

1 **Elevated antiviral, myeloid and endothelial inflammatory markers in severe COVID-19**

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25 Introductory paragraph

26 The mechanisms that underpin COVID-19 disease severity, and determine the outcome of infection,
27 are only beginning to be unraveled. The host inflammatory response contributes to lung injury, but
28 circulating mediators levels fall below those in classical ‘cytokine storms’. We analyzed serial plasma
29 samples from 619 patients hospitalized with COVID-19 recruited through the prospective
30 multicenter ISARIC clinical characterization protocol U.K. study and 39 milder community cases not
31 requiring hospitalization. Elevated levels of numerous mediators including angiopoietin-2, CXCL10,
32 and GM-CSF were seen at recruitment in patients who later died. Markers of endothelial injury
33 (angiopoietin-2 and von-Willebrand factor A2) were detected early in some patients, while
34 inflammatory cytokines and markers of lung injury persisted for several weeks in fatal COVID-19
35 despite decreasing antiviral cytokine levels. Overall, markers of myeloid or endothelial cell activation
36 were associated with severe, progressive, and fatal disease indicating a central role for innate
37 immune activation and vascular inflammation in COVID-19.

38 Main text

39 Fatal COVID-19 is associated with acute respiratory distress syndrome and raised systemic
40 inflammatory markers including IL-6 and C-reactive protein, often accompanied by neutrophilia and
41 lymphopenia¹. The beneficial effect of corticosteroid treatment in severe disease highlights the role
42 of steroid-responsive inflammation in pathogenesis^{2,3}, and post-mortem studies report pulmonary
43 vessel vasculitis (most commonly myeloid cells) and microthrombosis in fatal COVID-19^{4,5,6,7}. The
44 virus-induced inflammatory state has laboratory features that resemble secondary haemophagocytic
45 lymphohistiocytosis (sHLH)^{8,9,10} but the exact pattern and severity of inflammatory responses has
46 been only partially characterized. Levels of some inflammatory mediators, including IL-6, are
47 elevated in COVID-19, but are typically ten times lower than those reported in acute respiratory
48 distress syndrome (ARDS) and sepsis^{11,12,13}, suggesting that other factors may play a major role in
49 COVID-19 severity. Host genetic factors may also influence disease severity, with polymorphisms in

50 several regions, including the interferon pathway genes *IFNAR2* and *OAS1/2/3* recently associated
51 with enhanced disease severity¹⁴. Identification of such genetic inflammatory factors may define
52 a 'treatable trait'¹⁵, allowing both stratification of patients likely to benefit from therapies such as
53 dexamethasone and targeted biological anti-cytokine therapies, and design of novel therapeutics
54 targeting causative pathways.

55 Early clinical studies of COVID-19 identified elevated neutrophil counts and lymphopenia in
56 peripheral blood^{1, 16}, predominantly seen in late-stage disease and of limited prognostic value.

57 Peripheral blood neutrophilia is also seen in other severe respiratory viral¹⁷ and bacterial¹⁸
58 infections, suggesting that this is not a unique feature of COVID-19. Elevated levels of D-dimer, a
59 product of fibrin-degradation associated with thrombosis and inflammation, have also been
60 observed in COVID-19¹⁶, consistent with systemic inflammation and the high frequency of
61 macrovascular thrombotic complications in severe cases^{7, 19}. Post-mortem studies show that
62 thromboses and microthrombi within pulmonary vessels are common in fatal COVID-19 and are
63 associated with endothelial responses distinct from those that occur during fatal influenza A virus
64 infection^{4, 5, 7, 20}. However, the thrombotic aspects of life-threatening COVID-19, and the interaction
65 of this process with cytokine release have hitherto been described in relatively small groups of cases,
66 from single-center studies, or with a narrow range of disease severities.

67 Within the ISARIC4C study we obtained clinical data and 1,047 plasma samples from 619 hospitalized
68 patients with COVID-19^{21, 22}. Given the large number of cases, patients from the ISARIC4C database
69 could be stratified into five levels of severity according to their peak illness, in line with the World
70 Health Organization COVID-19 ordinal scale²³ (Supplementary Table 1): (1) no oxygen requirement
71 (Severity 3, n=169); (2) patients requiring oxygen by face mask (Severity 4, n=143); (3) patients
72 requiring high-flow nasal cannulae, a continuous positive airway pressure mask or other non-
73 invasive ventilation (Severity 5 n=99); (4) patients requiring invasive mechanical ventilation (Severity
74 6/7, n=113); and (5) fatal COVID-19 (Severity 8, n=95). The median duration of symptoms prior to

75 study enrollment was similar in all groups: Severity 3, 7 days; Severity 4, 9 days; Severity 5, 11 days;
76 Severity 6/7, 11 days; and Severity 8, 8 days. Some differences in routinely performed clinical
77 hematology and biochemistry measures were evident between clinical outcome groups at the time
78 of study enrolment: Lymphopenia was evident in groups 6/7 and 8, relative to 3, alongside
79 neutrophilia in 6/7 and 8 relative to 3 and 4 (Supplementary Fig. 1a and 1b, respectively). No
80 differences between groups were observed in ferritin levels, whilst LDH was elevated in groups 5,
81 6/7, and 8 relative to 3 (Supplementary Fig. 1c and 1d, respectively). Procalcitonin levels were
82 elevated in group 8 relative to 3 and 4, and in group 6/7 relative to 4 (Supplementary Fig. 1e). Partial
83 HScores²⁴ were calculated (fever, cytopenia, ferritin, triglycerides, and AST) but the only significant
84 difference between groups was between 6/7 and 4, indicating that sHLH is unlikely to be the
85 predominant pathophysiological mechanism in life-threatening COVID-19 (Supplementary Fig. 1f).
86 The ISARIC4C mortality scores²⁵ for these patients demonstrated an elevated risk of mortality,
87 calculated from admission data, in those that would progress to fatal disease (group 8) relative to all
88 other groups (Supplementary Fig. 1g), though there was considerable overlap between all groups.
89 Together, these data indicated limited clinical or biochemical differences between patient outcome
90 groups at the time of hospital admission.

91 We hypothesized that differences in the levels of plasma inflammatory mediators would reflect the
92 nature and scale of immunopathology in COVID-19 and would associate with different disease
93 outcomes. We therefore quantified 33 mediators in all available plasma samples using panels
94 designed to study a broad range of mediators that could be broadly categorized as having roles in
95 antiviral immunity, inflammation, or coagulation^{16, 19}. Analysis of plasma mediator levels at the time
96 of enrolment distinguished 3 clusters of patients that were associated with distinct patterns of
97 mediator levels (Fig. 1). The first of these clusters was enriched in patients from groups 6/7 and 8
98 and was associated with higher levels of CXCL10, GM-CSF, D-dimer, and vWF-A2. The second cluster
99 contained a more diverse mixture of severities and had a more pronounced pattern of coagulation
100 factor XIV and angiopoietin-2 containing mediator clusters, but lower levels of the CXCL10 containing

101 mediator cluster. The third patient cluster had lower levels of the CXCL10, D-dimer, and coagulation
102 factor XIV containing mediator clusters, but had a more varied pattern of other mediators including
103 IL-6R α , VEGF-D, and IL-4. Interestingly, this analysis did not indicate any obvious patterns of age,
104 symptom duration (onset), or sex in these plasma mediator levels. This analysis shows that, at the
105 time of enrolment, different COVID-19 outcome groups were already identifiable and associated
106 with distinct patterns of inflammatory mediators and that markers such as D-dimer, EN-RAGE,
107 CXCL10, and GM-CSF were particularly associated with enhanced disease severity. However, entry to
108 the study was determined by hospitalization which will be influenced by predisposing factors; these
109 factors may therefore not be evident in the data that we accumulate.

110 To further explore the relationship between the mediator levels and severity we analyzed plasma
111 from 15 healthy controls (7 males, median age 55, range 45-71) and 39 individuals recruited 7 days
112 after a SARS-CoV-2 positive PCR test who did not require hospitalization (15 males, median age 43,
113 range 27-62, termed group '1/2' as per the WHO scale ²³) and related these to hospitalized patients.
114 At the time of enrollment, numerous differences were evident between hospitalized COVID-19
115 patients and the control groups, along with many differences across the clinical outcome groups in
116 hospitalized patients (Fig. 2 and Supplementary Fig. 2). In contrast to other reports ²⁶ we found no
117 evident deficiency in IFN- α levels in those with severe disease (Fig. 2a). IFN- γ was elevated in
118 hospitalized COVID-19 patients relative to both healthy controls (HC) and group 1/2 (Fig. 2b) and was
119 elevated in the most severe outcome groups, relative to lower severity grades. The interferon-
120 induced chemokine CXCL10 was also substantially elevated in all hospitalized COVID-19 cases
121 relative to the control groups, with the most pronounced increases in groups 6/7 and 8 (Fig. 2c).
122 These results are in contrast to the decreased ISG gene expression in peripheral blood samples from
123 patients with severe COVID-19 ²⁶, showing that the gene expression pattern from blood does not
124 necessarily reflect the directly measured levels of gene product. We speculate that the abundance of
125 IFN- γ and CXCL10 results from release from the site of disease rather than from circulating cells,

126 though anti-IFN autoantibodies²⁷ and polymorphisms in IFN signaling¹⁴ may influence this pathway
127 in some patients.

