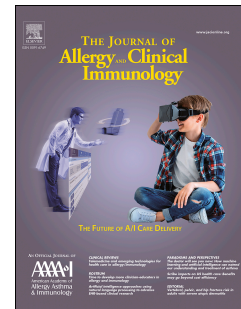


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Severe COVID-19 is associated with hyperactivation of the alternative complement pathway

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42 **Disclosure statement.** None of the authors has a relevant competing interest.

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ABSTRACT

Background: Severe coronavirus disease 2019 (COVID-19) is characterized by impaired type I interferon activity and a state of hyperinflammation leading to acute respiratory distress syndrome. The complement system has recently emerged as a key player in triggering and maintaining the inflammatory state, but the role of this molecular cascade in severe COVID-19 is still poorly characterized. **Objective:** We aimed at assessing the contribution of complement pathways at both protein and transcriptomic levels. **Methods:** To this end, we systematically assessed RNA levels of 28 complement genes in circulating whole blood of COVID-19 patients and healthy controls, including genes of the alternative pathway, for which data remain scarce. **Results:** We found differential expression of genes involved in the complement system, yet with various expression patterns: while patients displaying moderate disease had elevated expression of classical pathway genes, severe disease was associated with increased lectin and alternative pathway activation, which correlated with inflammation and coagulopathy markers. Additionally, properdin, a pivotal positive regulator of the alternative pathway, showed high RNA expression but was found at low protein concentrations in severe and critical patients, suggesting its deposition at the sites of complement activation. Notably, low properdin levels were significantly associated with the use of mechanical ventilation (AUC = 0.82, $p = 0.002$). **Conclusion:** This study sheds light on the role of the alternative pathway in severe COVID-19 and provides additional rationale for the testing of drugs inhibiting the alternative pathway of the complement system.

KEY MESSAGES

- The classical pathway is activated in all COVID-19 patients, while hyperactivation of the lectin and alternative pathways is associated with disease severity.
- Properdin RNA expression is increased in severe patients, yet its protein levels are decreased, suggesting its deposition on activating surfaces.
- Low properdin levels are associated with use of mechanical ventilation.

CAPSULE SUMMARY

We show that activation of the alternative complement pathway characterizes COVID-19 severity. Specifically, low properdin levels were associated with use of mechanical ventilation. This work provides a rationale for the specific inhibition of the alternative complement pathway.

KEYWORDS

Complement system, alternative pathway, COVID-19, SARS-CoV-2, immunology, hemostasis.

ABBREVIATIONS

ARDS (acute respiratory distress syndrome), C3G (C3 glomerulopathy), CRP (C-reactive protein), COVID-19 (coronavirus disease 2019), DAF (decay acceleration factor), IFN (interferon), MAC (membrane attack complex), SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2).

INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), has to date caused over 4 million deaths worldwide (1). While the majority of patients remain asymptomatic or show mild-to-moderate symptoms, approximately 5% of patients display severe disease, characterized by acute respiratory distress syndrome (ARDS), which can result in multi-organ failure and death (2,3). We previously demonstrated that severe and critical patients displayed an imbalanced immune response with impaired type I interferon (IFN) activity coupled to excessive inflammation (4). Glucocorticoids have shown to reduce COVID-19 mortality, yet complementary therapies could more specifically target certain members of the immune response (5). In this context, the complement system has emerged as an attractive candidate.

The complement system is a key player of innate immunity at the interface with the adaptive immune system (6). Activation of the complement cascade leads to the cleavage of C3 and the deposition of C3b on activating surfaces, triggering phagocytosis or cleavage of C5 into C5a and C5b, and subsequent formation of the membrane attack complex C5b9 (MAC), resulting in the perturbation of the cell membrane. Additionally, C3a and C5a are anaphylatoxins able to recruit and activate leukocytes, thereby bridging the gap between innate and adaptive immunity and promoting inflammation. The complement cascade can be activated through three different pathways, all converging to the cleavage of C3: (i) the classical pathway detects bound antibodies or other acute phase proteins via C1q; (ii) the lectin pathway recognizes carbohydrate structures in pathogens and damaged membranes; (iii) the alternative pathway is in a constant state of activation unless complement inhibitory proteins are presented, a process known as “tick over”, and can amplify C3b formation.

Complement activation has been associated with disease severity in bacterial and viral pneumonia, ARDS and multiorgan failure (7). As for SARS-CoV-2, the complement system was one of the most highly induced intracellular pathway in infected lung epithelial cells, driven by the transcription of C1r, C1s, factor B and C3 (8). Additionally, multiple products of the complement system, including sC5b9, C5a, C3bc, C3bBbP and C4d were found in COVID-19 patient sera (9). Accordingly, COVID-19 patients with severe disease displayed elevated C5a, C3a and sC5b9 plasma concentrations (9-13), and genetic defects in complement regulatory genes such as CD55 and factor H were associated with

disease severity (14). Additionally, anti-C5aR1 antibodies inhibited lung injury in human C5aR1 knock-in mice, indicating that targeting complement could reduce disease severity (10).

