Cycle threshold SARS-CoV-2 RT-PCR and bronchoalveolar cytokine concentrations redefine the COVID-19 phenotypes in critically ill patients

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Abstract

Abstract Rationale: Recent studies suggest that both hypo- and hyper-inflammatory ARDS phenotypes characterize severe COVID-19-related pneumonia. The role of lung SARS-CoV-2 viral load in contributing to these phenotypes remains unknown. Objectives: To redefine COVID-19 ARDS phenotypes when considering semi-quantitative SARS-CoV-2 RT-PCR in the bronchoalveolar lavage of intubated patients. To compare the relevance of deep respiratory samples vs plasma in linking the immune response and the semi-quantitative viral loads. Methods: Eligible subjects were adults diagnosed with COVID-19 ARDS who required mechanical ventilation and underwent bronchoscopy. We recorded the immune response in the bronchoalveolar lavage and plasma and semi-quantitative SARS-CoV-2 RT-PCR in the bronchoalveolar lavage. Hierarchical clustering on principal components was applied separately on the two compartments datasets. Baseline characteristics were compared between clusters. Measurements and Results: 20 subjects were enrolled between August 2020 and March 2021. Subjects underwent bronchoscopy on average 3.6 days after intubation. All subjects were treated with dexamethasone prior to bronchoscopy, 11 of 20 (55.6%) received remdesivir and 1 of 20 (5%) received tocilizumab. Adding viral load information to the classic two cluster model of ARDS revealed a new cluster characterized by hypo-inflammatory responses and high viral load in 23.1% of the cohort. Hyperinflammatory ARDS was noted in 15.4% of subjects. Bronchoalveolar lavage clusters were more stable compared to plasma. Conclusions: We identified a unique group of critically ill subjects with COVID-19 ARDS who exhibit hypo-inflammatory responses but high viral loads in the lower airways. Our approach adds the infection dimension to ARDS phenotypes described in COVID-19 pneumonia

Introduction

Acute respiratory distress syndrome (ARDS) requiring mechanical ventilation is the most severe manifestation of coronavirus disease 2019 (COVID-19) caused by the Severe Acute Respiratory Syndrome – Coronavirus 2 (SARS-CoV-2). Mortality has varied since initial reports but remains consistently high, above 40%, even in the most recent publications (1). A significant debate concerning the existence and effects of hyperinflammation in severe COVID-19 is ongoing (2, 3) and most of the therapeutic trials have focused on dampening the systemic immune response. Immune modulation has become the therapeutic norm even after non-corticosteroid immunosuppressive trials registered only small effect sizes. Clinical and treatment effect heterogeneity persists and only recent reports have overlapped the recognized ARDS phenotypes – hyper- and hypo-inflammatory – onto COVID-19 (4, 5). Both these phenotypes seem to respond to corticosteroids, suggesting treatment heterogeneity *within* the phenotypes.

There is also a signal that the SARS-CoV-2 viral load may play a role in the therapeutic response and ultimately the survival of critically ill patients with severe hypoinflammatory COVID-19 (5). Autopsy studies have not definitively determined the direct cause of demise in patients with severe COVID-19 ARDS and confirm that the abnormal pulmonary response in fatal COVID-19 can be triggered by direct viral cytopathic damage, an exaggerated immune infiltrate and even ventilator-induced lung injury. (6–8). It remains unclear how to best differentiate and treat patients who succumb to the uncontrolled viral replication vs an exaggerated, virus-independent immune response.

The main objective for this study was to identify groups of patients with severe COVID-19 with similar viral loads and immune response intensities using prespecified measures of viral load and immune markers in bronchoalveolar lavage (BAL) and plasma. We hypothesized that using surrogates of semi-quantitative SARS-CoV-2 RT-PCR in BAL would better define COVID-19 ARDS phenotypes, and that lower airways samples would provide a more accurate window into viral infection and the triggered host immune response when compared with matched plasma samples.

Methods

Study population and design

This was a pilot prospective observational cohort study at Washington University/ Barnes Jewish Hospital in St. Louis, MO between Aug 2020 and March 2021. This study was conducted according to the Declaration of Helsinki principles (as revised in 2013) and was approved by the Washington University Institutional Review Board (protocol # 202006151). Adult subjects with COVID-19 related pneumonia diagnosed by nasopharyngeal SARS-CoV-2 RT-PCR who required endotracheal intubation and mechanical ventilation for ARDS were considered for the study. Informed consent was obtained from the legally-authorized representative. ARDS was defined according to the Berlin criteria (9).