128 The fibrin degradation product D-dimer has been reported to be elevated in severe COVID-19¹⁶,
129 implicating thrombosis in disease severity^{4, 5, 20}. In agreement with these reports, D-dimer was
130 elevated in all hospitalized groups, but little difference was observed between the severity groups at
131 the time of enrolment (Fig. 2d). Given reports of the association between COVID-19 mortality and
132 pulmonary vasculitis⁴, we hypothesized that endothelial injury may be a feature of COVID-19,
133 potentially triggering coagulation and the thrombotic complications common in severe disease^{19, 28}.
134 Indeed, levels of angiopoietin-2, a marker of endothelial injury, were elevated in all hospitalized
135 patients relative to both control groups (Fig. 2e), with levels 5.6-fold higher in the mildest
136 hospitalized patients (group 3, median=1983pg/ml) than HCs (median=352pg/ml). Angiopoietin-2
137 levels were also significantly elevated in groups 6/7 and 8 relative to all other hospitalized COVID-19
138 outcome groups (Fig. 2e). As both angiopoietin-2 and vWF-A2 can enter the blood plasma through
139 exocytosis of endothelial cell Weibel-Palade bodies²⁹, we also quantified vWF-A2, which was
140 similarly elevated in hospitalized COVID-19 patients (Fig. 2f). In line with these markers of
141 endothelial injury and thrombosis, thrombomodulin, vWF-A2, and endothelin-1 were also elevated
142 in COVID-19, predominantly in those most severe patient outcome groups (Supplementary Fig. 2).
143 Elevations in these prothrombotic mediators were not counteracted by levels of the inhibitors
144 angiopoietin-1 or soluble Tie2, which were not significantly different between the tested groups (Fig.
145 S2). These results suggested that endothelial injury and coagulation are common features of patients
146 hospitalized with COVID-19 and that these are most pronounced in severe and fatal COVID-19.

147 In line with other reports^{1, 12}, we found that IL-6 was also significantly elevated in most hospitalized
148 groups relative to the controls (Fig. 2g), with a stepwise increase in levels with escalating severity. IL-
149 6 levels in groups 6/7 and 8 were significantly elevated above all other groups (all $P<0.0001$, Fig. 2g).
150 In agreement with the association of a strong inflammatory response with COVID-19 severity, GM-

151 CSF was similarly elevated in all hospitalized groups, relative to controls and was most pronounced
152 in the groups 6/7 and 8 (Fig. 2h). Numerous other inflammatory cytokines and chemokines showed
153 similar results including TNF- α , IL-2, GDF-15, G-CSF, and VEGF-D (Supplementary Fig. 2). EN-
154 RAGE/S100A12 has previously been characterized as a marker of respiratory damage in ARDS³⁰ and
155 indeed was elevated in groups 6/7 and 8 relative to most others (Fig. 2i). The neutrophil chemokine
156 IL-8 (CXCL8) was similarly elevated in severe disease, as was the neutrophil gelatinase associated
157 lipocalin (LCN-2/NGAL) (Supplementary Fig. 2), in line with the reported association between blood
158 neutrophilia and severity¹⁶ also seen in this cohort (Supplementary Fig. 1b).

159 Other immunological mediators (IL-6R α , IL-13, IL-17) were not significantly different between
160 groups, indicating that only limited aspects of the immune repertoire were active in COVID-19.
161 Interestingly, IL-4 levels were lower in the non-severe disease outcome groups (3, 4, and 5) relative
162 to both the control groups and the severe disease groups 6/7 and 8 (Supplementary Fig. 2),
163 indicating that suppression of the normal levels of type-2 cytokines may be associated with milder
164 COVID-19 disease, and that this mechanism is lost in severe disease. Similarly, IL-12p70, commonly
165 released by antigen presenting cells (APCs)³¹, was decreased in all hospitalized cases relative to the
166 HCs and group 1/2 (Figure S2), possibly owing to the trafficking of APCs to the site(s) of viral
167 infection.

168 To determine the strength of the relationships between these individual plasma mediators we
169 performed a hierarchical correlation matrix analysis of mediators from plasma samples collected at
170 the time of study enrolment. This identified a strongly correlated cluster of inflammatory mediators
171 including GM-CSF, CXCL10, vWF-A2, and IL-6 (Fig. 3a); increases in which were commonly associated
172 with the most severe COVID-19 outcome groups. Given the strong association between age and
173 COVID-19 severity²², and reports of increased inflammatory responses in males relative to females
174 with COVID-19³² we investigated the influence of these demographic factors on plasma mediators
175 levels in hospitalized patients. As the major effect in our cluster analysis was severity (Fig. 1), we

176 further stratified each of these severity groups by age (\geq or $<$ 70 years of age) and sex, to better
177 account for the influence of disease severity on plasma mediator levels. Following adjustment for
178 multiple testing, no mediator was found to be statistically different between males and females
179 within each outcome group (Supplementary Fig. 3). By contrast, several differences were evident
180 between those aged \geq 70 and $<$ 70 years, including elevated levels of D-dimer, CXCL10, and GM-CSF in
181 those aged \geq 70 years; IFN- γ levels were, by contrast, greater in younger patients within severity
182 group 4 (Fig. 3b and Supplementary Fig. 3).

183 We next sought to determine the changes in levels of some key plasma mediators from the time of
184 enrolment over the course of disease, by relating data to the patient reported duration of symptoms
185 at the time of each sample collection, including consecutive samples collected from individual
186 patients. This analysis indicated that many mediators were stable over the time-course of
187 hospitalization, supporting the validity of using samples from the time of enrolment to study the
188 immunologic basis of COVID-19. However, some mediators did change over time; for example, there
189 was a gradual decrease in IFN- γ and CXCL10 over time in most groups (Supplementary Fig. 4),
190 including group 8 (Fig. 4a and 4b, respectively). By contrast some other mediators remained
191 elevated or appeared to increase over the duration of symptoms in group 8, including angiopoietin-2
192 and D-dimer (Fig. 4c and 4d, respectively). Similarly, the inflammatory mediators GM-CSF and EN-
193 RAGE remained elevated or increased in group 8 in the latter stages of disease (Fig. 4e and 4f,
194 respectively). Together, these results indicated that the most severe outcomes of COVID-19 disease
195 were associated with persistent coagulation and inflammation, even as IFN levels declined.

196 Finally, we hypothesized that differences in plasma mediator levels between patients with Severe
197 (groups 6/7 and 8) and Non-severe (groups 3, 4, and 5) COVID-19 would be apparent within the first
198 few days of symptoms. Indeed, within the first 4 days of symptoms several mediators were
199 significantly elevated in the Severe group, relative to Non-severe, including IL-2, IL-6, and GM-CSF
200 (all $P < 0.0001$, Fig. 4g-i, respectively), indicating a pronounced inflammatory response early in Severe

201 disease. Similarly, many markers of coagulation and endothelial injury were elevated in Severe,
202 relative to Non-severe, including D-dimer and vWF-A2 ($P<0.0001$, Fig. 4j and 4k, respectively), in
203 addition to angiopoietin-2 and IL-1 α (which can be activated by thrombin ³³) (Supplementary Fig. 5).
204 By comparison the lung damage-associated marker EN-RAGE ³⁰ was not significantly different
205 between the Severe and Non-severe groups in the first 4 days of symptoms ($P=0.098$,
206 Supplementary Fig. 5). Together, these data indicated that severe COVID-19 is associated with
207 elevated levels of plasma mediators indicative of coagulation, endothelial activation and a broad
208 inflammatory response including CXCL10, GM-CSF, and IL-6. These differences were apparent within
209 the first days of symptoms, while markers of lung damage may only become elevated later in
210 disease, potentially indicating a pathological role for these processes and a window of opportunity
211 for early immunomodulation to prevent significant lung damage.

212 While markers of fibrinolysis have previously been associated with disease severity ¹⁶ and
213 thrombosis is common in severe and fatal COVID-19 ^{4, 5, 20} the causes of this manifestation of severe
214 disease are not known. We demonstrate that increasing disease severity is associated with broad
215 elevations in inflammatory mediator levels, alongside a signature of endothelial injury. This signal
216 was most pronounced in fatal COVID-19 and was apparent even in the early stages of disease.