What links the complement pathway to COVID-19 severity is still poorly understood, but one hypothesis lies in its association with coagulopathy (15). Severe COVID-19 has been shown to trigger thrombosis (16,17), and markers of coagulation have been associated with critical disease. Beyond identified connections between inflammation and coagulopathy, evidence suggests a crosstalk between the complement and coagulation cascades (18). Although studies showed association of complement activation with severe COVID-19, an integrative approach assessing the contribution of complement pathways at both protein and transcriptomic levels is lacking. To address this issue, we analyzed RNA and protein levels of components of the three complement pathways in COVID-19 patients and healthy controls.

RESULTS & DISCUSSION

We previously analyzed whole-blood transcriptomic data from 32 COVID-19 patients with various disease severity and 13 healthy controls (4). The main characteristics of these patients are described in Table I. To uncover the role of complement in disease severity, we determined the RNA levels of 28 complement genes with expression above the lower limit of quantification (Figure E1). Of those, 19 were differentially expressed depending on disease stage (Figure 1a). Hierarchical clustering identified two main gene groups (showing high intra-group correlation and including 17 of the 19 genes) displaying distinct patterns of expression: group 1 contained genes whose expressions peaked in moderate disease, whereas group 2 genes showed increased expression in severe and to a greater extent in critical patients, while patients with moderate disease had expression levels that were comparable to that of healthy controls (Figure 1b). Group 1 included genes belonging to the classical pathway (*C1QA* and *C1QB*) and both classical and lectin pathway (*C2* and *SERPING*, coding for C1 inhibitor), as well as the terminal phase (*C5*) (Figure 1c). In contrast, group 2 contained genes belonging to the lectin (*MBL2*, *MASP2* and *C4*, the latter also belonging to the classical pathway) and the alternative pathways (*C3*, its stabilizer CFP, coding for properdin, C3 receptors *ITGAM* and *ITGAX*, and C3 regulators *CR1*, *CD46*, *CD55*, *CD59*) (Figure 1c).

We next studied the correlation between complement gene expression and circulating CRP and IL-6 proteins on the one hand, and *PPBP* (encoding for platelet chemokine CXCL7) and *SELPLG* (encoding for PSGL-1) gene expression on the other hand, two markers of coagulopathy that we previously described as predictive of intubation and death (19) (Figure 2a, b). Additionally, we determined the 20 genes (among the 574-gene nCounter panel) showing highest correlation with group 1 or group 2 genes, respectively (bar graphs). Genes from group 1 moderately correlated with markers of inflammation, while they showed no correlation with coagulopathy markers. They most correlated with genes of the anti-viral response (e.g. *IFIH1*, encoding for MDA5, *IRF7*, an IFN regulator factor, and *BST2*, encoding for tetherin), consistent with the IFN response being a marker of moderate, but not severe, disease (4) (Figure 2a). Genes from group 2 showed moderate-to-high correlation with both inflammation and coagulopathy, and were most correlated to genes of inflammation (Figure 2b).

To more precisely characterize systemic complement activation in COVID-19, we measured complement protein levels from 33 COVID-19 patients with available sera (including 16 patients with

both RNA and protein data) (**Figure 3**). Levels of C1q, C1 inhibitor and sC5b9 were upregulated in all COVID-19 patients, regardless of severity grade, similarly to what was observed at the transcriptional level. C3 protein levels were upregulated in moderate and severe patients, while some critical patients showed lower levels of circulating C3. The lack of a significant decrease in C3 protein may be explained by higher C3 RNA levels and subsequent protein synthesis in critical patients, and as a result, C3 consumption is counterbalanced by C3 production. C4 remained within normal laboratory ranges. We found that properdin levels significantly decreased with disease severity, whereas its RNA levels inversely significantly increased (**Figure 1c**, group 2, *CFP*), suggesting the deposition of properdin to complement activating surface and the triggering of the alternative pathway. We confirmed this finding using plasma samples of an independent COVID intensive care unit cohort (13) (**Figure 3b**).

Finally, we analyzed the association of complement component with use of mechanical ventilation. ROC curve analysis demonstrated that decreased properdin levels were associated with use of mechanical ventilation (**Figure 4**, $AUC = 0.82$, $p = 0.002$, $q = 0.01$), with 83% positive predictive value and 73% negative predictive value using an optimal threshold of $25.5 \mu\text{g/mL}$.

