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

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42 ABSTRACT

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

59 KEY MESSAGES

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

65

66 CAPSULE SUMMARY

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

70

71 KEYWORDS

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

73

74 ABBREVIATIONS

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

78 INTRODUCTION

79 Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of coronavirus disease
80 2019 (COVID-19), has to date caused over 4 million deaths worldwide (1). While the majority of patients remain
81 asymptomatic or show mild-to-moderate symptoms, approximately 5% of patients display severe disease,
82 characterized by acute respiratory distress syndrome (ARDS), which can result in multi-organ failure and death
83 (2,3). We previously demonstrated that severe and critical patients displayed an imbalanced immune response
84 with impaired type I interferon (IFN) activity coupled to excessive inflammation (4). Glucocorticoids have shown
85 to reduce COVID-19 mortality, yet complementary therapies could more specifically target certain members of
86 the immune response (5). In this context, the complement system has emerged as an attractive candidate.

87 The complement system is a key player of innate immunity at the interface with the adaptive immune
88 system (6). Activation of the complement cascade leads to the cleavage of C₃ and the deposition of C_{3b} on
89 activating surfaces, triggering phagocytosis or cleavage of C₅ into C_{5a} and C_{5b}, and subsequent formation of
90 the membrane attack complex C_{5b9} (MAC), resulting in the perturbation of the cell membrane. Additionally,
91 C_{3a} and C_{5a} are anaphylatoxins able to recruit and activate leukocytes, thereby bridging the gap between
92 innate and adaptive immunity and promoting inflammation. The complement cascade can be activated
93 through three different pathways, all converging to the cleavage of C₃: (i) the classical pathway detects bound
94 antibodies or other acute phase proteins via C_{1q}; (ii) the lectin pathway recognizes carbohydrate structures in
95 pathogens and damaged membranes; (iii) the alternative pathway is in a constant state of activation unless
96 complement inhibitory proteins are presented, a process known as “tick over”, and can amplify C_{3b} formation.
97 Complement activation has been associated with disease severity in bacterial and viral pneumonia, ARDS and
98 multiorgan failure (7). As for SARS-CoV-2, the complement system was one of the most highly induced
99 intracellular pathway in infected lung epithelial cells, driven by the transcription of C_{1r}, C_{1s}, factor B and C₃ (8).
100 Additionally, multiple products of the complement system, including sC_{5b9}, C_{5a}, C_{3bc}, C_{3bBbP} and C_{4d} were
101 found in COVID-19 patient sera (9). Accordingly, COVID-19 patients with severe disease displayed elevated
102 C_{5a}, C_{3a} and sC_{5b9} plasma concentrations (9–13), and genetic defects in complement regulatory genes such
103 as CD55 and factor H were associated with disease severity (14). Additionally, anti-C_{5aR1} antibodies inhibited
104 lung injury in human C_{5aR1} knock-in mice, indicating that targeting complement could reduce disease severity
105 (10).

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

114 RESULTS & DISCUSSION

115 We previously analyzed whole-blood transcriptomic data from 32 COVID-19 patients with various disease
116 severity and 13 healthy controls (4). The main characteristics of these patients are described in **Table I**. To
117 uncover the role of complement in disease severity, we determined the RNA levels of 28 complement genes
118 with expression above the lower limit of quantification (**Figure E1**). Of those, 19 were differentially expressed
119 depending on disease stage (**Figure 1a**). Hierarchical clustering identified two main gene groups (showing high
120 intra-group correlation and including 17 of the 19 genes) displaying distinct patterns of expression: group 1
121 contained genes whose expressions peaked in moderate disease, whereas group 2 genes showed increased
122 expression in severe and to a greater extent in critical patients, while patients with moderate disease had
123 expression levels that were comparable to that of healthy controls (**Figure 1b**). Group 1 included genes
124 belonging to the classical pathway (*C1QA* and *C1QB*) and both classical and lectin pathway (*C2* and *SERPINC1*,
125 coding for C1 inhibitor), as well as the terminal phase (*C5*) (**Figure 1c**). In contrast, group 2 contained genes
126 belonging to the lectin (*MBL2*, *MASP2* and *C4*, the latter also belonging to the classical pathway) and the
127 alternative pathways (*C3*, its stabilizer CFP, coding for properdin, C3 receptors *ITGAM* and *ITGAX*, and C3
128 regulators *CR1*, *CD46*, *CD55*, *CD59*) (**Figure 1c**).