217 The elevation of angiopoietin-2, thrombomodulin, and vWF-A2 in fatal COVID-19 cases provides
218 evidence for the involvement of endothelial injury in COVID-19 severity. Endothelial injury following
219 inflammatory damage, including the increasingly recognized pulmonary artery vasculitis ^{4, 20} in
220 COVID-19, may result in the initiation of a pro-coagulant role for these cells ³⁴. Alternatively, this
221 response could be triggered by direct viral infection of vascular cells (though this has yet to be
222 conclusively determined ³⁴ viral replication in non-respiratory tissues is commonly observed at post-
223 mortem ^{4, 7}); or thrombin mediated activation of IL-1 α ³³. This pro-coagulant role could lead to the
224 deposition of microthrombi, evident in COVID-19 ⁴, the development of features of disseminated
225 intravascular coagulopathy (DIC) and ultimately elevated levels D-dimer through the degradation of

226 fibrin rich thrombi²⁸. Neutrophilic inflammation could have an etiological role in endothelial injury
227 though neutrophilia is predominantly a feature of the later phases of COVID-19¹, while endothelial
228 injury was evident in the first days of symptoms. However, the continued thrombosis in late stage
229 fatal COVID-19 may result from neutrophil mediated coagulation, observed in other settings^{35, 36, 37}
230 and recently demonstrated in COVID-19³⁸. Combined, these results indicate a multiplicity of possible
231 pro-coagulant triggers that may contribute to pathology at different stages of disease.

232 We found that the antiviral immune mediator CXCL10 and the myeloid cell growth factor GM-CSF,
233 were strikingly elevated in fatal cases of COVID-19. This is confirmed by a recent report describing
234 the potential utility of CXCL10 as an early prognostic marker of COVID-19 severity³⁹. An influx of
235 monocytes/macrophages has been described in the lung parenchyma in fatal COVID-19, combined
236 with a mononuclear cell pulmonary artery vasculitis⁶, and presence of pro-inflammatory monocyte-
237 derived macrophages in bronchoalveolar lavage fluid from patients with severe COVID-19^{4, 40}. The
238 elevation of CXCL10 and GM-CSF in severe disease reported here could contribute to monocyte
239 recruitment and activation leading to this vasculitis, alongside the role of GM-CSF in the recruitment
240 of neutrophils to the pulmonary vasculature⁴¹.

241 Large scale randomized clinical trials for IL-6 signaling antagonists are on-going, though early results
242 of the COVACTA trial of Tocilizumab found no improvement in clinical status or mortality⁴². Small
243 scale studies of anti-GM-CSF have shown promising results^{43, 44} but require formal testing in a
244 clinical trial. Given the role of GM-CSF in granulopoiesis and enhancement of neutrophil survival,
245 alongside the neutrophil activation observed in late stage fatal COVID-19, these trials may inform
246 our understanding of the importance of this pathway in COVID-19 immunopathogenesis⁴⁵. While
247 early studies demonstrated elevated GM-CSF levels in both ICU and non-ICU treated COVID-19
248 patients¹, we now demonstrate a positive association with disease severity and outcome, in
249 agreement with reports of elevated frequencies of GM-CSF⁺ Th1 cells in patients with COVID-19
250 requiring ICU treatment⁴⁶.

251 While many cytokines and other inflammatory mediators were most significantly elevated in fatal
252 and critical COVID-19, these data do not necessarily support the concept of a “cytokine storm” in
253 COVID-19^{12,13}. While some elements, such as elevated IL-6 and ferritin levels (reported in other
254 studies, but not seen here)^{8,9,10}, are reminiscent of sHLH, the relatively gradual clinical progression
255 and persistent elevation of some cytokines, even during the early stages of symptomatic disease, are
256 uncommon amongst conditions associated with cytokine storms such as toxic-shock syndrome and
257 bacterial sepsis.

258 To our knowledge, this is to date the largest study of inflammatory responses in COVID-19. The
259 multicenter nature of ISARIC4C adds to the ability to interpret and apply these results to other
260 settings. However, further studies are needed to determine the prognostic value of these key plasma
261 biomarkers, including multivariable analyses of biological data alongside clinical and demographic
262 data. This detailed level of analysis may also enable the phenotyping of patients most likely to
263 respond to individual therapies. Future analyses should focus on the biological features of patients
264 that respond to therapeutic interventions, such as dexamethasone^{2,3}, to enable mechanistic insight
265 and targeting of treatment. The clear distinction between patients that would progress to severe
266 COVID-19 and those that would not, even in the earliest stages of disease, indicates that early
267 therapeutic intervention may be crucial to limit mortality. Overall, these data indicate an early
268 inflammatory response in COVID-19, most prominent in those who will later suffer severe or fatal
269 disease. These responses may enable the development of prognostic biomarkers, inform our
270 understanding of immunopathogenesis in COVID-19 and enable novel approaches for therapeutic
271 intervention.

272 **Supplementary Methods**

273 **Study design and setting**

274 The ISARIC WHO Clinical Characterization Protocol for Severe Emerging Infections in the UK (CCP-UK)

275 is an ongoing prospective cohort study of hospitalized patients with COVID-19, which is recruiting in

276 258 hospitals in England, Scotland, and Wales (National Institute for Health Research Clinical

277 Research Network Central Portfolio Management System ID: 14152) ⁴⁷. The protocol, revision

278 history, case report form, patient information leaflets, consent forms and details of the Independent

279 Data and Material Access Committee are available online ⁴⁸ and published previously ²².

280 **Participants**

281 Hospitalized patients with PCR-proven or high likelihood of SARS-CoV-2 infection were recruited,

282 including both patients with community- and hospital-acquired COVID-19. This study analyzed

283 plasma from blood samples obtained on the day of enrolment to the study (day 1, Tier 1) and

284 additional serial samples obtained following a sampling schedule (Tier 2) harmonized with

285 international investigators to allow meaningful comparison of results between studies ²¹. Healthy

286 controls were recruited prior to December 2019 under approval from the London – Fulham Research

287 Ethics Committee (REC) (reference 14/LO/1023) or from healthy donors following informed consent

288 from a sub-collection of the Imperial College Healthcare NHS Trust National Institute for Health

289 Research Imperial Biomedical Research Centre Tissue Bank. Use of the sub-collection was approved

290 by the Tissue Bank Ethics Committee (Approval R12023). Samples from community managed COVID-

291 19 cases were collected through a subproject of Imperial College London Communicable Disease

292 Research Tissue Bank, under approval from the south central Oxford REC (reference 15/SC/0089).

293 **HScores**

294 To calculate partial HScores²⁴, ferritin, triglyceride and AST measurements from this study were
295 combined with recorded results from case report forms for temperature and routine hemoglobin,
296 white cell counts, and platelet counts.

297 **Immunoassays**

298 IFN- γ , TNF- α , IL-1 β , IL-2, IL-4, IL-6, CXCL8/IL-8, IL-10, IL-12p70 and IL-13 were quantified using MSD
299 (Mesoscale Diagnostics, Rockville, Maryland, USA) V-Plex proinflammatory plates on a SQ120
300 Quickplex instrument. IL-1 α , IL-1ra, IL-6R α , angiopoetin-1, angiopoetin-2, endothelin-1, VEGF-D, D-
301 dimer, thrombomodulin, Tie2, von-Willebrand Factor-A2 (vWF-A2), G-CSF, GM-CSF, IL-17A,
302 LCN2/NGAL, CXCL10/IP-10, CCL2, CCL3, CCL4 and CCL5 were quantified using a Bio Plex 200
303 instrument (Bio-Rad, Hercules, California, USA) with custom Luminex panel kits from Biotechne
304 (Minneapolis, Minnesota, USA) and MilliporeSigma (Burlington, Massachusetts, USA). IFN- α was
305 quantified using Quanterix (Billerica, Massachusetts, USA) IFN- α assay kits on the SIMOA platform.
306 All values at or below the lower limit of detection (LLOD) were replaced with the geometric mean of
307 the lower limits of detection across plates for each assay.

308 **Statistical analyses**

309 Statistical analyses used GraphPad Prism v8.3.0 (GraphPad, La Jolla, California, USA) R version 3.6.1
310 and Python 3.7.3 with Pandas 1.0.3 and Seaborn 0.10.0. Non-parametric mediator data (as
311 determined by D'Agostino and Pearson normality test) were analyzed by ANOVA using Kruskal-Wallis
312 tests with Dunn's test for multiple comparisons of patient groups within in time group. Non-
313 parametric two-way analyses were performed using Mann-Whitney U tests. Correlation matrix
314 analysis was performed using the R packages ggplot2 and ggcrrplot and Spearman's test for
315 correlation of non-parametric data, after P-value adjustment for multiple testing. The false discovery
316 rate, or expected proportion of discoveries which are falsely rejected, was controlled using the
317 methods of Benjamini and Hochberg. Heatmaps of scaled plasma mediator data were generated
318 using the ComplexHeatmap package in R with rows and columns split by K-means clustering and

319 dendrograms based on Ward's minimum variance method (ward.D2) and Spearmans rank
320 correlations. For heatmap analyses missing values were imputed by predictive mean matching using
321 the Multivariate Imputation by Chained Equations (MICE) package ⁴⁹.