This work constitutes the first study to our knowledge to simultaneously analyze the three complement pathways in COVID-19 patients at both the RNA and protein levels, unraveling properdin consumption as a feature of severe COVID-19. Lower properdin concentration in severe and critical COVID-19 patients cannot be explained by congenital properdin deficiency, a rare X-linked disorder associated with vulnerability for *Nessieria meningitidis*-driven meningitis (20), since these patients showed increased properdin RNA levels. Reduced properdin levels were found in various disease conditions, such as C3 glomerulopathy (C3G) and lupus nephritis, and was correlated with C5 convertase dysregulation in C3G (21,22). Complement alternative pathway activation in the context of severe COVID-19 may explain the excess soluble C5a and sC5b9 previously described (9,10). We confirmed increased sC5b9 in COVID-19 patients, albeit sC5B19 did not discriminate severity grades as potently as properdin. Accordingly, Ma et al. found only a weak association between sC5b9 levels and disease severity (12).

This study has limitations: it is a monocentric, cross-sectional analysis, with limited sample size, and longitudinal data will be needed to better characterize the sequential activation of complement during COVID-19. Additionally, most complement components in circulation are

synthesized by the liver, therefore whole blood RNA quantification may offer a limited view of the complement transcriptional regulation. However, several complement proteins are produced by a wide variety of cell types (23) and others mainly by leukocytes, such as C1q and properdin, the latter playing a key role in our data. An additional limitation is the substantial overlap in properdin levels between severity groups, suggesting that the impact of properdin may be driven by a subgroup of patients. While ROC analysis highlighted interesting positive and negative predictive values, future work specifically designed to evaluate the predictive potential of properdin are warranted. It will be important to assess the target population and better evaluate the contributions of potential confounding factors, such as comorbidities. Moreover, we do not provide mechanistic evidence of properdin consumption at local sites of infection, and studies that include analysis of pathological tissue samples are warranted to address this issue. Lastly, despite *in vitro* evidence that SARS-CoV-2 spike protein can inhibit factor H and prevent the decay of the alternative pathway C3 convertase (24), other mechanisms may be at play, such as inhibition or activation of other regulatory proteins, or non-specific inflammation-mediated hyperactivation, and this phenomenon could be observed in other infectious conditions with excess inflammation. Further work in other disease models will help delineate the specificity of this observation and dissect its underlying mechanism.

In summary, we showed that severe COVID-19 is characterized by increased activation of the complement alternative pathway, which correlated with inflammation and coagulopathy markers. Specific targeting of the alternative pathway rather than the classical pathway may prove useful to control disease severity without hampering essential anti-viral responses.

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TABLES

Table 1 | Patients' characteristics. Continuous variables are represented as median (interquartile range), and categorical data are shown as absolute numbers (%). Significance between severity groups was determined using the Kruskal-Wallis test (continuous variables) or Fisher's exact test or the χ^2 test of independence where applicable (categorical data). Percentages may not total 100 due to rounding. COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; MV, mechanical ventilation; NA, not assessed.

Characteristics	Healthy controls <i>n</i> = 13	All patients <i>n</i> = 32	Disease severity			<i>p</i> -value
			Moderate <i>n</i> = 11	Severe <i>n</i> = 10	Critical <i>n</i> = 11	
Age (years)	59.2 (45.2-60.0)	55.6 (51.2-64.8)	55.9 (44.7-65.1)	53.5 (48.02-61)	60.2 (54.8-71.65)	0.23
Male	10 (77)	24 (75)	8 (72.7)	9 (90)	7 (63.6)	0.37
Interval from first symptoms to admission (days)	-	10 (9-11)	9 (9-11)	10.5 (10-12)	9 (8-11)	0.06
Coexisting disorder						
Any	0 (0)	14 (44)	2 (18)	3 (30)	9 (82)	0.006
COPD	0 (0)	1 (3)	0 (0)	0 (0)	1 (9)	0.37
Diabetes	0 (0)	5 (16)	0 (0)	1 (10)	4 (36)	0.053
Hypertension	0 (0)	10 (31)	2 (18)	2 (20)	6 (55)	0.12
Cardiovascular disease	0 (0)	3 (9)	0 (0)	0 (0)	3 (27)	0.042
Cancer or hemopathy	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	-
Chronic renal disease	0 (0)	1 (3)	0 (0)	0 (0)	1 (9)	0.37
Overweight	0 (0)	2 (6)	1 (9)	1 (10)	0 (0)	0.5
Fever on admission						
Median temperature (°C)	NA	38.9 (38.5-39.4)	38.8 (38.2-39.5)	38.6 (38.5-39.7)	39.0 (38.5-39.4)	-
Symptoms on admission						

