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

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Introductory paragraph

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

Main text

Fatal COVID-19 is associated with acute respiratory distress syndrome and raised systemic inflammatory markers including IL-6 and C-reactive protein, often accompanied by neutrophilia and lymphopenia [1]. The beneficial effect of corticosteroid treatment in severe disease highlights the role of steroid-responsive inflammation in pathogenesis [2, 3], and post-mortem studies report pulmonary vessel vasculitis (most commonly myeloid cells) and microthrombosis in fatal COVID-19 [4, 5, 6, 7]. The virus-induced inflammatory state has laboratory features that resemble secondary haemophagocytic lymphohistiocytosis (sHLH) [8, 9, 10] but the exact pattern and severity of inflammatory responses has been only partially characterized. Levels of some inflammatory mediators, including IL-6, are elevated in COVID-19, but are typically ten times lower than those reported in acute respiratory distress syndrome (ARDS) and sepsis [11, 12, 13], suggesting that other factors may play a major role in COVID-19 severity. Host genetic factors may also influence disease severity, with polymorphisms in

several regions, including the interferon pathway genes *IFNAR2* and *OAS1/2/3* recently associated with enhanced disease severity [14]. Identification of such genetic of inflammatory factors may define a 'treatable trait' [15], allowing both stratification of patients likely to benefit from therapies such as dexamethasone and targeted biological anti-cytokine therapies, and design of novel therapeutics targeting causative pathways.

Early clinical studies of COVID-19 identified elevated neutrophil counts and lymphopenia in peripheral blood [1, 16], predominantly seen in late-stage disease and of limited prognostic value. Peripheral blood neutrophilia is also seen in other severe respiratory viral [17] and bacterial [18] infections, suggesting that this is not a unique feature of COVID-19. Elevated levels of D-dimer, a product of fibrin-degradation associated with thrombosis and inflammation, have also been observed in COVID-19 [16], consistent with systemic inflammation and the high frequency of macrovascular thrombotic complications in severe cases [7, 19]. Post-mortem studies show that thromboses and microthrombi within pulmonary vessels are common in fatal COVID-19 and are associated with endothelial responses distinct from those that occur during fatal influenza A virus infection [4, 5, 7, 20]. However, the thrombotic aspects of life-threatening COVID-19, and the interaction of this process with cytokine release have hitherto been described in relatively small groups of cases, from single-center studies, or with a narrow range of disease severities.

Within the ISARIC4C study we obtained clinical data and 1,047 plasma samples from 619 hospitalized patients with COVID-19 [21, 22]. Given the large number of cases, patients from the ISARIC4C database could be stratified into five levels of severity according to their peak illness, in line with the World Health Organization COVID-19 ordinal scale [23] (Supplementary Table 1): (1) no oxygen requirement (Severity 3, n=169); (2) patients requiring oxygen by face mask (Severity 4, n=143); (3) patients requiring high-flow nasal cannulae, a continuous positive airway pressure mask or other non-invasive ventilation (Severity 5 n=99); (4) patients requiring invasive mechanical ventilation (Severity 6/7, n=113); and (5) fatal COVID-19 (Severity 8, n=95). The median duration of symptoms prior to

study enrollment was similar in all groups: Severity 3, 7 days; Severity 4, 9 days; Severity 5, 11 days; Severity 6/7, 11 days; and Severity 8, 8 days. Some differences in routinely performed clinical hematology and biochemistry measures were evident between clinical outcome groups at the time of study enrolment: Lymphopenia was evident in groups 6/7 and 8, relative to 3, alongside neutrophilia in 6/7 and 8 relative to 3 and 4 (Supplementary Fig. 1a and 1b, respectively). No differences between groups were observed in ferritin levels, whilst LDH was elevated in groups 5, 6/7, and 8 relative to 3 (Supplementary Fig. 1c and 1d, respectively). Procalcitonin levels were elevated in group 8 relative to 3 and 4, and in group 6/7 relative to 4 (Supplementary Fig. 1e). Partial HScores [24] were calculated (fever, cytopenia, ferritin, triglycerides, and AST) but the only significant difference between groups was between 6/7 and 4, indicating that sHLH is unlikely to be the predominant pathophysiological mechanism in life-threatening COVID-19 (Supplementary Fig. 1f). The ISARIC4C mortality scores [25] for these patients demonstrated an elevated risk of mortality, calculated from admission data, in those that would progress to fatal disease (group 8) relative to all other groups (Supplementary Fig. 1g), though there was considerable overlap between all groups. Together, these data indicated limited clinical or biochemical differences between patient outcome groups at the time of hospital admission.

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

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

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

126 though anti-IFN autoantibodies [27] and polymorphisms in IFN signaling [14] may influence this pathway
127 in some patients.

128 The fibrin degradation product D-dimer has been reported to be elevated in severe COVID-19 [16],
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 vasculitis [4], 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). Angiopoetin-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 bodies [29], 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-

CSF was similarly elevated in all hospitalized groups, relative to controls and was most pronounced in the groups 6/7 and 8 (Fig. 2h). Numerous other inflammatory cytokines and chemokines showed similar results including TNF-α, IL-2, GDF-15, G-CSF, and VEGF-D (Supplementary Fig. 2). EN-RAGE/S100A12 has previously been characterized as a marker of respiratory damage in ARDS [30] and indeed was elevated in groups 6/7 and 8 relative to most others (Fig. 2i). The neutrophil chemokine IL-8 (CXCL8) was similarly elevated in severe disease, as was the neutrophil gelatinase associated lipocalin (LCN-2/NGAL) (Supplementary Fig. 2), in line with the reported association between blood neutrophilia and severity [16] also seen in this cohort (Supplementary Fig. 1b).