322 Figure legends

323 **Fig. 1 – Plasma mediators at the time of study enrollment demonstrate a broad exaggerated**
324 **immune response in patients hospitalized with COVID-19.** Clustered heatmap of 33 immune
325 mediators in plasma samples collected from patients hospitalized with COVID-19 at the time of study
326 enrolment. Values for each mediator were scaled and rows and columns were split by K-means
327 clustering. Each patients' column is additionally annotated with data on disease outcome
328 ("Severity") as one of the following outcome groups: not requiring oxygen support ('3', n=128),
329 requiring oxygen via a face mask ('4', n=103), requiring non-invasive ventilation or high-flow nasal
330 canulae ('5', n=78), requiring invasive mechanical ventilation ('6/7', n=87) or fatal disease ('8', n=69).
331 Columns are additionally annotated with patient age, sex and duration of illness at the time of
332 sample collection ("Onset").

333 **Fig. 2 – Antiviral, coagulation, and inflammation associated mediators distinguish severity groups**
334 **early in disease.** Plasma samples from the time of study enrolment were analyzed for levels of the
335 antiviral cytokines a) IFN- α , b) IFN- γ , and c) the interferon-induced chemokine CXCL10 in healthy
336 control (HC, n=15), patients with COVID-19 not requiring hospitalization ('1/2', n=39), and
337 hospitalized patients with COVID-19 that would: not require oxygen support ('3', n=32-128), require
338 an oxygen face mask ('4', n=23-103), require non-invasive ventilation or high-flow nasal cannulae
339 ('5', n=19-78), require invasive mechanical ventilation ('6/7', n=19-87) or progress to fatal disease
340 ('8', n=14-69). Mediators associated with coagulation and endothelial injury were also quantified in
341 these plasma samples; d) D-dimer, e) Angiopoietin-2, and f) von-Willebrand factor A2 (vWF-A2).
342 Similarly, mediators associated with inflammation were quantified: g) IL-6; h) GM-CSF; and i) EN-

343 RAGE/S100A12. Data were analyzed for statistical significance using Kruskal-Wallis tests with Dunn's
344 tests for multiple comparisons between all groups. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

345 **Fig. 3 – Plasma mediators in COVID-19 are coordinated around GM-CSF and influenced by age.** a)
346 Correlogram of the association between plasma mediator levels at the time of enrolment in all
347 patients hospitalized with COVID-19 (n=465). b) Inflammatory mediator levels within an outcome
348 group, stratified as those \geq or < than 70 years of age. Data in panel a were analyzed using
349 Spearman's rank correlations with correction for multiple testing; significant correlations are
350 denoted by a circle, the color of which denotes the Spearman's R value. Data in panel b were
351 analyzed using Mann-Whitney U tests with P -value adjustment for false discovery rate.

352 **Fig. 4 – Longitudinal analysis of plasma mediator levels demonstrate a progressive immune**
353 **response and an exaggerated signature of endothelial injury and inflammation early in fatal**
354 **COVID-19.** Plasma levels of a) IFN- γ , b) CXCL10, c) Angiopoietin-2, d) D-dimer, e) GM-CSF, and f) EN-
355 RAGE/S100A12 over the course of disease in patients with fatal COVID-19. Plasma mediator levels of
356 g) IL-2, h) IL-6, i) GM-CSF, j) D-dimer, and k) von-Willebrand factor A2 (vWF-A2) within the first 4
357 days of symptom onset in patients in severity groups 6/7 or 8 ("Severe", n=22) and groups 3, 4, or 5
358 ("Non-Severe", n=54). Linear regressions with 95% confidence intervals are shown in panels a-f. Data
359 in panels g-k were analyzed for statistical significance using Mann-Whitney U tests, where thick
360 horizontal dashed lines denote the median values and thin horizontal dashed lines denote the
361 interquartile ranges.

362 **Supplementary table 1 – Clinical demographics, hematology, and biochemistry data of patients**
363 **hospitalized with COVID-19 at the time of study enrolment**

364 **Supplementary Fig. 1 – Clinical hematology, biochemistry, and severity scores of patients**
365 **hospitalized with COVID-19 at enrolment.** a) Peripheral blood lymphocyte count, b) neutrophil
366 count, c) ferritin levels, d) lactate dehydrogenase (LDH) levels, e) procalcitonin levels, f) partial
367 HScores, and g) ISARIC4C mortality scores at the time of enrolment in hospitalized patients with

368 COVID-19 that would: not require oxygen support ('3', n=9-93); require an oxygen face mask ('4',
369 n=22-71); require non-invasive ventilation or high-flow nasal cannulae ('5', n=15-63); require
370 invasive mechanical ventilation ('6/7', n=19-91); or progress to fatal disease ('8', n=15-63). Data
371 were analyzed for statistical significance using Kruskal-Wallis tests with Dunn's tests for multiple
372 comparisons between all groups. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

373 **Supplementary Fig. 2 – Plasma mediators at the time of enrolment in patients hospitalized with**
374 **COVID-19.** Mediator levels were quantified from plasma collected at the point of study enrolment
375 from hospitalized patients with COVID-19 that would: not require oxygen support ('3', n=128),
376 require an oxygen face mask ('4', n=103), require non-invasive ventilation or high-flow nasal
377 cannulae ('5', n=78), require invasive mechanical ventilation ('6/7', n=87) or progress to fatal disease
378 ('8', n=69). Data were analyzed for statistical significance using Kruskal-Wallis tests with Dunn's tests
379 for multiple comparisons between all groups. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

380 **Supplementary Fig. 3 – Age, but not sex, is associated with differences in plasma cytokine levels**
381 **within COVID-19 disease outcome groups.** Heatmap of false-discovery rate adjusted P-values for
382 each plasma mediator between males and females ("Sex") and those aged ≥70 years and <70 years
383 ("Age") within each disease outcome group ('8'=Red, '6/7'=Orange, '5'=Purple, '4'=Dark blue,
384 '3'=Cyan). Data were analyzed using Mann-Whitney U tests with P-value adjustment for false
385 discovery rate.

386 **Supplementary Fig. 4 – Longitudinal analysis of selected plasma mediators within each disease**
387 **outcome group.** All data within each severity group was related to the duration of symptoms at the
388 time of sample collection ("Onset to sample", measured in days) for each plasma mediator.
389 Generalized additive modelling was used to fit a restricted cubic spline which is plotted together
390 with the standard error (grey).

391 **Supplementary Fig. 5 – Longitudinal analysis of selected plasma mediators within each disease**
392 **outcome group.** Levels of immune mediators collected within the first 4 days of symptom onset in

393 patients in the groups 6/7 or 8 ("Severe", n=22) and groups 3, 4, or 5 ("Non-Severe", n=54). Data
394 were analyzed for statistical significance using Mann-Whitney U tests, where thick horizontal dashed
395 lines denote the median values and thin horizontal dashed lines denote the interquartile ranges.

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485 Supplementary table 1

	Total N (%)	levels	Severity 8	Severity 6/7	Severity 5	Severity 4	Severity 3	Total
Total N (%)			95 (15.3)	113 (18.3)	99 (16.0)	143 (23.1)	169 (27.3)	619
Age on admission (years)	607 (98.1)	<50	5 (5.3)	25 (22.7)	18 (18.4)	32 (22.7)	65 (39.6)	145 (23.9)
		50-69	42 (44.7)	73 (66.4)	61 (62.2)	67 (47.5)	67 (40.9)	310 (51.1)
		70-79	32 (34.0)	12 (10.9)	14 (14.3)	26 (18.4)	17 (10.4)	101 (16.6)
		80+	15 (16.0)	0 (0.0)	5 (5.1)	16 (11.3)	15 (9.1)	51 (8.4)
Sex at Birth	619 (100.0)	Male	76 (80.0)	79 (69.9)	63 (63.6)	84 (58.7)	92 (54.4)	394 (63.7)
		Female	19 (20.0)	34 (30.1)	35 (35.4)	59 (41.3)	77 (45.6)	224 (36.2)
		Not specified	0 (0.0)	0 (0.0)	1 (1.0)	0 (0.0)	0 (0.0)	1 (0.2)
Ethnicity	591 (95.5)	Aboriginal/First Nations	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Arab	0 (0.0)	2 (1.9)	4 (4.4)	0 (0.0)	1 (0.6)	7 (1.2)
		Black	4 (4.4)	14 (13.3)	3 (3.3)	8 (5.7)	5 (3.0)	34 (5.8)
		East Asian	1 (1.1)	2 (1.9)	2 (2.2)	3 (2.1)	4 (2.4)	12 (2.0)
		Latin American	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