We collected demographic and clinical variables of interest from the electronic health records including age, gender, race, weight, body mass index, comorbidities, prior use of immunosuppressive medications, treatments administered, BAL and laboratory results and relevant outcomes (mortality, duration of ventilation and length of stay in the hospital). Subjects were enrolled if they had undergone bronchoscopy with BAL performed for clinical reasons, most commonly to rule out bacterial pneumonia. Immune response markers and cycle threshold (Ct) SARS-CoV-2 RT-PCR were obtained from the subjects' plasma and BAL fluid. We excluded intubated subjects who did not undergo planned clinical bronchoscopy and those subjects who we could not obtain informed consent in sufficient time to analyze / store the fresh samples.

Procedures

On the same day as the bronchoscopy, we collected blood samples into EDTA-anticoagulated tubes. Plasma was frozen at -80^{0}C until analysis. The BAL fluid was collected by wedging the bronchoscope within the right middle lobe and instilling 100ml of sterile normal saline and collecting the return fluid (>30ml). Two subjects underwent bronchoscopies of the right lower lobe based on radiographic imaging. 1ml of the BAL sample was analyzed in the main clinical laboratory for SARS-CoV2- RT-PCR and 10-15 ml were kept on ice until processing for cytokine quantification in the research laboratory.

SARS-CoV-2 RT-PCR

The test was performed on the DiaSorin Molecular Simplexa COVID-19 Direct (https://www.fda.gov/media/136286/download) under an FDA EUA for BAL samples. Fifty microliters of the sample were directly analyzed, without a separate extraction step, for 2 different targets - SARS-CoV-2 *orf1a/b* and *S* genes. The instrument provides the Ct value (number of PCR cycles required to amplify the targeted viral material to a detectable level). We used the Ct value as surrogate for the amount of virus present in BAL specimens. Tests were run on fresh BAL samples immediately after the bronchoscopy, except for three subjects who had their samples frozen at -80^{0}C prior to testing.

Cytokine quantification

Plasma and BAL samples were analyzed using a human magnetic cytokine panel providing parallel measurement of 35 cytokines and chemokines (Thermo Fisher Scientific). The assay was performed according to the manufacturer's instructions with the following modification: the samples were fixed with 100 μL of 1% paraformaldehyde at room temperature for 60 min on a shaker at 700 RPM and washed once before the final resuspension. All samples were run in duplicate. The samples were analyzed on a Luminex FLEXMAP 3D instrument.

Statistical analysis

We first selected a set of cytokines most closely related to the viral immune response based on our previous analyses and published literature. These included IFN- γ, IL-1β, IL-6, IL-8, IL-10, IL-12, MIP-1β, MCP-1, IP-10 (10, 11). Given the temporal variation for both the cytokine concentrations and semi-quantitative SARS-CoV-2 RT-PCR, we standardized and rescaled the values from 0 to 1 per week. The BAL SARS-CoV-2 RT-PCR Ct values and the prespecified set of cytokines in BAL and plasma were included in the clustering analyses. We used a hierarchical clustering on principal components approach. This was employed separately for plasma and BAL with complete linkage and Euclidean distances predefining the number of clusters as 2 and 3. The number of clusters was a priori specified given the known partitioning into 2 clusters of subjects with COVID-19 ARDS and our hypothesis on the role of semi-quantitative SARS-CoV-2 RT-PCR in differentiating a unique cluster. Hierarchical clustering is a common unsupervised machine learning technique that aims to group similar subjects by measuring the distance between them. The variables of interest (i.e. SARS-CoV-2 Ct values and cytokine concentrations) define a multidimensional space where the distances between subjects are measured. We analyzed the variables explaining the variance between the clusters and containing the most information in the data by using principal component analysis. This was important as the pro-inflammatory cytokines are correlated with each other. We retained the first 3 principal components and the explanatory variables were represented graphically as component loadings. The clinical, laboratory data, immune characteristics and outcomes of the clusters were listed. We compared the BAL and the plasma clusters by using the Dunn index. Finally, we juxtaposed the semi-quantitative SARS-CoV-2 RT-PCR and cytokine concentrations per week for both plasma and BAL. Analyses were performed in R 4.0.3 (R Core Team. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