Fever	NA	32 (100)	11 (100)	10 (100)	11 (100)	-
Dyspnea	NA	32 (100)	11 (100)	10 (100)	11 (100)	-
Cough	NA	31 (97)	10 (91)	10 (100)	11 (100)	0.37
Fatigue	NA	31 (97)	10 (91)	10 (100)	11 (100)	0.37
Myalgia	NA	20 (63)	8 (73)	8 (10)	4 (36)	0.082
Diarrhea	NA	11 (34)	4 (36)	5 (50)	1 (9)	0.091
Oxygen requirement (L/min)	NA	—	1.5 (1-3)	5 (4-6)	MV	-
Clinical outcomes						
Thrombotic events	NA	3 (9)	0 (0)	1 (10)	2 (18)	0.34
MV	NA	-	0 (0)	5 (50)	-	-
Death	NA	5 (15.6)	0 (0)	0 (0)	5 (45.5)	<0.001
Laboratory findings on admission						
Leukocytes ($\times 10^9/L$)	NA	6.7 (4.31-8.82)	4.71 (3.78-5.68)	7.78 (6.46-8.43)	9.38 (5.48–10.49)	0.038
Neutrophils ($\times 10^9/L$)	NA	5.08 (3.12-7.37)	3.25 (2.07-3.44)	5.81 (4.74-6.36)	7.69 (4.32-9.13)	0.022
Lymphocytes ($\times 10^9/L$)	NA	0.84 (0.56-1.13)	1.00 (0.84-1.40)	0.88 (0.57-1.12)	0.65 (0.45-0.84)	0.031
Monocytes ($\times 10^9/L$)	NA	0.41 (0.23-0.52)	0.40 (0.26-0.52)	0.42 (0.27-0.51)	0.33 (0.12-1.05)	0.95
Platelets ($\times 10^9/L$)	NA	249 (159-298)	166 (112-251)	229 (170-282)	313 (199-352)	0.007
CRP (mg/L)	0.7 (0.0-0.8)	118 (55-242)	30 (14-76)	169 (136-249)	159 (109-308)	<0.001
Lactate dehydrogenase (U/L)	169 (155-224)	424 (346-574)	262 (196-454)	411 (396-623)	504 (426-614)	0.10

FIGURES LEGENDS

Figure 1 | Complement genes show distinct patterns of RNA expression in whole blood.

a | Heatmap representation of RNA levels of the complement genes with differential expression in at least one severity group, measured by the Nanostring nCounter technology. **b |** Main two gene groups, as determined by hierarchical clustering, and their expression pattern (determined as their mean expression across all individuals from a severity group). **c |** Individual dot plots showing normalized counts for each patient and gene of interest. Significance was determined using the Kruskal-Wallis test (comparing the four groups); p denotes uncorrected p -values, while q corresponds to false-discovery rates (corrected p -values). Groups: healthy controls, $n = 13$; moderate, $n = 11$; severe, $n = 10$; critical, $n = 11$.