		Other	8 (8.9)	11 (10.5)	8 (8.9)	6 (4.3)	11 (6.6)	44 (7.4)
		South Asian	7 (7.8)	4 (3.8)	4 (4.4)	13 (9.3)	5 (3.0)	33 (5.6)
		West Asian	0 (0.0)	0 (0.0)	1 (1.1)	0 (0.0)	1 (0.6)	2 (0.3)
		White	70 (77.8)	72 (68.6)	68 (75.6)	110 (78.6)	139 (83.7)	459 (77.7)
Chronic cardiac disease	609 (98.4)	Yes	37 (38.9)	13 (11.9)	13 (13.3)	34 (24.5)	27 (16.1)	124 (20.4)
		No	58 (61.1)	96 (88.1)	85 (86.7)	105 (75.5)	141 (83.9)	485 (79.6)
Chronic kidney disease	608 (98.2)	Yes	15 (16.0)	4 (3.6)	3 (3.1)	14 (10.1)	14 (8.3)	50 (8.2)
		No	79 (84.0)	106 (96.4)	95 (96.9)	124 (89.9)	154 (91.7)	558 (91.8)
Malignant neoplasm	606 (97.9)	Yes	5 (5.3)	1 (0.9)	5 (5.2)	6 (4.3)	3 (1.8)	20 (3.3)
		No	89 (94.7)	109 (99.1)	92 (94.8)	132 (95.7)	164 (98.2)	586 (96.7)
Moderate or severe liver disease	607 (98.1)	Yes	2 (2.1)	0 (0.0)	2 (2.0)	4 (2.9)	2 (1.2)	10 (1.6)
		No	92 (97.9)	109 (100.0)	96 (98.0)	134 (97.1)	166 (98.8)	597 (98.4)
Obesity (as defined by clinical staff)	589 (95.2)	Yes	11 (12.4)	21 (20.2)	16 (16.8)	20 (14.7)	15 (9.1)	83 (14.1)
		No	78 (87.6)	83 (79.8)	79 (83.2)	116 (85.3)	150 (90.9)	506 (85.9)

Chronic pulmonary disease (not asthma)	609 (98.4)	Yes	12 (12.6)	8 (7.3)	8 (8.1)	14 (10.1)	14 (8.3)	56 (9.2)
		No	83 (87.4)	101 (92.7)	91 (91.9)	124 (89.9)	154 (91.7)	553 (90.8)
Diabetes (without complications)	604 (97.6)	Yes	26 (27.7)	21 (19.3)	14 (14.3)	18 (13.1)	23 (13.9)	102 (16.9)
		No	68 (72.3)	88 (80.7)	84 (85.7)	119 (86.9)	143 (86.1)	502 (83.1)
Respiratory Rate	585 (94.5)	Median (IQR)	24.0 (8.8)	24.0 (10.0)	24.0 (10.0)	21.0 (5.0)	19.0 (4.0)	22.0 (8.0)
Oxygen saturation	580 (93.7)	Median (IQR)	93.0 (6.0)	93.0 (8.0)	94.0 (4.0)	96.0 (4.0)	97.0 (3.0)	95.0 (5.0)
Systolic blood pressure	594 (96.0)	Median (IQR)	129.0 (30.0)	124.0 (24.0)	133.0 (30.5)	130.0 (26.0)	129.0 (26.0)	129.0 (28.0)
Diastolic blood pressure	594 (96.0)	Median (IQR)	74.0 (16.0)	73.0 (18.0)	75.0 (15.0)	78.0 (16.0)	77.5 (19.0)	76.0 (17.0)
Temperature	591 (95.5)	Median (IQR)	37.3 (1.5)	37.4 (1.6)	37.6 (1.6)	37.3 (1.2)	36.9 (1.3)	37.3 (1.5)
Heart Rate	598 (96.6)	Median (IQR)	88.0 (26.0)	98.0 (30.0)	95.5 (21.2)	91.0 (28.0)	85.5 (22.0)	90.0 (27.0)
Glasgow Coma Score:	510 (82.4)	Median (IQR)	15.0 (12.0)	4.0 (12.0)	15.0 (0.0)	15.0 (0.0)	15.0 (0.0)	15.0 (0.0)
FiO2 (0.21-1.0)	249 (40.2)	Median (IQR)	0.6 (0.3)	0.5 (0.3)	0.4 (0.3)	0.2 (0.1)	0.2 (0.0)	0.4 (0.4)
PaO2:	146 (23.6)	Median (IQR)	9.3 (3.4)	9.2 (4.9)	8.2 (1.6)	7.9 (7.6)	3.9 (6.1)	8.9 (3.8)
PCO2	148 (23.9)	Median (IQR)	6.1 (2.0)	6.5 (2.7)	4.7 (1.0)	4.6 (1.0)	5.3 (2.2)	5.5 (2.3)

pH	132 (21.3)	Median (IQR)	7.4 (0.1)	7.4 (0.2)	7.5 (0.0)	7.5 (0.1)	7.4 (0.1)	7.4 (0.1)
HCO3-	141 (22.8)	Median (IQR)	24.6 (5.1)	25.0 (5.8)	25.2 (3.6)	25.0 (1.6)	23.7 (7.9)	25.0 (5.3)
Urine flow rate:	88 (14.2)	Median (IQR)	1312.5 (1286.2)	1325.0 (971.0)	1475.0 (387.5)	915.0 (0.0)	1250.0 (822.2)	1337.5 (1040.8)
If yes, were infiltrates present?	270 (43.6)	YES	36 (66.7)	51 (73.9)	32 (66.7)	33 (68.8)	18 (35.3)	170 (63.0)
		NO	18 (33.3)	16 (23.2)	15 (31.2)	15 (31.2)	33 (64.7)	97 (35.9)
		N/A	0 (0.0)	2 (2.9)	1 (2.1)	0 (0.0)	0 (0.0)	3 (1.1)
Haemoglobin	419 (67.7)	Median (IQR)	124.0 (33.0)	125.0 (22.0)	136.0 (23.5)	133.0 (26.0)	136.0 (28.0)	130.0 (26.5)
WBC count	418 (67.5)	Median (IQR)	9.3 (5.5)	8.1 (4.6)	7.3 (3.8)	6.6 (4.0)	5.5 (3.1)	7.1 (4.6)
Neutrophil count	396 (64.0)	Median (IQR)	7.6 (5.0)	7.2 (4.1)	5.7 (3.8)	4.9 (3.6)	3.5 (2.3)	5.2 (4.5)
Lymphocyte count	397 (64.1)	Median (IQR)	0.8 (0.6)	0.8 (0.4)	0.9 (0.4)	1.1 (0.7)	1.2 (0.8)	0.9 (0.6)
Haematocrit	332 (53.6)	Median (IQR)	23.0 (38.4)	0.4 (34.2)	36.0 (41.6)	0.4 (38.3)	0.5 (40.6)	0.4 (38.6)
Platelet Count	413 (66.7)	Median (IQR)	230.5 (131.0)	233.0 (110.0)	237.0 (124.0)	218.5 (168.2)	226.0 (102.0)	230.0 (122.0)
PT	216 (34.9)	Median (IQR)	13.4 (3.6)	13.2 (2.4)	13.1 (2.2)	12.6 (2.1)	13.0 (1.8)	13.1 (2.4)
APTT/APTR	200 (32.3)	Median (IQR)	33.5 (9.9)	32.0 (10.0)	30.2 (11.1)	31.1 (4.9)	31.5 (6.7)	31.9 (9.4)
Sodium	407 (65.8)	Median (IQR)	137.0 (7.2)	138.0 (5.0)	137.0 (4.0)	138.0 (5.0)	139.0 (5.0)	138.0 (5.0)

Potassium	394 (63.7)	Median (IQR)	4.3 (1.1)	4.2 (0.7)	4.0 (0.5)	4.0 (0.5)	4.1 (0.5)	4.1 (0.6)
Total Bilirubin	381 (61.6)	Median (IQR)	12.0 (9.0)	10.0 (8.0)	11.0 (5.0)	8.0 (4.2)	8.0 (5.0)	9.0 (7.0)
ALT / SGPT	360 (58.2)	Median (IQR)	34.0 (23.5)	42.0 (24.5)	38.0 (38.0)	29.0 (34.0)	26.0 (25.8)	32.5 (32.0)
AST/SGOT	189 (30.5)	Median (IQR)	49.0 (39.0)	44.0 (33.5)	43.0 (30.0)	31.0 (24.0)	25.0 (12.0)	36.0 (32.0)
Lactate dehydrogenase (LDH)	53 (8.6)	Median (IQR)	591.5 (348.5)	579.0 (396.0)	576.0 (469.0)	307.5 (46.0)	316.0 (347.0)	536.0 (434.0)
Glucose	165 (26.7)	Median (IQR)	9.7 (4.5)	8.5 (3.5)	6.7 (2.7)	6.3 (1.9)	5.9 (2.0)	7.6 (3.8)
Blood Urea Nitrogen (urea)	372 (60.1)	Median (IQR)	8.2 (7.5)	5.9 (5.1)	5.0 (3.3)	4.7 (3.6)	4.7 (2.9)	5.3 (4.3)
Creatinine	412 (66.6)	Median (IQR)	91.5 (79.2)	80.0 (37.2)	73.0 (25.0)	70.0 (30.0)	72.5 (24.8)	76.0 (33.2)
Lactate	142 (22.9)	Median (IQR)	1.4 (0.7)	1.3 (0.8)	1.0 (0.7)	1.2 (0.5)	1.7 (1.4)	1.3 (0.8)
Procalcitonin	23 (3.7)	Median (IQR)	1.0 (3.0)	0.7 (2.6)	NA (NA)	3.9 (0.0)	NA (NA)	0.7 (3.3)
C-reactive protein (CRP)	386 (62.4)	Median (IQR)	165.5 (197.2)	199.3 (162.5)	106.5 (100.0)	85.0 (85.1)	34.0 (100.5)	99.0 (151.4)