Results

We prospectively enrolled 19 subjects with severe COVID-19 related pneumonia who required endotracheal intubation and mechanical ventilation for acute hypoxemic respiratory failure between Aug 2020 and March 2021 when the alpha variant was in widespread circulation. One subject was prospectively enrolled into a separate study in May 2020 and his BAL sample and plasma samples were available for use in future studies. All subjects had positive nasopharyngeal SARS-CoV-2 RT-PCR and two subjects had negative viral RT-PCRs on evaluation of the bronchoalveolar lavage (BAL). The 18 subjects with positive BAL had a mean age of 68 years (SD 7.8 years) and were predominantly white (83.3%) (Table 1 and supplemental Table E1). Males and females were equally distributed. 33.3% (6 of 20) of subjects were considered immunocompromised: three were solid organ transplant recipients and three suffered from rheumatologic conditions. Chronic immunosuppressive medications in these six subjects included mycophenolic acid, prednisone, tacrolimus, sirolimus and azathioprine. Subjects underwent bronchoscopy 3.6 days (SD 3.6 days) after intubation and 13.7 (SD 7.8) days after development of symptoms (3 subjects in week 1, 7 in week 2, 6 in week 3 and 2 in week 5). All eighteen subjects received corticosteroids prior to bronchoscopy, 17/18 received remdesivir and one subject received tocilizumab 8 days prior to the BAL. Mortality was 55.6% with 1.8 average ventilator free days (SD 2.9 days).

Survivors and non-survivors had similar age and immuncompromising comorbidities. The majority of non-survivors were admitted from outside hospitals and had a longer duration of intubation.

BAL Hierarchical clustering and Principal Component Analysis

13 subjects had complete BAL and plasma immune markers and available Ct SARS-CoV-2 RT-PCR. Immune markers IL-1β IL-10, IL-6, IL-12, MCP-1, IFN-γ and IP-10 were the most relevant variables in separating subjects into 2 clusters: cluster 1 comprised the majority of the subjects (11 patients 84.6%) while cluster 2 was characterized by higher inflammatory markers (2 patients, 15.4%). The model with 3 clusters further divided cluster 1, i.e. the hypoinflammatory cluster, into low and high viral load as measured by the Ct SARS-CoV-2 RT-PCR: the new cluster 3 (3 of 13 patients, 23.1%) had higher semi-quantitative SARS-CoV-2 RT-PCR compared to the new cluster 1 (8 of 13, 61.5%) (Fig. 1 Panels A and B).

Overall, 88% of the variance was accounted for by the first 3 principal components (PC) (Figs.1 and 2 and supplemental Table E2). PC1 explains the highest variance at 63% with IFN-γ, IL-10, IL-6, IL-1β, IL-12, IP-10 and MCP-1, having the highest loadings (negatively correlated). The largest group of subjects (84.6%), clusters 1 and 3 (i.e. the hypoinflammatory clusters), are located on the positive side of PC1. PC2 explains 15% of the variance and it is mainly based on the Ct values (positively correlated). Clusters 2 and 3 are located on opposite sides of PC2. PC3 explained 10% of the variance with IL-10, Ct and MCP-1 being the highest contributors (positively correlated). Overall, we can label cluster 1 as hypoinflammatory low viral load, cluster 2 as hyperinflammatory and cluster 3 as hypoinflammatory high viral load.

Tables 1 and E1 illustrate the distribution of clinical variables, laboratory results and outcomes between the entire cohort and the BAL clusters. The frequency of immunocompromising conditions, prior administration of remdesivir and dexamethasone were similar in all groups. Cytokine concentrations were higher in the hyperinflammatory cluster 2. This was also characterized by higher use of vasopressors, lower PaO2/FiO2 ratio and neutrophilic predominance of the BAL. Subjects in the hypoinflammatory high viral load cluster (Cluster 3) had bronchoscopy performed more rapidly following intubation, had a predominantly lymphocytic BAL and had higher mortality.

Plasma Hierarchical clustering and PCA

In contrast to BAL, plasma clusters were less well defined. When plotting IL-6 as a marker of immune inflammatory activation and Ct values, the BAL clusters were better delineated (Fig.3). A full description of the plasma clusters is provided in the supplemental material.