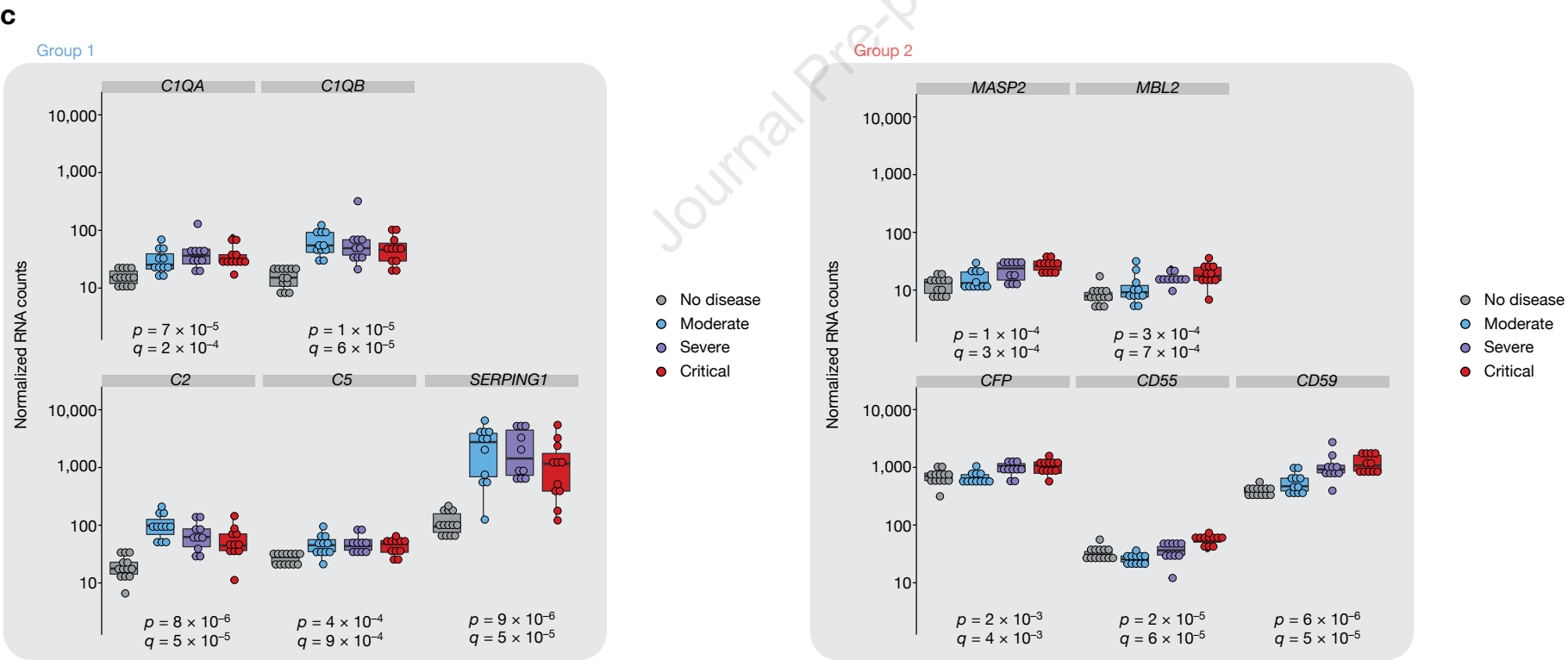
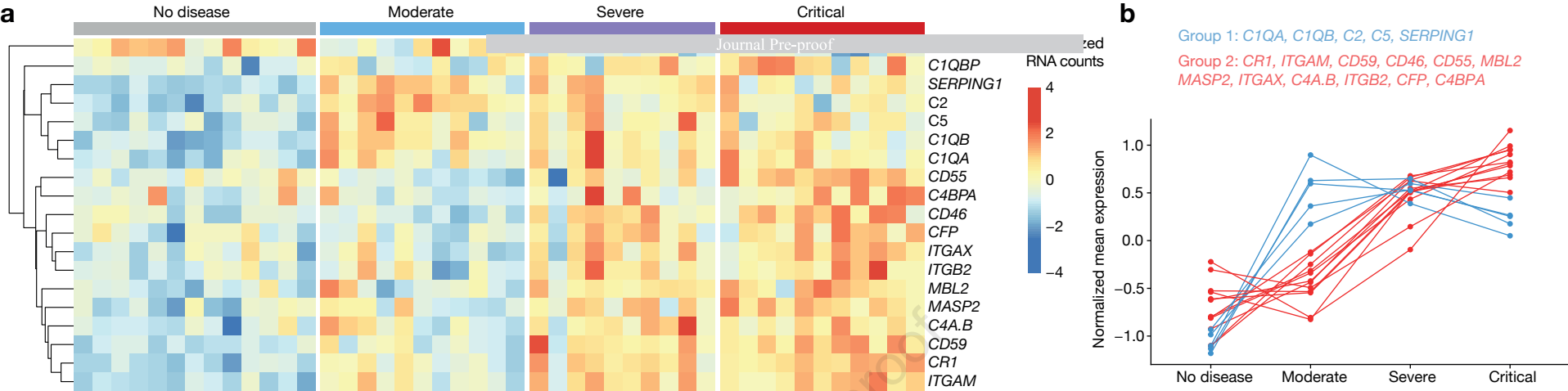
Figure 2 | Correlations of complement RNA expression with inflammation and coagulopathy markers. a | Correlation matrix (with coefficients determined by Spearman's correlation, left), and 20 genes with highest mean correlation with group 1 genes, among the 574-gene dataset. **b |** As in a, for group 2 genes. Asterisks denote significant results (corrected $p < 0.05$), determined by the multiple testing procedure for correlation developed by Cai and Liu (25).

Figure 3 | Complement protein levels in circulating blood. a | Protein concentrations of C1q, C1inh, C3, and C4 in the same samples, measured by ELISA. **b |** Serum concentration of sC5b9 and properdin in the same samples, as well as samples from an independent cohort of critical COVID-19, and healthy controls. Significance was determined using the Kruskal-Wallis test (comparing the three groups); p denotes uncorrected p -values, while q corresponds to false-discovery rates (corrected p -values). NS, not significant. Groups: healthy controls, $n = 73$; moderate, $n = 5-8$; severe, $n = 4-8$; critical, $n = 13-17$; critical (repetition), $n = 46$. Laboratory norms are indicated with a grey bar.

Figure 4 | Properdin is associated with use of mechanical ventilation. a | Protein levels of non-severe patients at admission depending on future use of mechanical ventilation. Dashed lines represent optimal threshold obtained by maximization of Youden's index for the significant area under the curve (AUC) (properdin). **b |** Associated ROC curves, with AUC and p -values; q corresponds to false-discovery rates (corrected p -values). **c |** Bar graphs indicating the number of patients in each

307 group depending on normalized RNA levels of a single gene with respect to the optimal threshold
308 (properdin only). Groups: no intubation ($n = 10-14$) and intubation ($n = 13-19$).