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

To determine the strength of the relationships between these individual plasma mediators we performed a hierarchical correlation matrix analysis of mediators from plasma samples collected at the time of study enrolment. This identified a strongly correlated cluster of inflammatory mediators including GM-CSF, CXCL10, vWF-A2, and IL-6 (Fig. 3a); increases in which were commonly associated with the most severe COVID-19 outcome groups. Given the strong association between age and COVID-19 severity [22], and reports of increased inflammatory responses in males relative to females with COVID-19 [32] we investigated the influence of these demographic factors on plasma mediators 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 (≥ 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 ≥70 and <70 years, including elevated levels of D-dimer, CXCL10, and GM-CSF in
181 those aged ≥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

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

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

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

fibrin rich thrombi [28]. Neutrophilic inflammation could have an etiological role in endothelial injury though neutrophilia is predominantly a feature of the later phases of COVID-19 [1], while endothelial injury was evident in the first days of symptoms. However, the continued thrombosis in late stage fatal COVID-19 may result from neutrophil mediated coagulation, observed in other settings [35, 36, 37] and recently demonstrated in COVID-19 [38]. Combined, these results indicate a multiplicity of possible pro-coagulant triggers that may contribute to pathology at different stages of disease.

We found that the antiviral immune mediator CXCL10 and the myeloid cell growth factor GM-CSF, were strikingly elevated in fatal cases of COVID-19. This is confirmed by a recent report describing the potential utility of CXCL10 as an early prognostic marker of COVID-19 severity [39]. An influx of monocytes/macrophages has been described in the lung parenchyma in fatal COVID-19, combined with a mononuclear cell pulmonary artery vasculitis [6], and presence of pro-inflammatory monocyte-derived macrophages in bronchoalveolar lavage fluid from patients with severe COVID-19 [4, 40]. The elevation of CXCL10 and GM-CSF in severe disease reported here could contribute to monocyte recruitment and activation leading to this vasculitis, alongside the role of GM-CSF in the recruitment of neutrophils to the pulmonary vasculature [41].

Large scale randomized clinical trials for IL-6 signaling antagonists are on-going, though early results of the COVACTA trial of Tocilizumab found no improvement in clinical status or mortality [42]. Small scale studies of anti-GM-CSF have shown promising results [43, 44] but require formal testing in a clinical trial. Given the role of GM-CSF in granulopoiesis and enhancement of neutrophil survival, alongside the neutrophil activation observed in late stage fatal COVID-19, these trials may inform our understanding of the importance of this pathway in COVID-19 immunopathogenesis [45]. While early studies demonstrated elevated GM-CSF levels in both ICU and non-ICU treated COVID-19 patients [1], we now demonstrate a positive association with disease severity and outcome, in agreement with reports of elevated frequencies of GM-CSF^{+} Th1 cells in patients with COVID-19 requiring ICU treatment [46].

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

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

Supplementary Methods

Study design and setting

The ISARIC WHO Clinical Characterization Protocol for Severe Emerging Infections in the UK (CCP-UK) is an ongoing prospective cohort study of hospitalized patients with COVID-19, which is recruiting in 258 hospitals in England, Scotland, and Wales (National Institute for Health Research Clinical Research Network Central Portfolio Management System ID: 14152) [47]. The protocol, revision history, case report form, patient information leaflets, consent forms and details of the Independent Data and Material Access Committee are available online [48] and published previously [22].

Participants

Hospitalized patients with PCR-proven or high likelihood of SARS-CoV-2 infection were recruited, including both patients with community- and hospital-acquired COVID-19. This study analyzed plasma from blood samples obtained on the day of enrolment to the study (day 1, Tier 1) and additional serial samples obtained following a sampling schedule (Tier 2) harmonized with international investigators to allow meaningful comparison of results between studies [21]. Healthy controls were recruited prior to December 2019 under approval from the London – Fulham Research Ethics Committee (REC) (reference 14/LO/1023) or from healthy donors following informed consent from a sub-collection of the Imperial College Healthcare NHS Trust National Institute for Health Research Imperial Biomedical Research Centre Tissue Bank. Use of the sub-collection was approved by the Tissue Bank Ethics Committee (Approval R12023). Samples from community managed COVID-19 cases were collected through a subproject of Imperial College London Communicable Disease Research Tissue Bank, under approval from the south central Oxford REC (reference 15/SC/0089).

HScores

To calculate partial HScores [24], ferritin, triglyceride and AST measurements from this study were combined with recorded results from case report forms for temperature and routine hemoglobin, white cell counts, and platelet counts.

Immunoassays

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

Statistical analyses

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

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

Figure legends

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

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

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

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

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

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

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

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

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

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

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

Supplementary Fig. 5 – Longitudinal analysis of selected plasma mediators within each disease 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.