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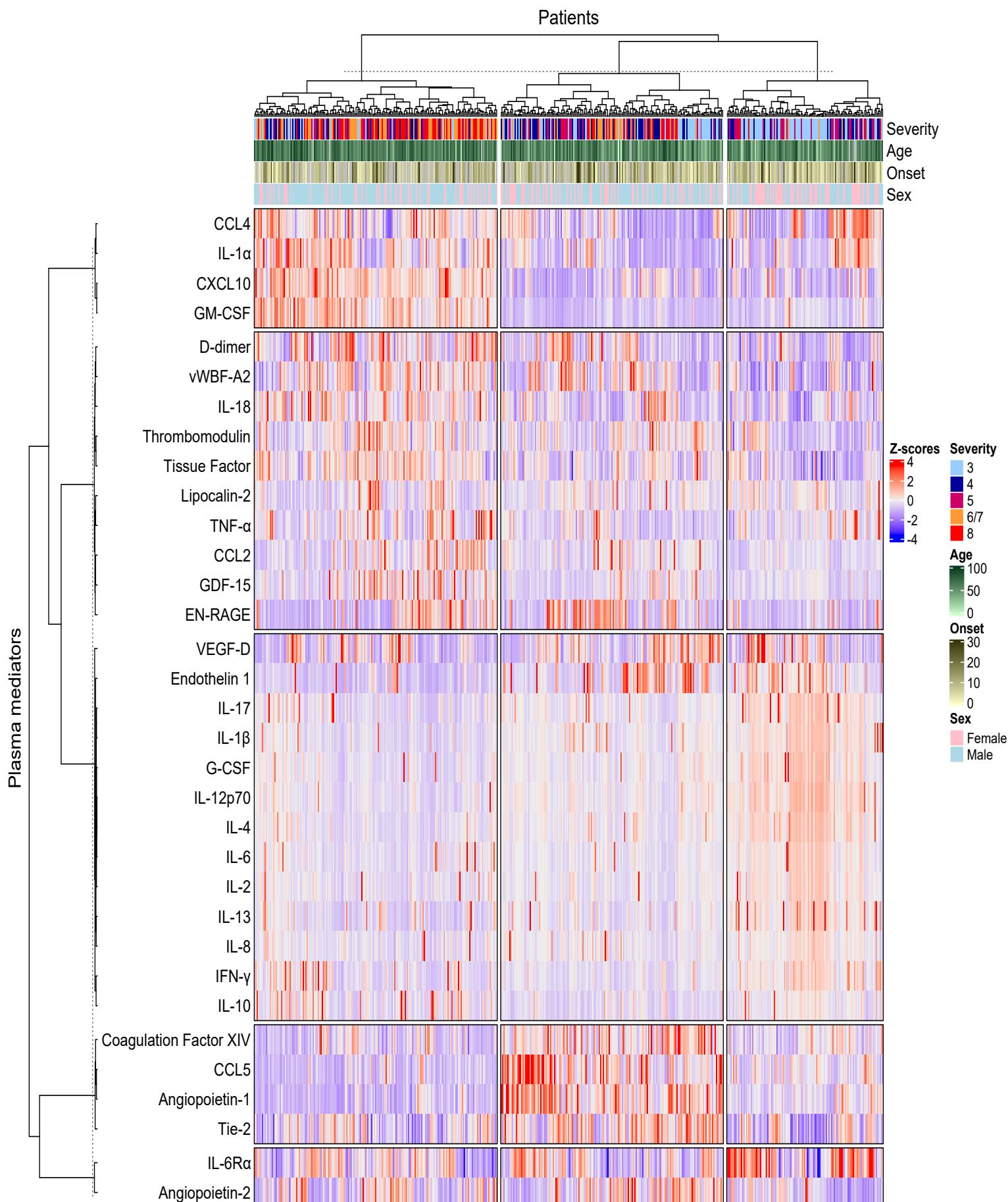


Figure 2

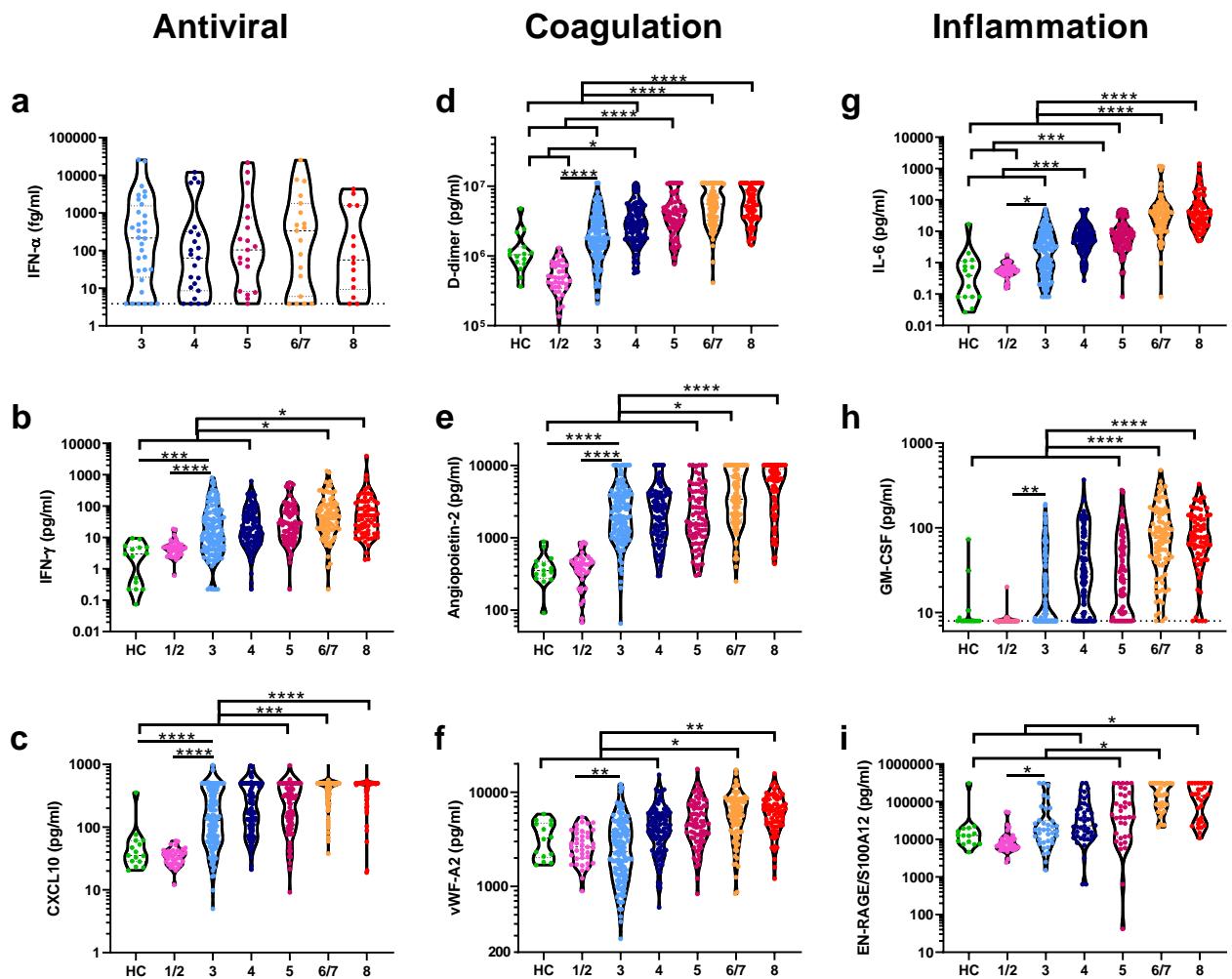
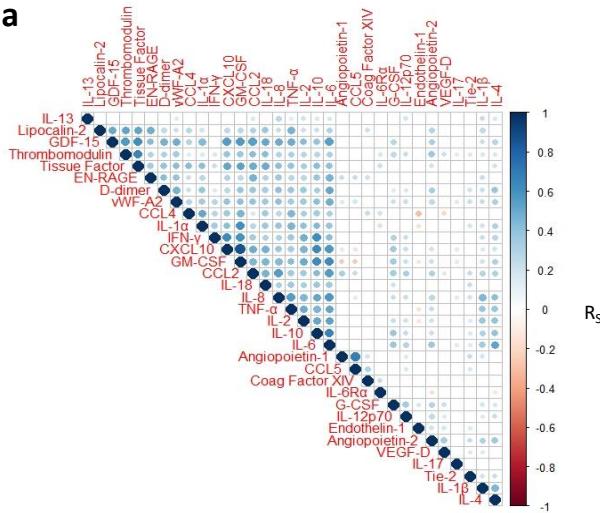