When comparing plasma and BAL cytokine concentrations, most cytokines were higher in the lung compartment except for IL-6 and IL-12. The ratio between plasma and BAL concetrations was not consistent across cytokines. The Dunn index was higher for the BAL clusters (1.2) compared to the plasma clusters (0.76).

Cytokine concentrations and SARS-CoV-2 Ct values per week

The immune inflammatory response was not directly proportional to the intensity of the viral infection as measured by the BAL SARS-CoV-2 RT-PCR Ct value per week (see online supplemental Fig. E3 – panels A-D)

Discussion

In addition to re-establishing the hypo- and hyper-inflammatory COVID-19 ARDS phenotypes, our results segregate the hypoinflammatory group into low and high viral load subphenotypes. We have also transitioned to a more accurate clustering by sampling the lung compartment as opposed to the plasma. The potential advantage of our approach is obtaining hypothesis-driven clusters by linking the semi-quantitative viral loads and immune activation status. This may permit the treating physician to utilize a different balance between immune modulators and antiviral therapies as patients in the hypoinflammatory high viral load group may exhibit pathophysiology secondary to direct viral cytopathic effects rather than immunopathology. Inhibiting the immune responses further in these patients may ultimately prove detrimental.

Few studies have comprehensively described the clinical phenotypes in COVID-19 ARDS. Sinha et al confirmed the traditional hypo- and hyperinflammatory phenotypes in a cohort of 39 patients by using clinical variables and plasma IL-6 and TNFR1 concentrations (4). Cluster 2, the hyperinflammatory phenotype,

was observed in 10-21% of the patients and was characterized by higher organ failure and mortality. In a subsequent study, the authors overlapped the ARDS phenotypes defined by systemic inflammation and multiorgan failure to severe COVID-19 and reassessed the presence of the 2 phenotypes by using latent class analysis (5). 14% and 19% of patients from the 2 clusters were misclassified pointing towards noteworthy overlap between the clusters. The hyperinflammatory phenotype had higher proinflammatory markers, lactate and lower bicarbonate. The response to corticosteroids (nonrandomized, various formulations) was more prominent in the hyperinflammatory group. Interestingly, the nasopharyngeal SARS-CoV-2 RT-PCR Ct was associated with mortality only in the hypoinflammatory phenotype. Our study defines the high viral load subgroup part of the hypoinflammatory phenotype which may be a contributor to detrimental outcomes.

Bos et al employed group - based trajectory models to define COVID-19 ARDS phenotypes by looking at longitudinal data (12). Subphenotype 2 (33% of the patients) was characterized by higher minute ventilation - a marker of increased dead-space-, more venous thrombotic events, acute kidney injury and higher mortality. Cytokine concentrations were not available, but IL-6 has been linked with endothelial dysfunction and thrombosis in previous studies.

The main debate in severe COVID-19 remains: what is the driver of ARDS and bad outcomes? An exaggerated immune response or uncontrolled viral replication similar to other respiratory viral infections such as RSV?(13) It is known that coronaviruses have the capability to inhibit and delay the type I IFN immune response. In turn, this will promote continued viral replication and possibly immunopathology (14). Several autopsy reports have contributed to this debate: studies confirmed the presence of SARS-CoV-2 in lung specimens while others failed to find significant direct viral cytopathic effects (6–8).

The immune inflammatory response has been sought as a viable explanation and potential treatment target in severe COVID-19. Yet, numerous studies failed to detect a "cytokine" storm fingerprint in the plasma of patients with severe COVID-19 (3, 15). Usually, the plasma cytokine concentrations were similar or even 10 times lower in COVID-19 patients than in septic or ARDS patients (3). In a comprehensive study, Wauters et al analyzed the interplay between the innate and adaptative immune responses using single cell transcriptomics on BALs and found abundant and dysregulated T cells along with hyperinflammatory monocytes in critical COVID-19 (16). Viral- RNA tracking demonstrated infected lung epithelial cells along with neutrophils and macrophages involved in viral clearance.