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

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

142 and protein data) (**Figure 3**). Levels of C1q, C1 inhibitor and sC5b9 were upregulated in all COVID-19 patients,
143 regardless of severity grade, similarly to what was observed at the transcriptional level. C3 protein levels were
144 upregulated in moderate and severe patients, while some critical patients showed lower levels of circulating
145 C3. The lack of a significant decrease in C3 protein may be explained by higher C3 RNA levels and subsequent
146 protein synthesis in critical patients, and as a result, C3 consumption is counterbalanced by C3 production. C4
147 remained within normal laboratory ranges. We found that properdin levels significantly decreased with disease
148 severity, whereas its RNA levels inversely significantly increased (**Figure 1c**, group 2, *CFP*), suggesting the
149 deposition of properdin to complement activating surface and the triggering of the alternative pathway. We
150 confirmed this finding using plasma samples of an independent COVID intensive care unit cohort (13) (**Figure**
151 **3b**).

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

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

167 This study has limitations: it is a monocentric, cross-sectional analysis, with limited sample size, and
168 longitudinal data will be needed to better characterize the sequential activation of complement during COVID-
169 19. Additionally, most complement components in circulation are synthesized by the liver, therefore whole
170 blood RNA quantification may offer a limited view of the complement transcriptional regulation. However,

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

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

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254 **TABLES**

255 **Table 1 | Patients' characteristics.** Continuous variables are represented as median (interquartile range), and categorical data are shown as absolute numbers (%).
 256 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
 257 applicable (categorical data). Percentages may not total 100 due to rounding. COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; MV, mechanical
 258 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

260 FIGURES LEGENDS

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

262 **a |** Heatmap representation of RNA levels of the complement genes with differential expression in at least one
263 severity group, measured by the Nanostring nCounter technology.

264 **b |** Main two gene groups, as determined by hierarchical clustering, and their expression pattern (determined
265 as their mean expression across all individuals from a severity group). **c |** Individual dot plots showing

266 normalized counts for each patient and gene of interest. Significance was determined using the Kruskal–Wallis
267 test (comparing the four groups); p denotes uncorrected p -values, while q corresponds to false-discovery rates
268 (corrected p -values). Groups: healthy controls, $n = 13$; moderate, $n = 11$; severe, $n = 10$; critical, $n = 11$.

269

270 **Figure 2 | Correlations of complement RNA expression with inflammation and coagulopathy markers. a**

271 **|** Correlation matrix (with coefficients determined by Spearman’s correlation, left), and 20 genes with highest

272 mean correlation with group 1 genes, among the 574-gene dataset. **b |** As in a, for group 2 genes. Asterisks

273 denote significant results (corrected $p < 0.05$), determined by the multiple testing procedure for correlation
274 developed by Cai and Liu (25).

275

276 **Figure 3 | Complement protein levels in circulating blood. a |** Protein concentrations of C1q, C1inh, C3, and

277 C4 in the same samples, measured by ELISA. **b |** Serum concentration of sC5b9 and properdin in the same

278 samples, as well as samples from an independent cohort of critical COVID-19, and healthy controls. Significance

279 was determined using the Kruskal–Wallis test (comparing the three groups); p denotes uncorrected p -values,

280 while q corresponds to false-discovery rates (corrected p -values). NS, not significant. Groups: healthy controls,

281 $n = 73$; moderate, $n = 5–8$; severe, $n = 4–8$; critical, $n = 13–17$; critical (repetition), $n = 46$. Laboratory norms are

282 indicated with a grey bar.