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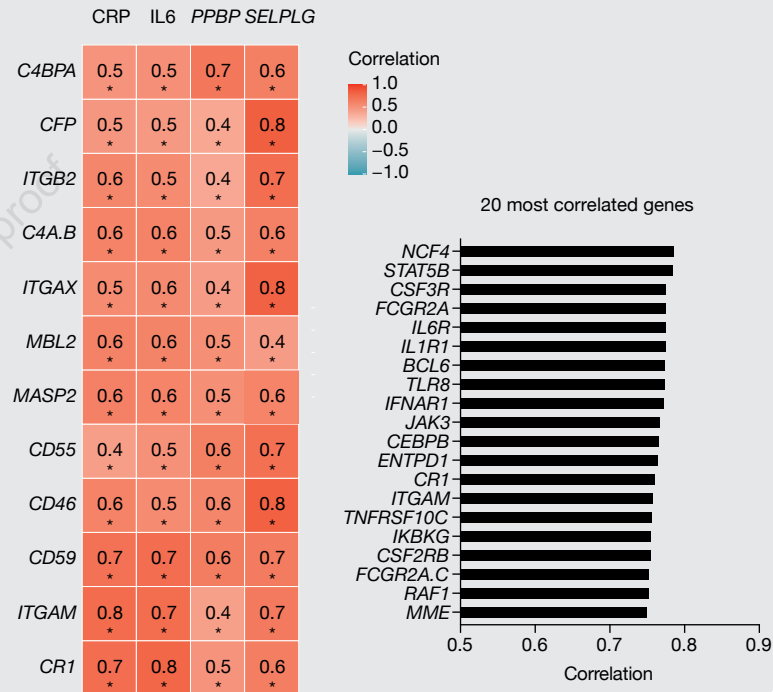


a

Group 1

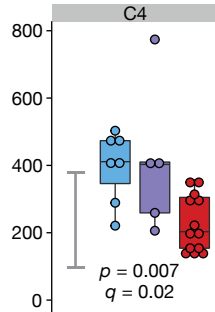
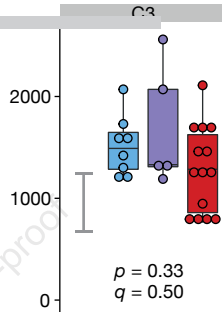
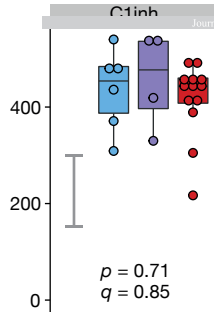
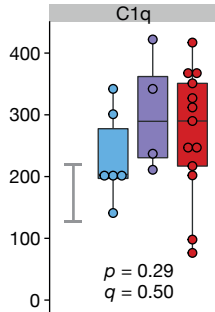
**b**

Group 2

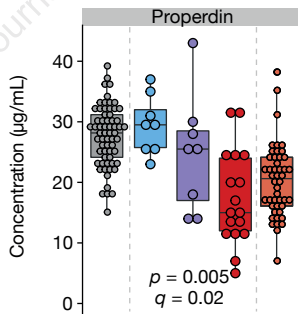
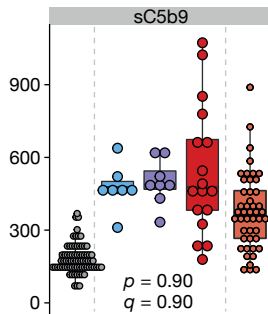


a

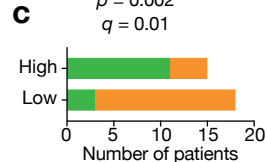
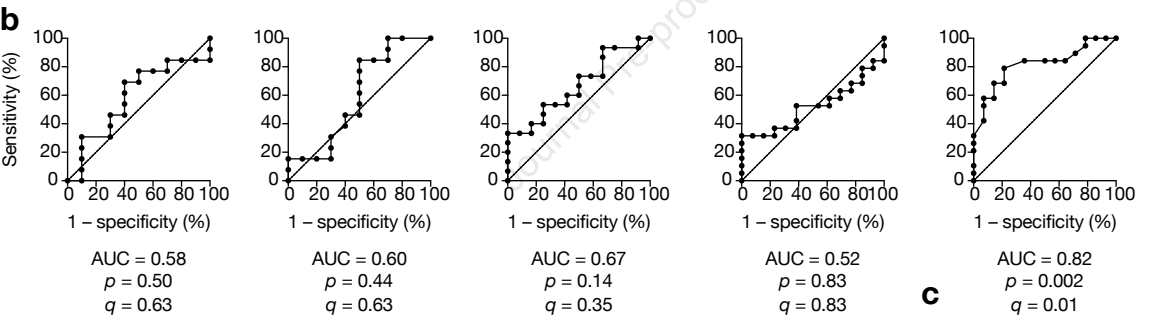
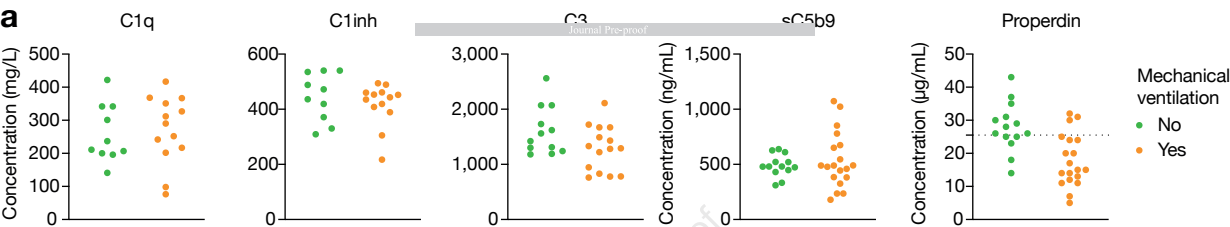
Concentration (mg/L)