396 The ISARIC4C Investigators

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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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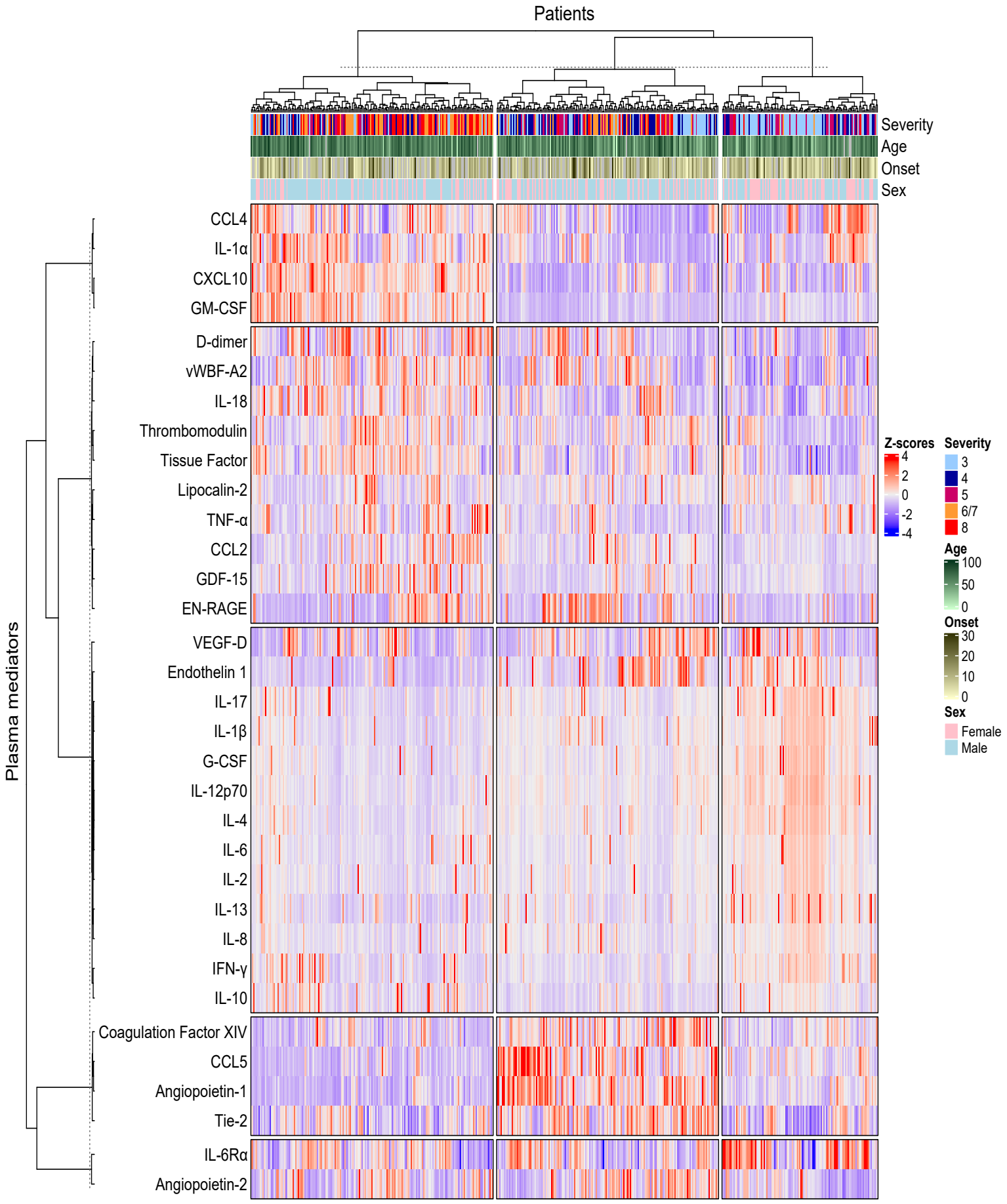