283

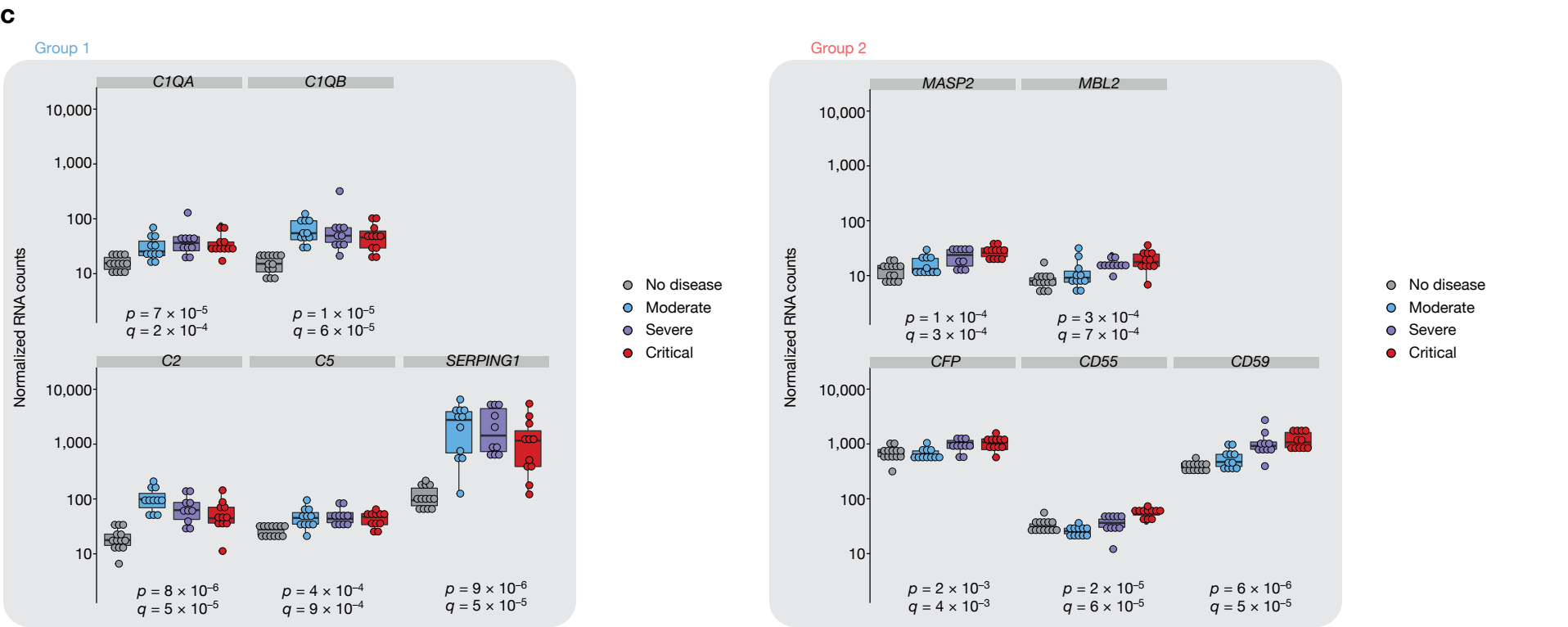
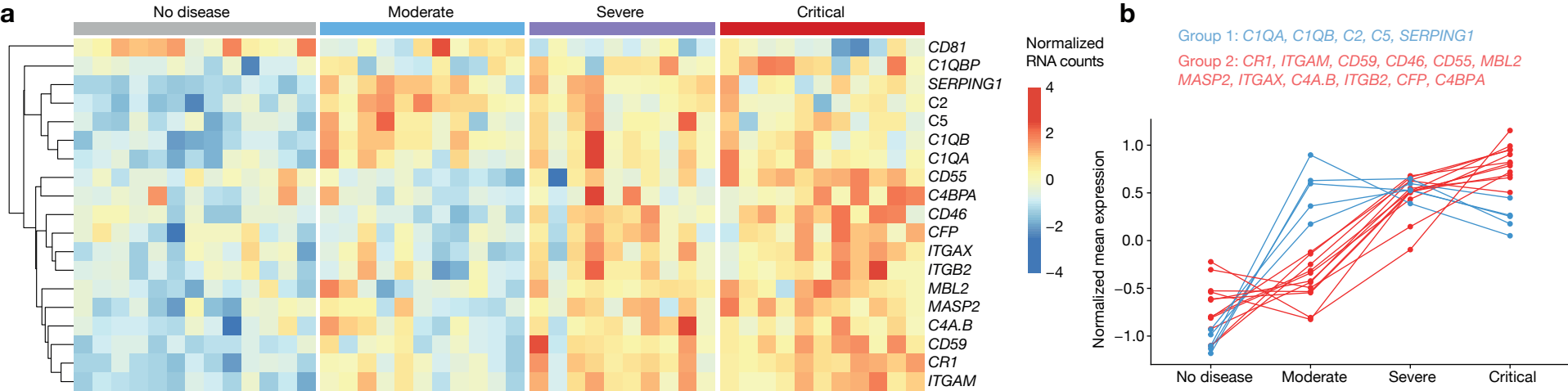
284 **Figure 4 | Properdin is associated with use of mechanical ventilation. a |** Protein levels of non-severe patients

285 at admission depending on future use of mechanical ventilation. Dashed lines represent optimal threshold

286 obtained by maximization of Youden’s index for the significant area under the curve (AUC) (properdin). **b**

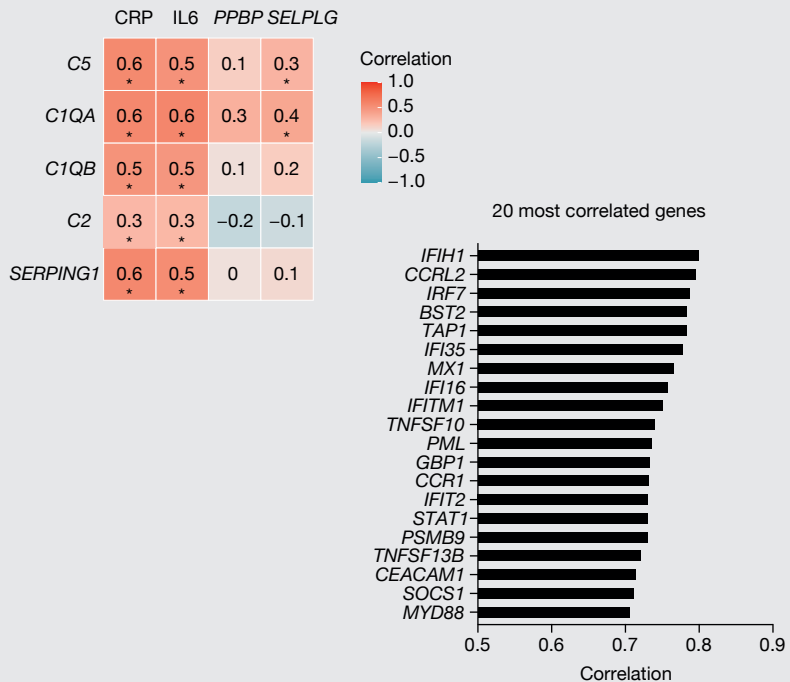
287 **|** Associated ROC curves, with AUC and p -values; q corresponds to false-discovery rates (corrected p -values). **c**