Figure 3

a



b

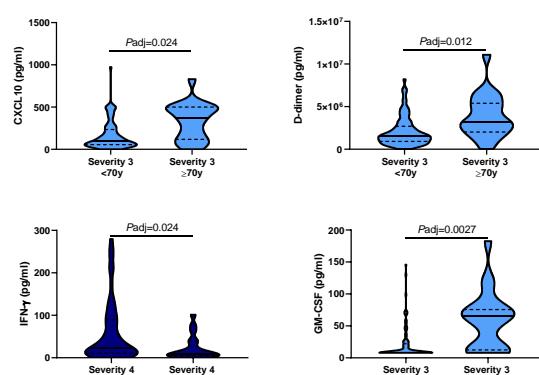


Figure 4

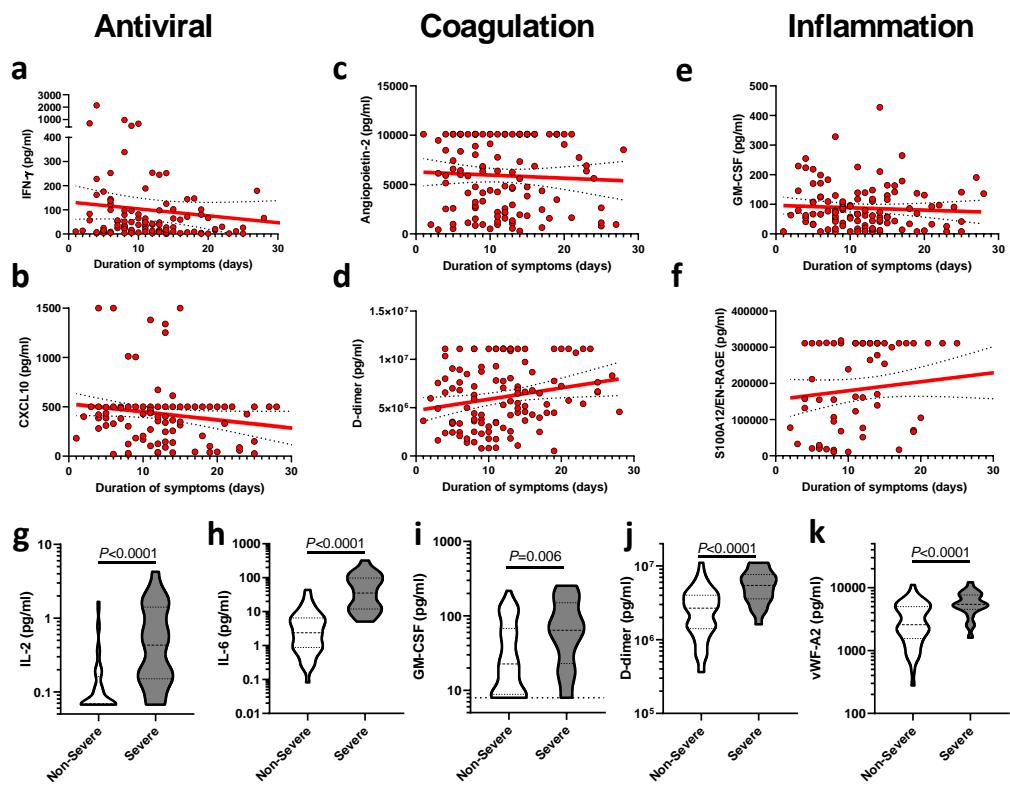


Figure S1 - Severity at V1

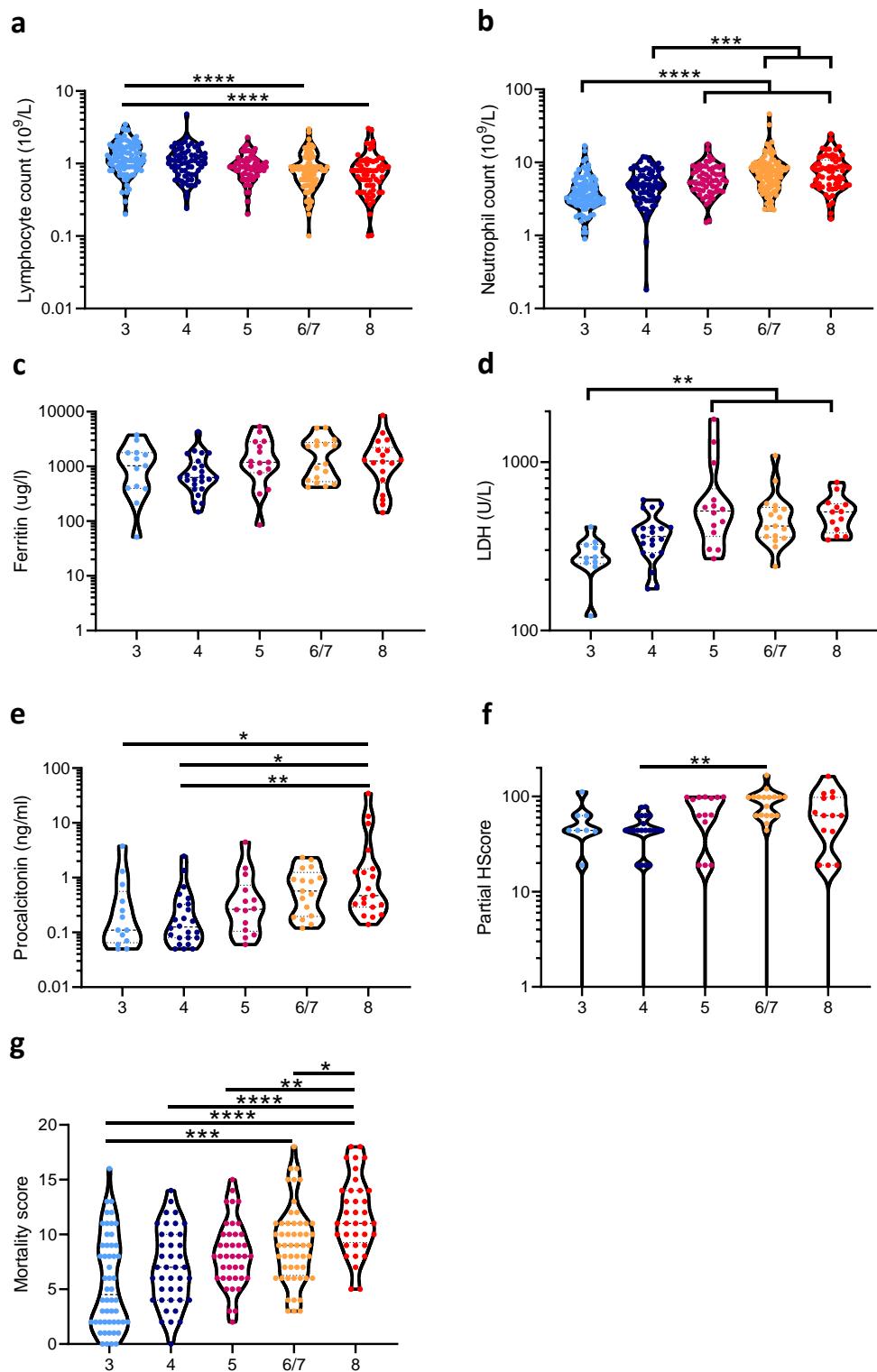