Several other authors have argued instead for stimulating the immune response in critically ill patients with COVID-19 and not suppressing it. Remy et al compared the T cell subsets and quantified the T cell IFN-γ and monocyte TNF-α production in patients with severe COVID-19, sepsis, critically ill non-septic and healthy volunteers (2). Patients with severe COVID-19 were characterized by impaired immune effector cell function which was partially restored *ex vivo* by administering IL-7. Analyzing these discrepancies and also the improved mortality with several immune modulators prompted our second hypothesis that using the bronchoalveolar lavage will give us a more accurate image of the lung-centric immunity.

In our previous study, we described the inflammatory immune dysregulation occurring in the lower airways where 28/35 cytokines had elevated levels. Among those only IP-10 BAL levels correlated with plasma levels (11). Therefore, most peripheral blood cytokine concentrations serve as inaccurate/ weak markers in quantifying the intensity of the immune response during severe COVID-19. This may contribute to the discrepancy between low blood cytokine concentrations but positive clinical response to corticosteroids and other immune modulators. The RECOVERY trial was the first randomized controlled trial to show a significant mortality reduction in patients treated with dexamethasone (17). A randomized Bayesian trial showing a trend towards improved organ support- free days was stopped early after the results of the RECOVERY trial were released (18). Given these results and the fact that the hyperinflammatory phenotype characterizes only a minority of patients (4), it results that dexamethasone improved mortality in patients with hypoinflammatory phenotype as well. This opens the hypothesis of treatment heterogeneity *within* the phenotypes, hypothesis that our study tried to ellucidate.

The second most studied class of immune modulators in COVID-19 are the IL-6 blockers. Many studies

missed to detect a significant IL-6 concentration in plasma (5, 15, 19–21). Multiple non-randomized studies of IL-6 blockade suggested a benefit in reducing intubation rates and even mortality (22, 23) yet the initial randomized controlled trials failed to prove a benefit in moderate/ severe COVID-19 pneumonia (24, 25). Meta-analyses found that IL-6 blockers decreased the need for mechanical ventilation and mortality in both critically ill and non-critically ill patients albeit with a small effect size with mortality risk ratio of 0.89, (95% CI 0.82 to 0.96) (26, 27).

After dexamethasone became the standard of care in patients diagnosed with COVID-19 requiring oxygen, several studies tried to quantify the benefit of adding a second immune modulator agent to corticosteroids. Despite the debate on systemic inflammation, several other immune modulators acting on distinct pathways have shown additional benefits in hospitalized patients with COVID-19 treated with steroids, mainly in reducing rates of intubation. These include tocilizumab, an antagonist of the IL-6 receptor (28), baricitinib, a JAK 2 inhibitor (29), lenzilumab, a Granulocyte-macrophage colony-stimulating factor (GM-CSF) blocking antibody (30) and anakinra, an IL-1 blocker (31).

Similar to the small signal to harm in the hypoinflammatory phenotype (5), the small effect sizes and partial lack of replication point towards lack of power or heterogeneity of treatment effects and mixing responders and non-responders as possible causes (32). This may be explained by differences in semi-quantitative SARS-CoV-2 RT-PCR. So far, the SARS-CoV-2 RT-PCR tests have been developed as qualitative tests and the relationship between Ct and viral load at very low and very high viral loads is not linear. Ct thresholds have been linked to severity of illness, transmissibility and differentiating replicating virus from persistent shedding after a resolved infection (33, 34). We used the same test in all of our patients and also a very standardized bronchoscopy procedure and sampling technique. The results for the*S* and *Orf* gene targets were very similar in our sample with less than 0.5 variation in Ct. While the qualitative outputs of the nasopharyngeal swabs and BAL correlate in terms of positive and negative, no published reports establish the correspondence between Ct values in the upper vs lower airways (35, 36). As such, we elected to use SARS-CoV-2 RT-PCR Ct in the BAL as a surrogate marker for the amount of virus.