Figure 2

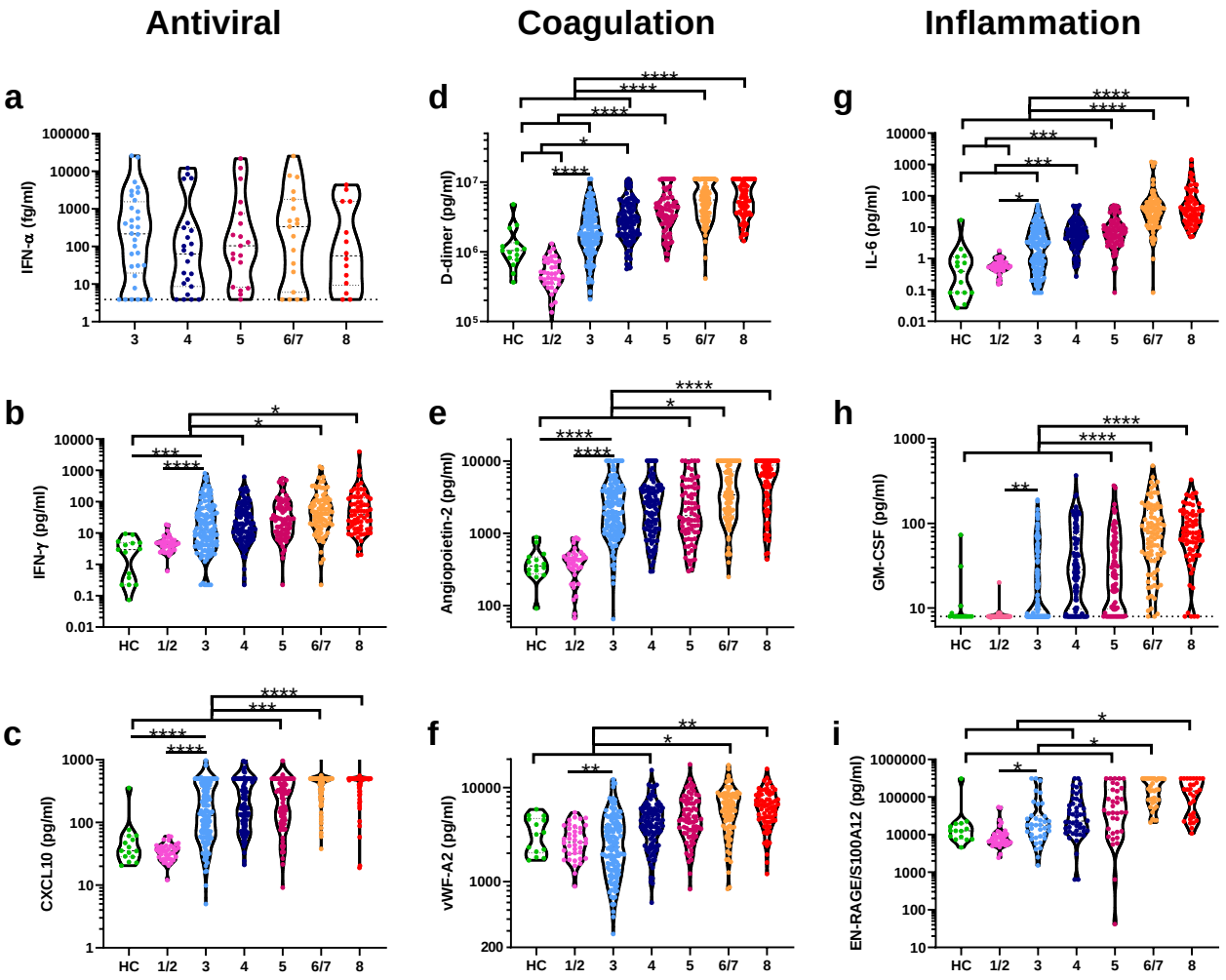

Antiviral
Coagulation
Inflammation
a
IFN-α (fg/ml)
b
IFN-γ (pg/ml)
c
CXCL10 (pg/ml)
d
D-dimer (pg/ml)
e
Angiopoietin-2 (pg/ml)
f
vWF-A2 (pg/ml)
g
IL-6 (pg/ml)
h
GM-CSF (pg/ml)
i
EN-RAGE/S100A12 (pg/ml)
HC
1/2
3
4
5
6/7
8

Figure 3

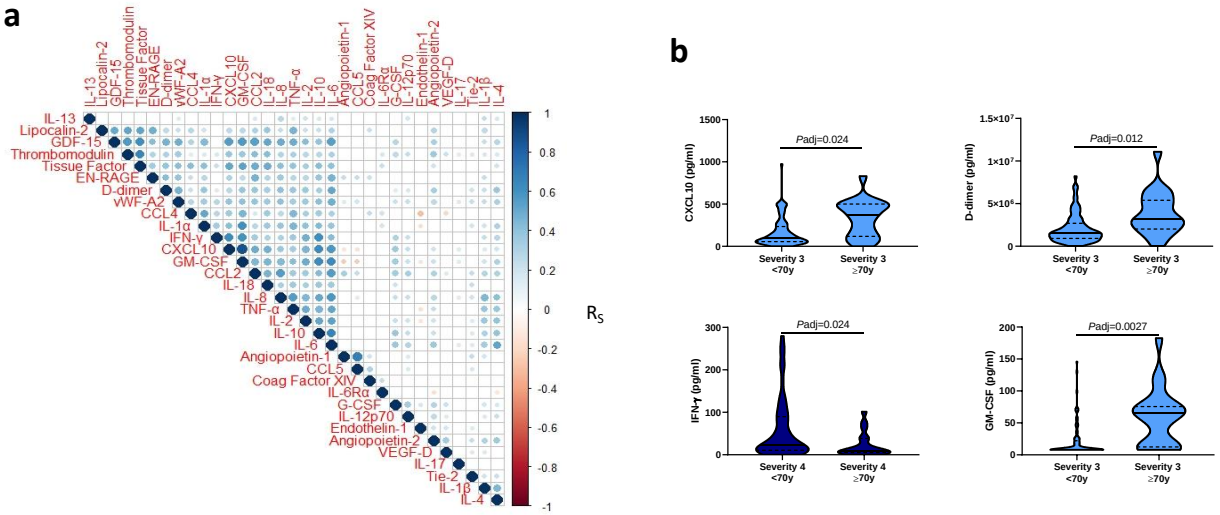

a
IL-13
Lipocalin-2
GDF-15
Thrombomodulin
Tissue Factor
EN-RAGE
D-dimer
vWF-A2
CCL4
IL-1α
IFN-γ
CXCL10
GM-CSF
CCL2
IL-18
IL-8
TNF-α
IL-2
IL-10
IL-5
Angiopoietin-1
CCL5
Coag Factor XIV
IL-6Rα
G-CSF
IL-12p70
Endothelin-1
Angiopoietin-2
VEGF-D
IL-17
Tie-2
IL-1β
IL-4
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
Rs
b
CXCL10 (pg/ml)
Padj=0.024
Severity 3 <70y
Severity 3 ≥70y
D-dimer (pg/ml)
Padj=0.012
IFN-γ (pg/ml)
Padj=0.024
Severity 4 <70y
Severity 4 ≥70y
GM-CSF (pg/ml)
Padj=0.0027

