have influenced complement activation products differentially, a factor that is not considered. Although the study includes the first three waves of COVID-19, we have no data on the effects of new SARS-CoV-2 variants on systemic complement activation. A significant proportion of follow-up samples were missing within the total study population, with only 284 and 41 individuals providing samples at 3 months and 1 year, respectively. Nonetheless, after excluding patients who died within 60 days, the occurrence of the composite endpoint (ICU/RF) remained similar in individuals with and without follow-up samples both

at 3 months (38% in both groups) and after 1 year (37% and 39%, respectively). Thus, in terms of the primary ICU/RF outcome, the longitudinal sampling populations appear to adequately represent the entire study population. Furthermore, the study population is from Norway, and thus, we cannot fully exclude that other ethnical populations may have behaved differently. However, as the complement system is among the most conserved defence systems throughout evolution, it suggests that significant variations are less probable across different ethnicities. The study also has several strengths, including two well-characterized prospective cohorts with common and high-quality standardized protocols for biobanking, facilitating the collection of serial samples for comprehensive complement analyses, strictly obtained, prepared and stored according to complement analysis guidelines within a large study population. Additionally, the study includes a long-term follow-up involving clinical assessment.

In conclusion, the present study demonstrates significant systemic complement activation in SARS-CoV-2 hospitalized patients, with more pronounced activation observed in the most severely ill individuals. In-hospital escalated levels of TCC and C4d were associated with an increased risk of 60-day mortality, whereas admission levels of these products were not. Future studies are warranted to clarify risk stratification and time for optimal therapeutic complement inhibitory intervention.

AUTHOR CONTRIBUTIONS

Thor Ueland, Andreas Barratt-Due, Tom Eirik Mollnes, Bente Halvorsen and Pål Aukrust were responsible for the study conception and execution of the present sub-study and securing the financial support. Andreas Barratt-Due, Marius Trøseid, Bente Halvorsen, Anne Ma Dyrhol-Riise, Tuva Børresdatter Dahl and Pål Aukrust were responsible for the management, coordination, research activity planning and execution of the NOR-solidarity trial. Jan Cato Holter, Lars Heggelund, Anders Benjamin Kildal, Kristian Tonby, Aleksander Rygh Holten and Anne Ma Dyrhol-Riise were responsible for the management, coordination, research activity, planning and execution of the Norwegian SARS-CoV-2 study. Tøri Vigeland Lerum, Ole Henning Skjønsberg and Trond M Aaløkken were responsible for the 3-month follow-up protocol for pulmonary function and CT scan. Tuva Børresdatter Dahl, Bente Halvorsen and Beate Kiland Granerud coordinated the collection and storage of the biobank material. Tom Eirik Mollnes, Camilla Schjalm, Kristin Pettersen and Renathe H. Grønli were responsible for the complement analyses. Thor Ueland was responsible for bioinformatics. Andreas Barratt-Due, Thor Ueland, Pål Aukrust, Tom Eirik Mollnes and Bente Halvorsen drafted the manuscript. All authors revised and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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DATA AVAILABILITY STATEMENT

Regarding data sharing, Norwegian institutional data privacy regulations prohibit deposition of individual level data to public repositories. Participant written consent also does not cover public sharing of data for use for unknown purposes. However, upon contact with Andreas Barratt-Due (andreas.barrattdue@gmail.com) or Bente Halvorsen (b.e.halvorsen@medisin.uio.no),

an institutional data transfer agreement can be established, and data shared if the aims of data use are covered by ethical approval and patient consent. The process entails obtaining an update for ethical approval undergoing review by legal departments at both institutions. Typically, this procedure is expected to take 1–2 months from the initial contact. The sharing of data will be facilitated through a secure online procedure. The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Correspondence: Andreas Barratt-Due, Department of Anesthesia and Intensive Care Medicine, Oslo University Hospital, Oslo, Norway.
Email: andreas.barrattdue@gmail.com

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplemental Table 1. Demographic, clinical, and biochemical characteristics in 459 patients hospitalised for COVID-19, stratified by two large multicentre cohorts in Norway.

Supplemental Table 2. Systemic concentrations of complement activation products during hospitalisation according to treatment modalities.

Supplemental Table 3. Admission demographic, clinical, and biochemical characteristics in 97 patients who assessed pulmonary function and chest CT at 3-month follow-up, stratified according if they had DLCO<LLN or reversible CT-changes.

Supplemental Table 4. Temporal profiles of complement activation products during the acute phase in hospitalised COVID-19 patients in relation to pulmonary function and CT findings at 3 months.