288 | Bar graphs indicating the number of patients in each group depending on normalized RNA levels of a single
289 gene with respect to the optimal threshold (properdin only). Groups: no intubation ($n = 10-14$) and intubation
290 ($n = 13-19$).

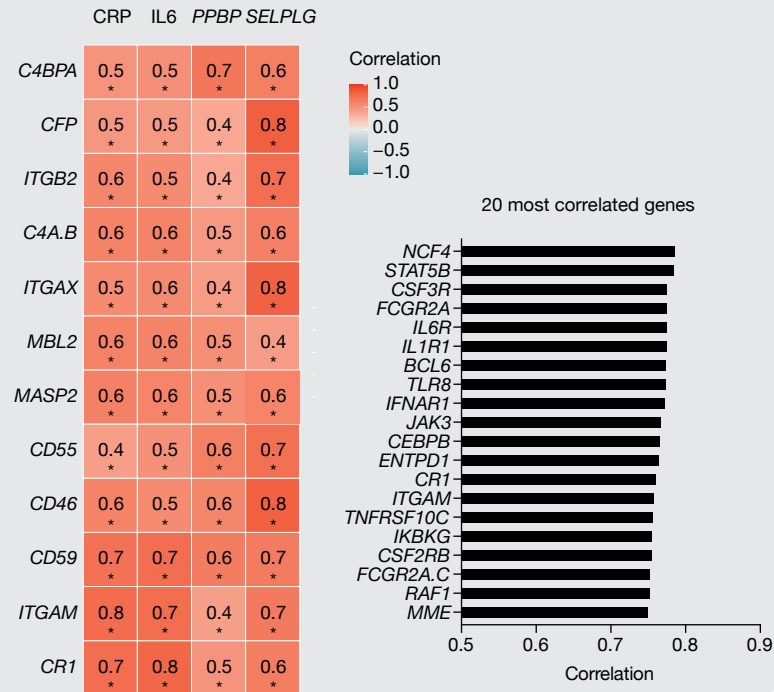


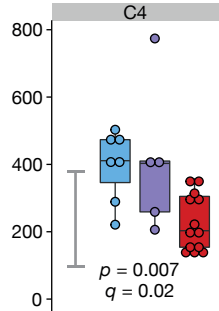
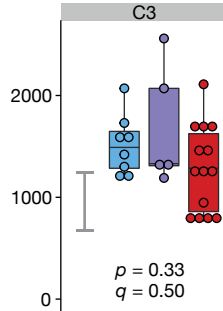
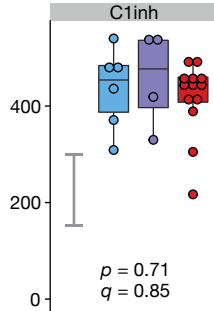
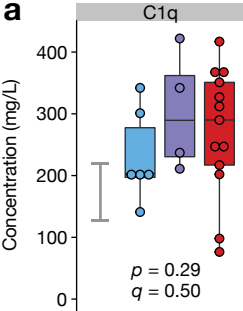
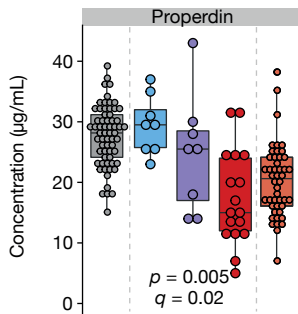
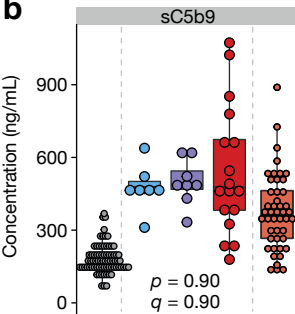
a







Group 1

**b**

Group 2



a**b**

-  Laboratory norm
-  No disease
-  Moderate
-  Severe
-  Critical
-  Critical (repetition)