Figure 4

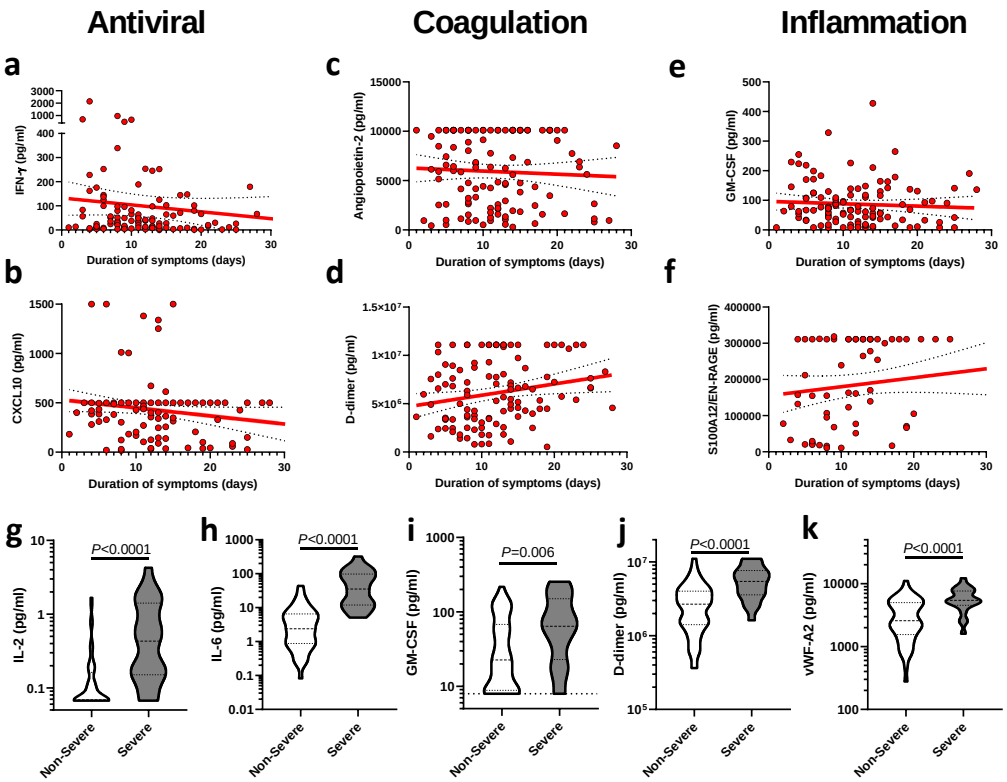

Antiviral
Coagulation
Inflammation
a
IFN-γ (pg/ml)
Duration of symptoms (days)
b
CXCL10 (pg/ml)
Duration of symptoms (days)
c
Angiopoietin-2 (pg/ml)
Duration of symptoms (days)
d
D-dimer (pg/ml)
Duration of symptoms (days)
e
GM-CSF (pg/ml)
Duration of symptoms (days)
f
S100A12/EN-RAGE (pg/ml)
Duration of symptoms (days)
g
IL-2 (pg/ml)
P<0.0001
Non-Severe
Severe
h
IL-6 (pg/ml)
P<0.0001
Non-Severe
Severe
i
GM-CSF (pg/ml)
P=0.006
Non-Severe
Severe
j
D-dimer (pg/ml)
P<0.0001
Non-Severe
Severe
k
vWF-A2 (pg/ml)
P<0.0001
Non-Severe
Severe