Figure S2

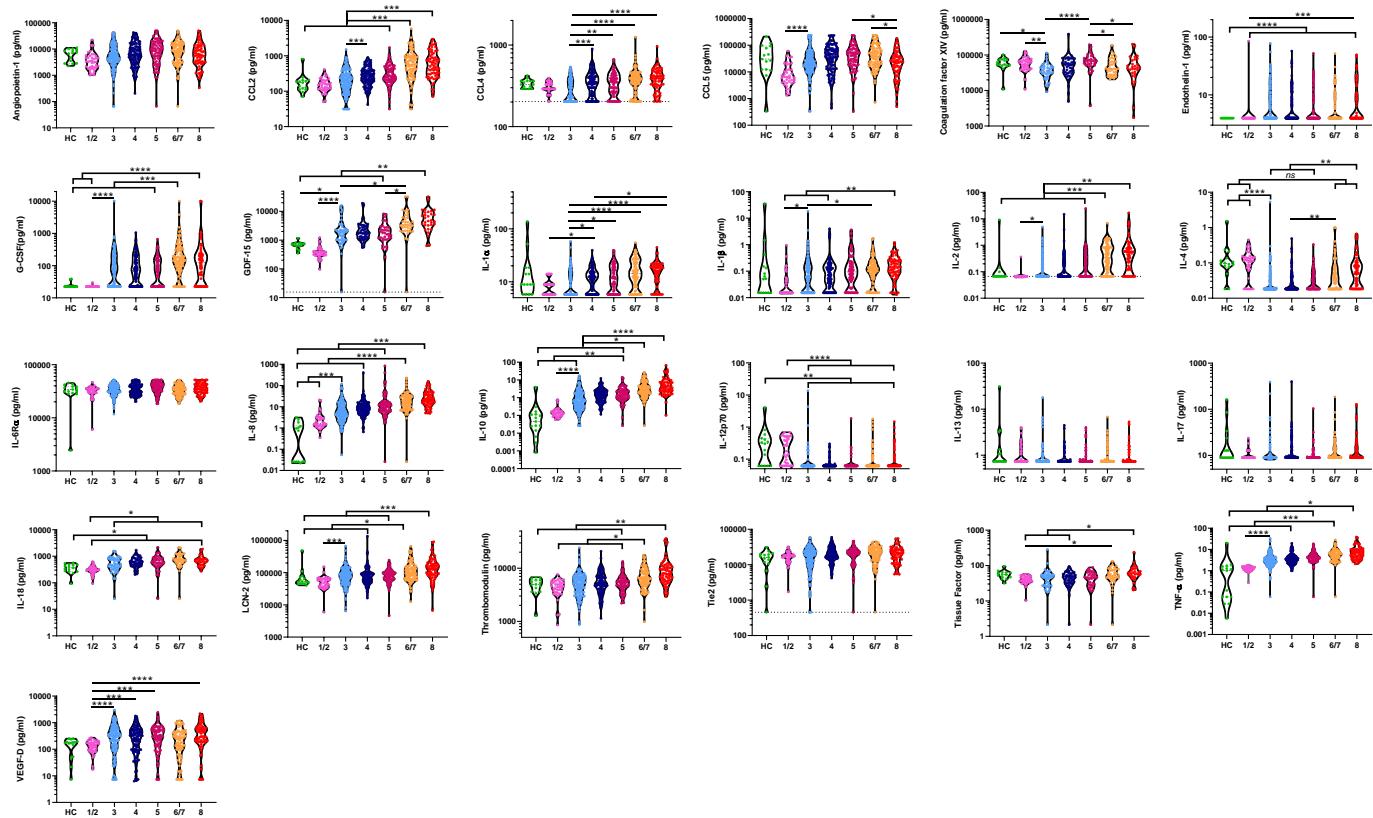
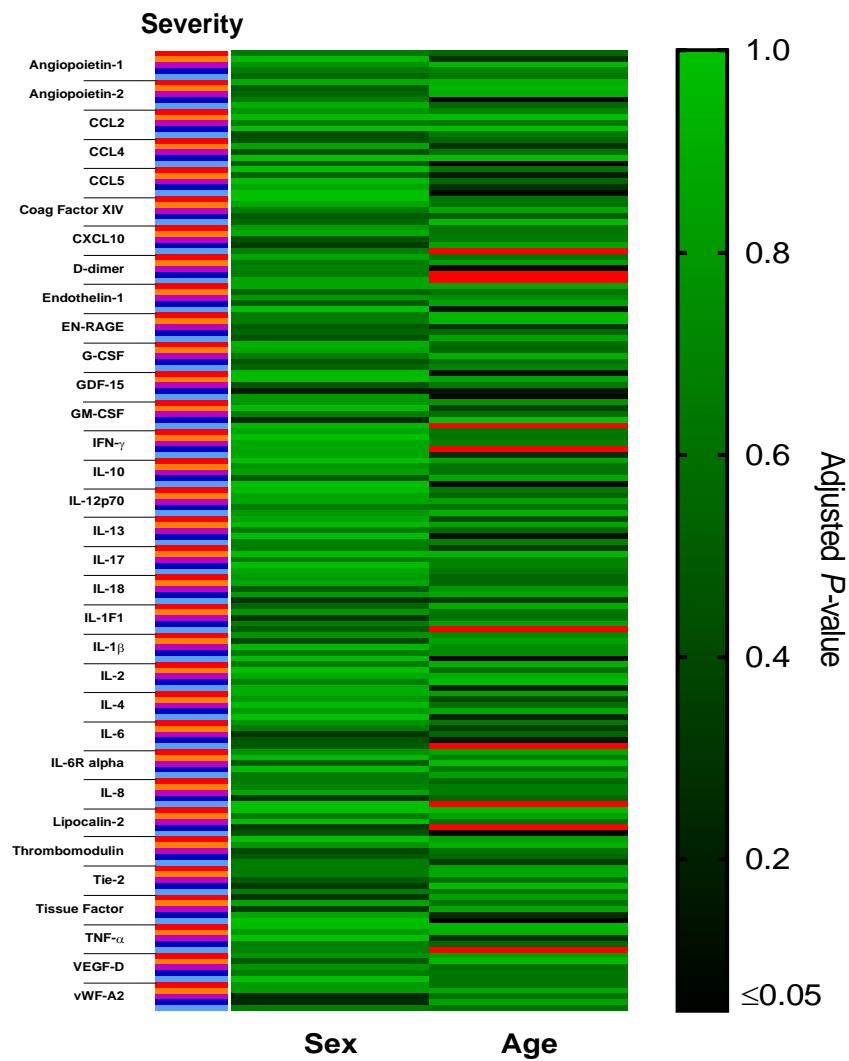


Figure S3



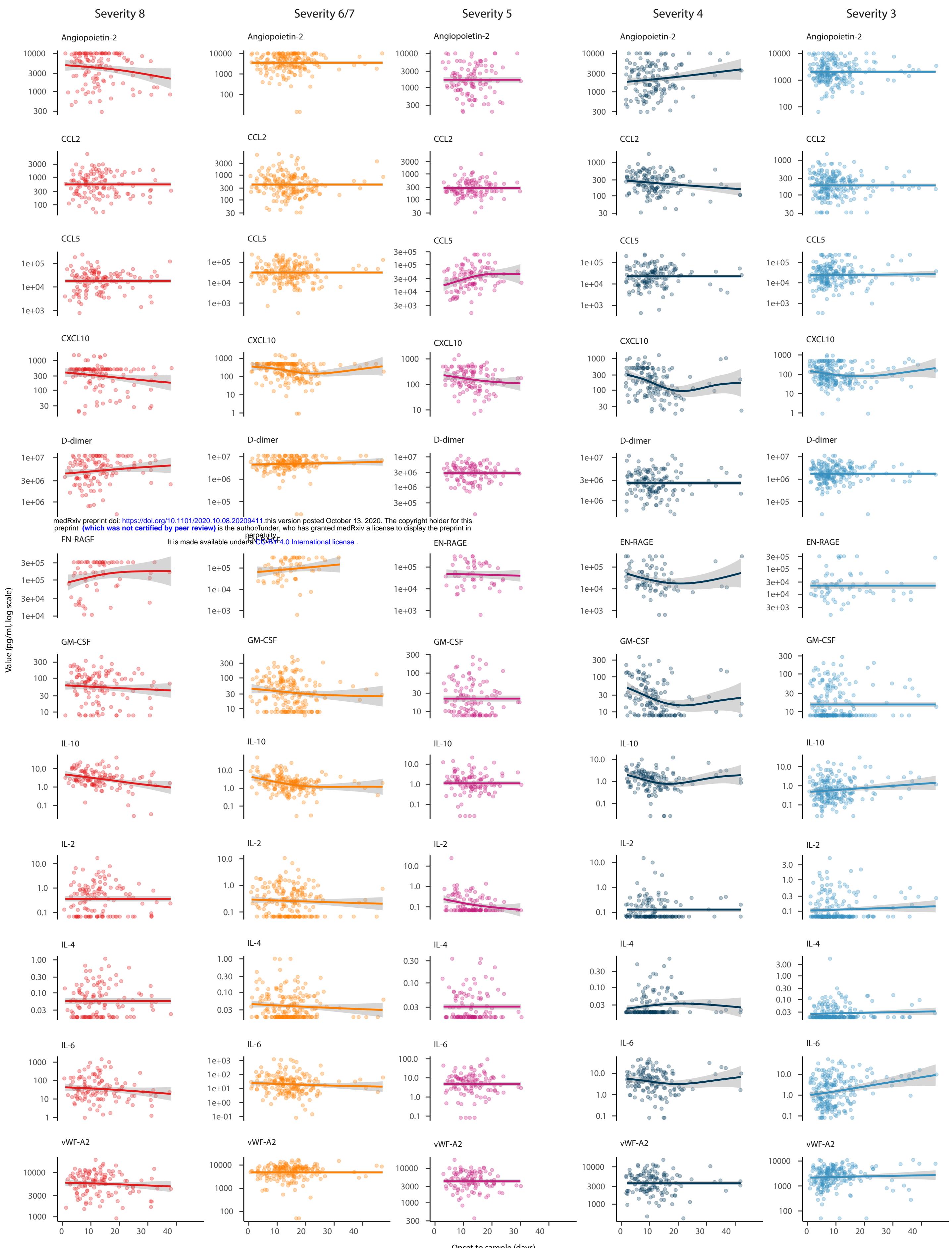


Figure S5