The most important limitation of our study is the small sample size. It is possible that differences between groups may have been missed. The similar distribution and signal described in other published related studies lends external validity to our results. As it has been noted, identifying groups based on theory should take precedence since clustering/ phenotyping techniques will always find groups in the data (37). A recent study by Sinha et al questions the use of machine learning without biomarker data in grouping patients to explain heterogeneity of treatment effects (38). When analyzing 3 RCT in ARDS by using 9 clustering methodologies, the authors found a wide variation to identify clusters which was only exacerbated in the absence of biomarkers. Our study pushes phenotyping one step further by using a biologically plausible hypothesis (i.e. the infection matters in explaining heterogeneity of treatment effects) and providing a measurable marker in semi-quantitative SARS-CoV-2 in BAL. Our results expand on previous studies by showing the role of SARS-CoV-2 RT-PCR in phenotyping patients with COVID-19 ARDS and providing evidence that the lower airways are the best compartment to sample for accurately assessing the intensity of the immune response. Since we do not have BAL or plasma samples prior to dexamethasone to verify the "native" clusters, it is possible that more patients could have been grouped under the "hyperinflammatory" phenotype earlier in the disease process. We provide a homogenous cohort of mechanically ventilated patients with COVID-19 ARDS but this may also be labelled as a convenience sample since these were patients undergoing bronchoscopy based on clinical reasons. Yet, only 1 patient in cluster 1 had cryptococcal pneumonia and 1 in cluster 2 had *Staphylococcus aureus* pneumonia, no patients in cluster 3 exhibited positive BAL cultures. It is unlikely that the use of remdesivir altered our results as previous reports did not find a difference in nasopharygeal Ct values when patients were treated with remdesivir (39). We counteracted the different timeline of sampling by standardizing all variables per week.

Conclusion: Our hypothesis-driven study adds the infection dimension to ARDS phenotypes. We identified a unique group of hypoinflammatory with high viral load patients that is biologically plausible and where additional immune modulators may have a detrimental role. Future studies are needed to establish

a threshold for SARS-CoV-2 RT-PCR and to assess the immune cascade before and after administration of additional immune-modulating therapies.

References

1. Patel BV, Haar S, Handslip R, Auepanwiriyakul C, Lee TM-L, Patel S, Harston JA, Hosking-Jervis F, Kelly D, Sanderson B, Borgatta B, Tatham K, Welters I, Camporota L, Gordon AC, Komorowski M, Antcliffe D, Prowle JR, Puthucheary Z, Faisal AA, Sanderson B, Patel B, Hosking-Jervis F, Auepanwiriyakul C, Komorowski M, Addie E, Borgatta B, Chisholm R, Crocokft A, *et al.* Natural history, trajectory, and management of mechanically ventilated COVID-19 patients in the United Kingdom.*Intensive Care Med* 2021;47:549–565.

2. Remy KE, Mazer M, Striker DA, Ellebedy AH, Walton AH, Unsinger J, Blood TM, Mudd PA, Yi DJ, Mannion DA, Osborne DF, Martin RS, Anand NJ, Bosanquet JP, Blood J, Drewry AM, Caldwell CC, Turnbull IR, Brakenridge SC, Moldwawer LL, Hotchkiss RS. Severe immunosuppression and not a cytokine storm characterizes COVID-19 infections. *JCI Insight*2020;5:140329.

3. Leisman DE, Ronner L, Pinotti R, Taylor MD, Sinha P, Calfee CS, Hirayama AV, Mastroiani F, Turtle CJ, Harhay MO, Legrand M, Deutschman CS. Cytokine elevation in severe and critical COVID-19: a rapid systematic review, meta-analysis, and comparison with other inflammatory syndromes. *Lancet Respir Med* 2020;8:1233–1244.

4. Sinha P, Calfee CS, Cherian S, Brealey D, Cutler S, King C, Killick C, Richards O, Cheema Y, Bailey C, Reddy K, Delucchi KL, Shankar-Hari M, Gordon AC, Shyamsundar M, O'Kane CM, McAuley DF, Szakmany T. Prevalence of phenotypes of acute respiratory distress syndrome in critically ill patients with COVID-19: a prospective observational study. *Lancet Respir Med* 2020;8:1209–1218.

5. Sinha P, Furfaro D, Cummings MJ, Abrams D, Delucchi K, Maddali MV, He J, Thompson A, Murn M, Fountain J, Rosen A, Robbins-Juarez SY, Adan MA, Satish T, Madhavan M, Gupta A, Lyashchenko AK, Agerstrand C, Yip NH, Burkart KM, Beitler JR, Baldwin MR, Calfee CS, Brodie D, O'Donnell MR. Latent Class Analysis Reveals COVID-19–related Acute Respiratory Distress Syndrome Subgroups with Differential Responses to Corticosteroids. *Am J Respir Crit Care Med* 2021;204:1274–1285.