Figure S1 - Severity at V1

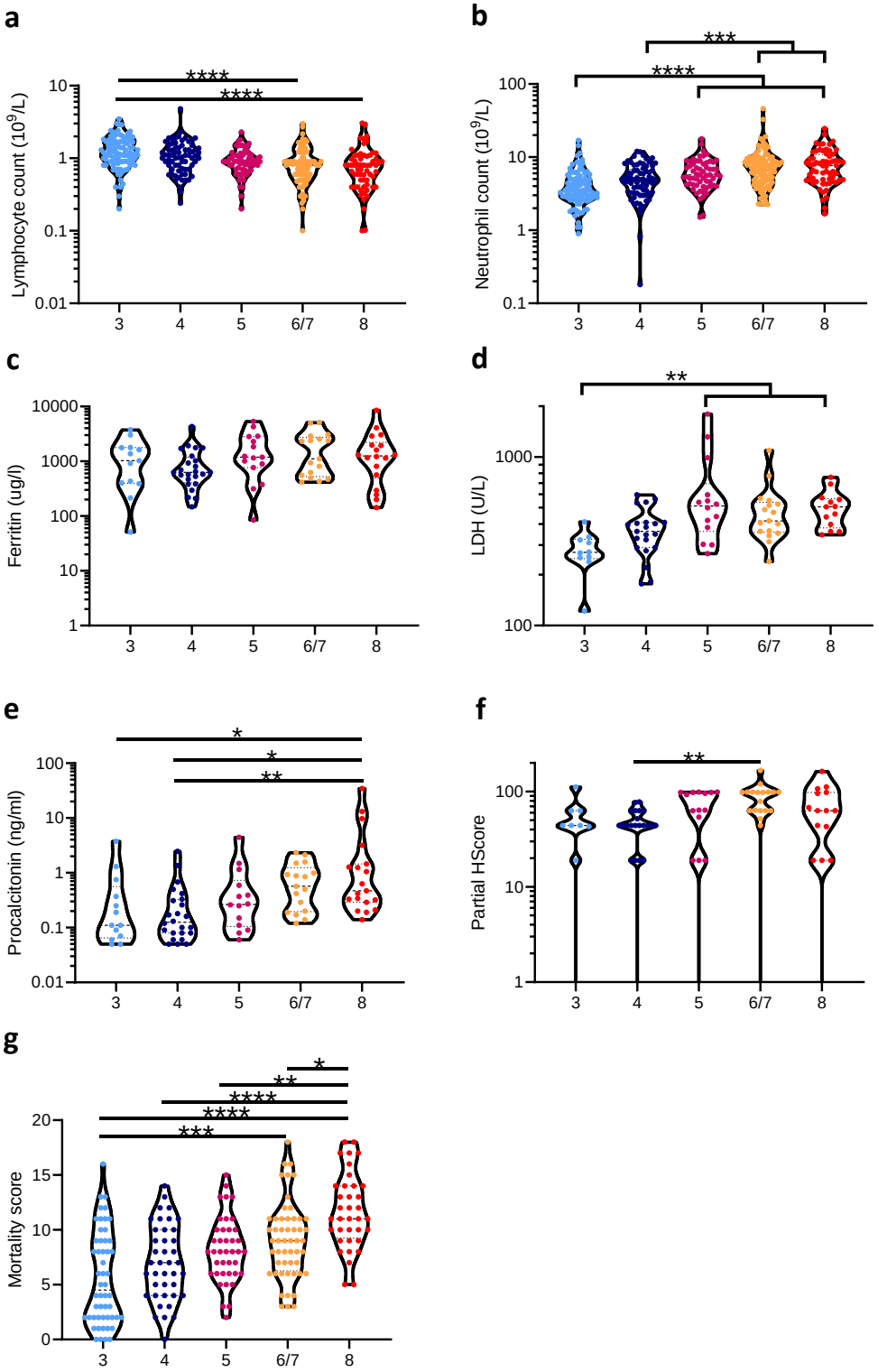


Figure S2

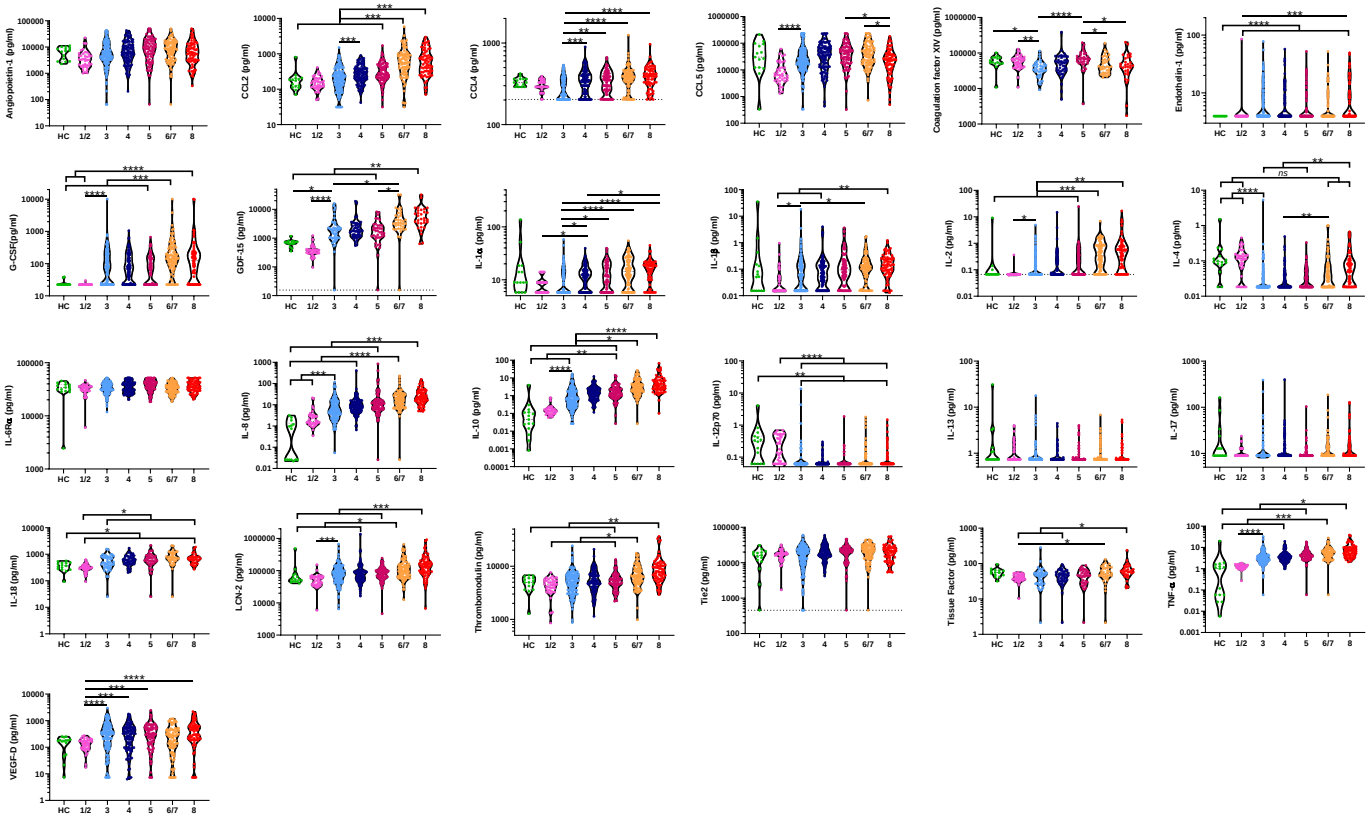
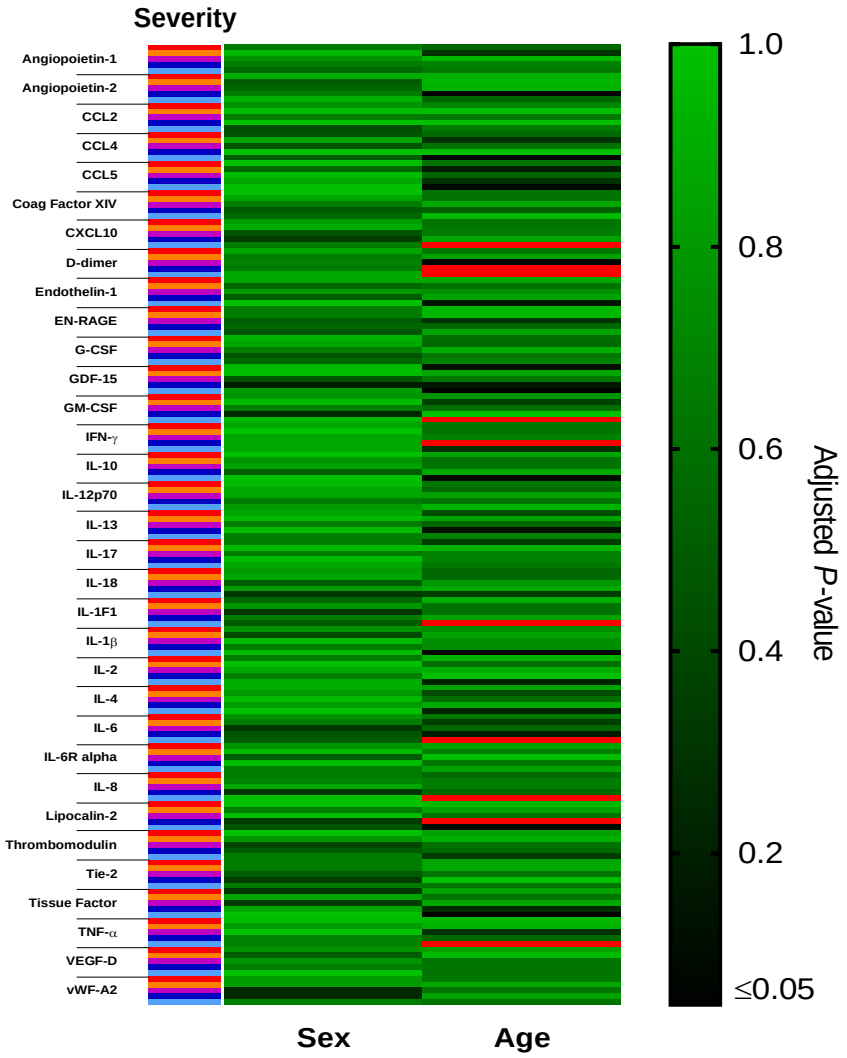