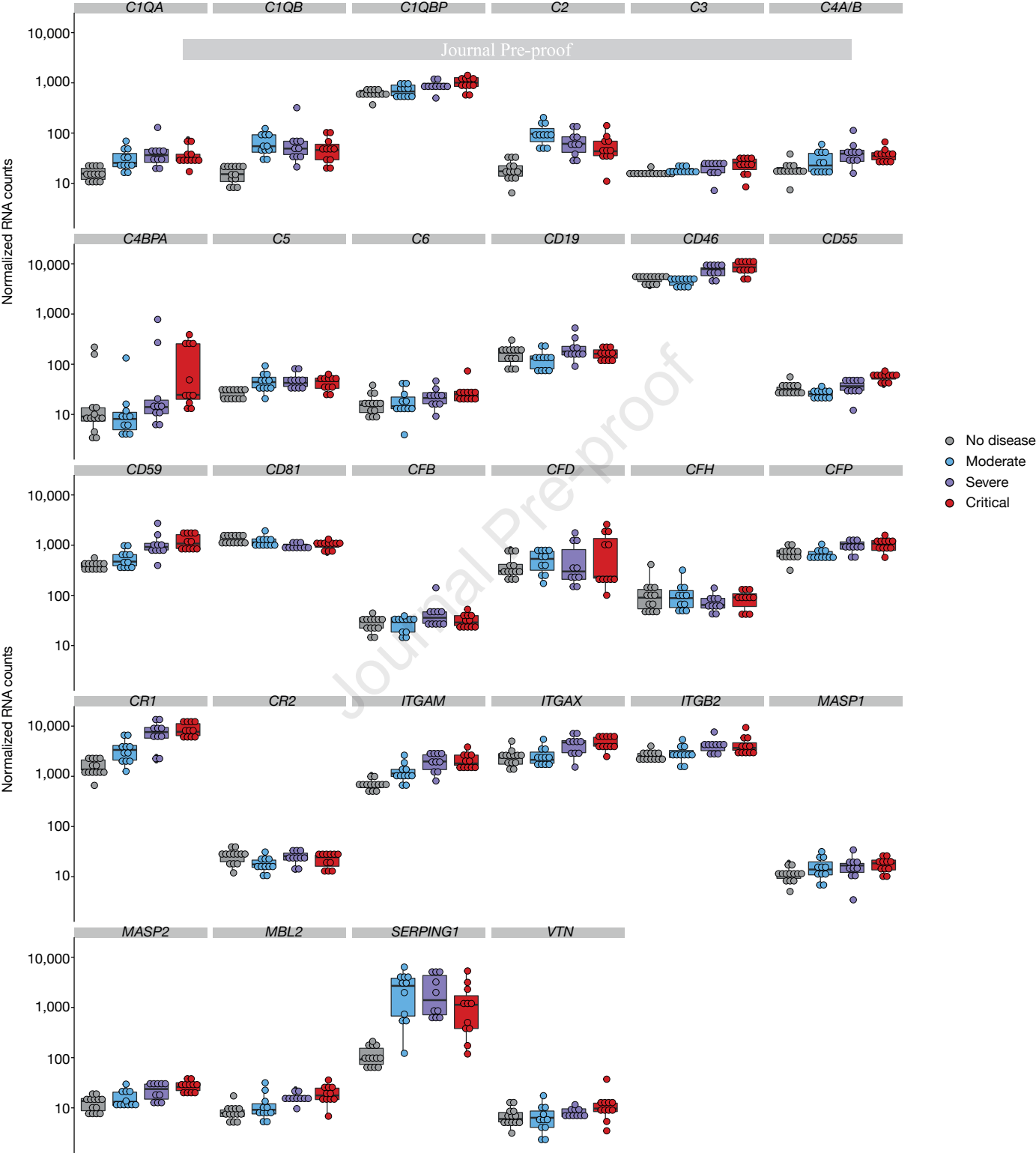
**b**

Concentration (ng/mL)



Laboratory norm
 No disease
 Moderate
 Severe
 Critical
 Critical (repetition)





METHODS

Patient characteristics

Recruitment was conducted between 19 March 2020 and 3 April 2020 in Cochin Hospital (Paris, France). Inclusion criteria for COVID-19 patients were: age 18-80 years old, COVID-19 diagnosis according to WHO interim guidance, and positive RT-PCR for SARS-CoV-2 on a respiratory sample (nasopharyngeal swab or invasive respiratory sample). Disease severity was classified at the time of admission using the adaptation of the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance. Mild-to-moderate (referred to as “moderate” in this article) disease was defined as either mild clinical symptoms (fever, myalgia, fatigue, diarrhea) and no sign of pneumonia on thoracic computed tomography (CT) scan, or dyspnea and radiological findings of pneumonia on thoracic CT scan, with a requirement of ≤ 3 L/min of oxygen, stable for at least the following 24 hours. Severe disease was defined as respiratory distress with requirement of >3 L/min of oxygen and no other organ failure, stable for at least the following 24 hours. Critical cases were defined as respiratory failure requiring mechanical ventilation, shock and/or other organ failure requiring an ICU.