6. Casadevall A, Pirofski L. In fatal COVID-19, the immune response can control the virus but kill the patient. *Proceedings of the National Academy of Sciences* 2020;117:30009–30011.

7. D'Agnillo F, Walters K-A, Xiao Y, Sheng Z-M, Scherler K, Park J, Gygli S, Rosas LA, Sadtler K, Kalish H, Blatti CA, Zhu R, Gatzke L, Bushell C, Memoli MJ, O'Day SJ, Fischer TD, Hammond TC, Lee RC, Cash JC, Powers ME, O'Keefe GE, Butnor KJ, Rapkiewicz AV, Travis WD, Layne SP, Kash JC, Taubenberger JK. Lung epithelial and endothelial damage, loss of tissue repair, inhibition of fibrinolysis, and cellular senescence in fatal COVID-19. *Science Translational Medicine* 13:eabj7790.

8. Schaller T, Hirschbühl K, Burkhardt K, Braun G, Trepel M, Märkl B, Claus R. Postmortem Examination of Patients With COVID-19. *JAMA*2020;323:2518–2520.

9. The ARDS Definition Task Force*. Acute Respiratory Distress Syndrome: The Berlin Definition. *JAMA* 2012;307:2526–2533.

10. Chen Y, Wang J, Liu C, Su L, Zhang D, Fan J, Yang Y, Xiao M, Xie J, Xu Y, Li Y, Zhang S. IP-10 and MCP-1 as biomarkers associated with disease severity of COVID-19. *Mol Med* 2020;26:97.

11. Reynolds D, Vazquez Guillamet C, Day A, Borcherding N, Vazquez Guillamet R, Choreño-Parra JA, House SL, O'Halloran JA, Zúñiga J, Ellebedy AH, Byers DE, Mudd PA. Comprehensive Immunologic Evaluation of Bronchoalveolar Lavage Samples from Human Patients with Moderate and Severe Seasonal Influenza and Severe COVID-19. *J Immunol*2021;207:1229–1238.

12. Bos LDJ, Sjoding M, Sinha P, Bhavani SV, Lyons PG, Bewley AF, Botta M, Tsonas AM, Serpa Neto A, Schultz MJ, Dickson RP, Paulus F. Longitudinal respiratory subphenotypes in patients with COVID-

19-related acute respiratory distress syndrome: results from three observational cohorts. *Lancet Respir Med* 2021;9:1377–1386.

13. Lee N, Chan MCW, Lui GCY, Li R, Wong RYK, Yung IMH, Cheung CSK, Chan ECY, Hui DSC, Chan PKS. High Viral Load and Respiratory Failure in Adults Hospitalized for Respiratory Syncytial Virus Infections. *J Infect Dis* 2015;212:1237–1240.

14. Brodin P. Immune determinants of COVID-19 disease presentation and severity. *Nat Med* 2021;27:28–33.

15. Wilson JG, Simpson LJ, Ferreira A-M, Rustagi A, Roque J, Asuni A, Ranganath T, Grant PM, Subramanian A, Rosenberg-Hasson Y, Maecker HT, Holmes SP, Levitt JE, Blish CA, Rogers AJ. Cytokine profile in plasma of severe COVID-19 does not differ from ARDS and sepsis. *JCI Insight*2020;5:140289.

16. Wauters E, Van Mol P, Garg AD, Jansen S, Van Herck Y, Vanderbeke L, Bassez A, Boeckx B, Malengier-Devlies B, Timmerman A, Van Brussel T, Van Buyten T, Schepers R, Heylen E, Dauwe D, Dooms C, Gunst J, Hermans G, Meersseman P, Testelmans D, Yserbyt J, Tejpar S, De Wever W, Matthys P, CONTAGIOUS collaborators, Neyts J, Wauters J, Qian J, Lambrechts D. Discriminating mild from critical COVID-19 by innate and adaptive immune single-cell profiling of bronchoalveolar lavages. *Cell Res*2021;31:272–290.