Angiopoietin-1 (pg/ml)
CCL2 (pg/ml)
CCL4 (pg/ml)
CCL5 (pg/ml)
Coagulation factor XIV (pg/ml)
Endothelin-1 (pg/ml)
G-CSF(pg/ml)
GDF-15 (pg/ml)
IL-1α (pg/ml)
IL-1β (pg/ml)
IL-2 (pg/ml)
IL-4 (pg/ml)
IL-6Rα (pg/ml)
IL-8 (pg/ml)
IL-10 (pg/ml)
IL-12p70 (pg/ml)
IL-13 (pg/ml)
IL-17 (pg/ml)
IL-18 (pg/ml)
LCN-2 (pg/ml)
Thrombomodulin (pg/ml)
Tie2 (pg/ml)
Tissue Factor (pg/ml)
TNF-α (pg/ml)
VEGF-D (pg/ml)
HC
1/2
3
4
5
6/7
8
ns

Figure S3


Severity
Angiopoietin-1
Angiopoietin-2
CCL2
CCL4
CCL5
Coag Factor XIV
CXCL10
D-dimer
Endothelin-1
EN-RAGE
G-CSF
GDF-15
GM-CSF
IFN-γ
IL-10
IL-12p70
IL-13
IL-17
IL-18
IL-1F1
IL-1β
IL-2
IL-4
IL-6
IL-6R alpha
IL-8
Lipocalin-2
Thrombomodulin
Tie-2
Tissue Factor
TNF-α
VEGF-D
vWF-A2
Sex
Age
1.0
0.8
0.6
0.4
0.2
≤0.05
Adjusted P-value