Blood sampling was performed after a median of 10 days (interquartile range 9-11) after onset of first symptoms, and before the initiation of any antiviral or anti-inflammatory treatment, and before use of mechanical ventilation. Time interval from first symptoms to admission to hospital (which coincided for most patients with blood sampling) are specified in Table I. No longitudinal sampling was performed, but patients were followed up for at least 30 days. For more detailed clinical and immunological characterization of the cohort, refer to (1). Clinical, epidemiological, demographic, laboratory, treatment, and outcome data were all extracted from electronic medical records using standardized data collection form.

To repeat protein findings, we included plasma samples from a previously described cohort of COVID-19 ICU patients (2).

Gene expression analysis

Detailed methods were previously reported (1). Briefly, we analyzed 100 ng (5 μ l) of total RNA from each sample using the Nanostring Human Immunology kit v2, following manufacturer’s instructions. Raw RNA counts were adjusted using five housekeeping genes selected from the 15 candidate control

genes provided by Nanostring, using the geNorm method. Normalized counts were $\log_{10}$ -transformed for all subsequent analyses. Thirty-five genes belonging to the complement system were identified, of which 28 were expressed above the lower limit of quantification in at least one sample. Among those, 19 were differentially expressed in one group, as determined by uncorrected p -value < 0.05 of one-way analysis of variance. Heatmap displaying these 19 genes (Figure 1a) was obtained using pheatmap (package pheatmap), with data centered to 0 and scaled to unit variance for each gene. Hierarchical clustering of these 19 genes was performed using hclust with default distance matrix, and revealed 2 groups with lower intra-group variance, when the number of clusters was set to 4, leaving out two genes with different patterns (*CD81* and *C1QBP*).

Genes with highest correlation to a given group (Figure 2, bar graphs) were determined using as a starting gene set all genes among the 574 genes of the nCounter kit with at least one value above lower limit of quantification, at the exception of those belonging to the complement gene group of interest. For each gene of the starting set, mean Spearman's correlation coefficient with respect to genes of the group of interest was computed. Genes were ordered by decreasing coefficients, and the first 20 genes are represented.

Protein concentrations

Plasma concentrations of C1q, C1 inhibitor, C3 and C4 were measured by nephelometry (Siemens), and sC5b9 levels were assessed using Microvue Complement sC5b-9 Plus kit (Quidel). Levels of properdin were determined using a home-made enzyme-linked immunosorbent assay (sheep anti-human properdin, the Binding Site, PC116X).

Statistical analyses

Continuous variables were compared among groups using the Kruskal-Wallis test, while categorical variables were compared using Fisher's exact test or the χ^2 test of independence, where applicable. No exact a priori sample size was determined, for lack of prior knowledge of the expected effect size in such an exploratory study. However, we calculated that $n = 10$ in each group would allow to detect a Cohen's effect size of 1.5 (i.e. a difference in means of 1.5 times the standard deviation) with a power of 90% and a 5% significance level. Correlations coefficients were determined using Spearman's method to detect monotonic relationships. ROC AUC p -values were calculated using GraphPad Prism's

default method(3). Specifically, following Hanley's computation (4), assuming the null hypothesis, the standard error is $\sqrt{0.25 + (n_1 + n_2 - 2)/(12 n_1 n_2)}$, where n_1 and n_2 are the number of patients in each group. The z ratio is then $(AUC - 0.5)/SE$, which follows a two tailed normal distribution, from which the p -value is derived. Optimal thresholds were obtained by maximization of Youden's index. All tests were two-sided and a p -value < 0.05 was considered statistically significant. Correction for multiple testing was obtained using false discovery rates algorithms: Cai and Liu's procedure (5) in the case of correlation data (adapted to the high level of dependencies), and Benjamini and Hochberg's procedure for all other instances (shown as q -values). Analyses were performed using R v. 3.4.3 (CRAN) and Prism v. 8 (Graphpad).

SUPPLEMENTARY FIGURES

Figure E1 | RNA expression of complement genes in whole blood. Individual dot plots showing normalized counts for each patient and all 28 complement genes with at least one measurement above the lower limit of quantification. Groups: healthy controls, $n = 13$; moderate, $n = 11$; severe, $n = 10$; critical, $n = 11$.

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