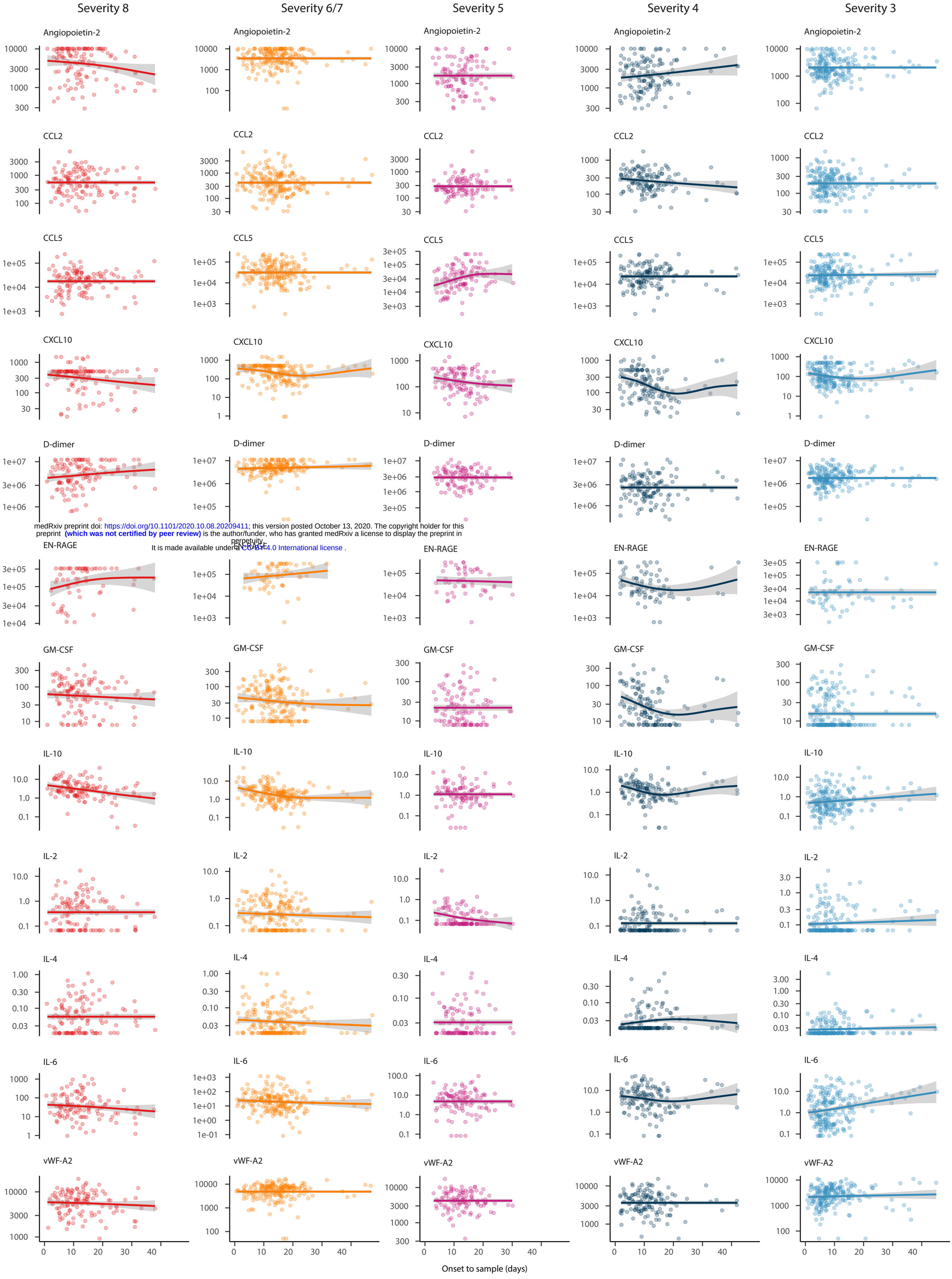
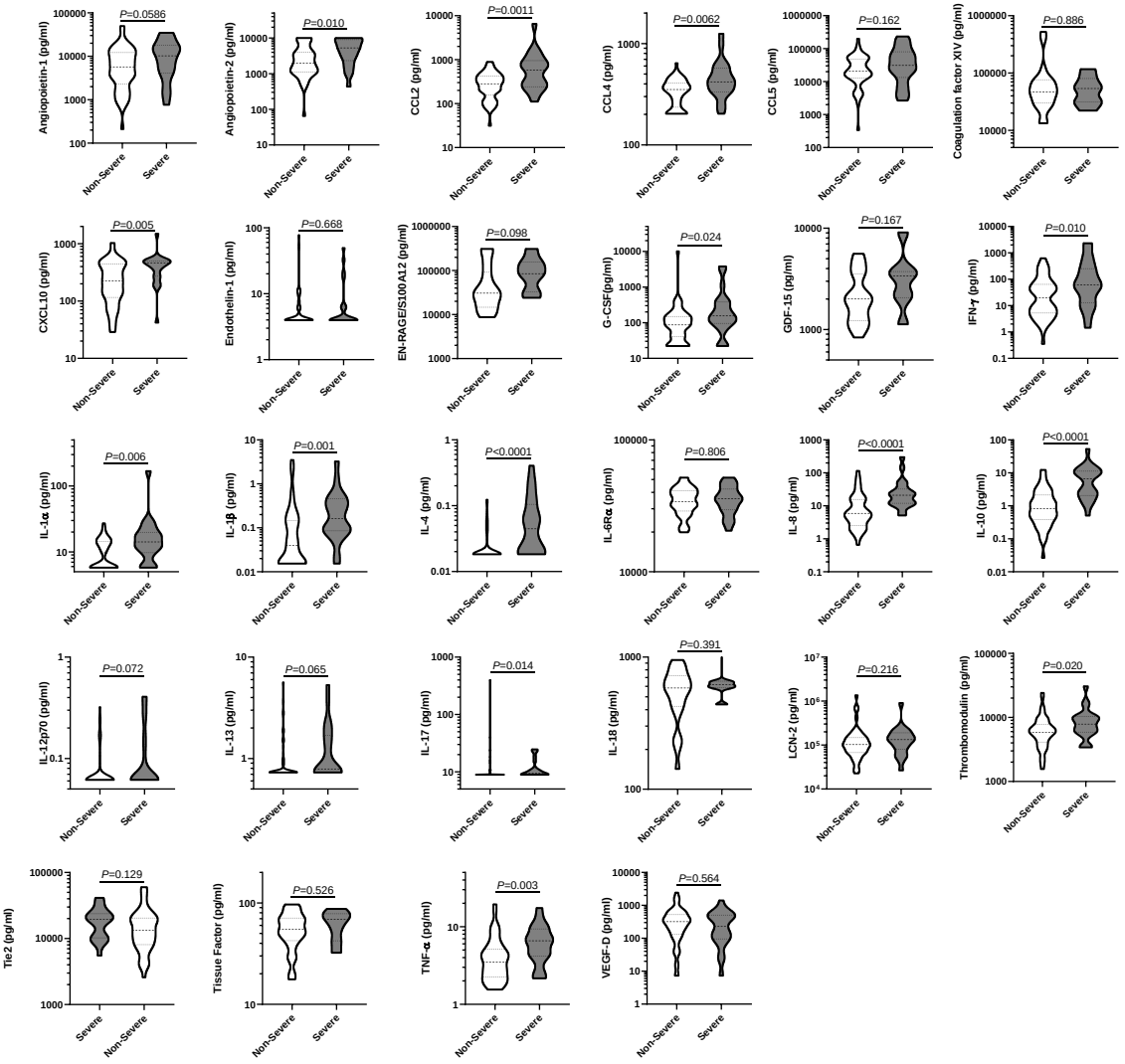


Figure S5


Angiopoietin-1 (pg/ml)
P=0.0586
Angiopoietin-2 (pg/ml)
P=0.010
CCL2 (pg/ml)
P=0.0011
CCL4 (pg/ml)
P=0.0062
CCL5 (pg/ml)
P=0.162
Coagulation factor XIV (pg/ml)
P=0.886
CXCL10 (pg/ml)
P=0.005
Endothelin-1 (pg/ml)
P=0.668
EN-RAGE/S100A12 (pg/ml)
P=0.098
G-CSF(pg/ml)
P=0.024
GDF-15 (pg/ml)
P=0.167
IFN-γ (pg/ml)
P=0.010
IL-1α (pg/ml)
P=0.006
IL-1β (pg/ml)
P=0.001
IL-4 (pg/ml)
P<0.0001
IL-6Rα (pg/ml)
P=0.806
IL-8 (pg/ml)
P<0.0001
IL-10 (pg/ml)
P<0.0001
IL-12p70 (pg/ml)
P=0.072
IL-13 (pg/ml)
P=0.065
IL-17 (pg/ml)
P=0.014
IL-18 (pg/ml)
P=0.391
LCN-2 (pg/ml)
P=0.216
Thrombomodulin (pg/ml)
P=0.020
Tie2 (pg/ml)
P=0.129
Tissue Factor (pg/ml)
P=0.526
TNF-α (pg/ml)
P=0.003
VEGF-D (pg/ml)
P=0.564
Non-Severe
Severe