17. RECOVERY Collaborative Group, Horby P, Lim WS, Emberson JR, Mafham M, Bell JL, Linsell L, Staplin N, Brightling C, Ustianowski A, Elmahi E, Prudon B, Green C, Felton T, Chadwick D, Rege K, Fegan C, Chappell LC, Faust SN, Jaki T, Jeffery K, Montgomery A, Rowan K, Juszczak E, Baillie JK, Haynes R, Landray MJ. Dexamethasone in Hospitalized Patients with Covid-19. *N Engl J Med* 2021;384:693–704.

18. Angus DC, Derde L, Al-Beidh F, Annane D, Arabi Y, Beane A, van Bentum-Puijk W, Berry L, Bhimani Z, Bonten M, Bradbury C, Brunkhorst F, Buxton M, Buzgau A, Cheng AC, de Jong M, Detry M, Estcourt L, Fitzgerald M, Goossens H, Green C, Haniffa R, Higgins AM, Horvat C, Hullegie SJ, Kruger P, Lamontagne F, Lawler PR, Linstrum K, *et al.* Effect of Hydrocortisone on Mortality and Organ Support in Patients With Severe COVID-19: The REMAP-CAP COVID-19 Corticosteroid Domain Randomized Clinical Trial. *JAMA* 2020;324:1317–1329.

19. Van Singer M, Brahier T, Ngai M, Wright J, Weckman AM, Erice C, Meuwly J-Y, Hugli O, Kain KC, Boillat-Blanco N. COVID-19 risk stratification algorithms based on sTREM-1 and IL-6 in emergency department. *J Allergy Clin Immunol* 2021;147:99-106.e4.

20. Tan L, Kang X, Ji X, Li G, Wang Q, Li Y, Wang Q, Miao H. Validation of Predictors of Disease Severity and Outcomes in COVID-19 Patients: A Descriptive and Retrospective Study. *Med (N Y)* 2020;1:128-138.e3.

21. Saji R, Nishii M, Sakai K, Miyakawa K, Yamaoka Y, Ban T, Abe T, Ohyama Y, Nakajima K, Hiromi T, Matsumura R, Suzuki N, Taniguchi H, Otsuka T, Oi Y, Ogawa F, Uchiyama M, Takahashi K, Iwashita M, Kimura Y, Fujii S, Furuya R, Tamura T, Ryo A, Takeuchi I. Combining IL-6 and SARS-CoV-2 RNAaemia-based risk stratification for fatal outcomes of COVID-19. *PLoS One* 2021;16:e0256022.

22. Gupta S, Wang W, Hayek SS, Chan L, Mathews KS, Melamed ML, Brenner SK, Leonberg-Yoo A, Schenck EJ, Radbel J, Reiser J, Bansal A, Srivastava A, Zhou Y, Finkel D, Green A, Mallappallil M, Faugno AJ, Zhang J, Velez JCQ, Shaefi S, Parikh CR, Charytan DM, Athavale AM, Friedman AN, Redfern RE, Short SAP, Correa S, Pokharel KK, *et al.* Association Between Early Treatment With Tocilizumab and Mortality Among Critically Ill Patients With COVID-19. *JAMA Internal Medicine* 2021;181:41–51.

23. Tleyjeh IM, Kashour Z, Damlaj M, Riaz M, Tlayjeh H, Altannir M, Altannir Y, Al-Tannir M, Tleyjeh R, Hassett L, Kashour T. Efficacy and safety of tocilizumab in COVID-19 patients: a living systematic review and meta-analysis. *Clin Microbiol Infect* 2021;27:215–227.

24. Stone JH, Frigault MJ, Serling-Boyd NJ, Fernandes AD, Harvey L, Foulkes AS, Horick NK, Healy BC, Shah R, Bensaci AM, Woolley AE, Nikiforow S, Lin N, Sagar M, Schrager H, Huckins DS, Axelrod M, Pincus MD, Fleisher J, Sacks CA, Dougan M, North CM, Halvorsen Y-D, Thurber TK, Dagher Z, Scherer

A, Wallwork RS, Kim AY, Schoenfeld S, *et al.*Efficacy of Tocilizumab in Patients Hospitalized with Covid-19.*New England Journal of Medicine* 2020;383:2333–2344.

25. Hermine O, Mariette X, Tharaux P-L, Resche-Rigon M, Porcher R, Ravaud P, CORIMUNO-19 Collaborative Group. Effect of Tocilizumab vs Usual Care in Adults Hospitalized With COVID-19 and Moderate or Severe Pneumonia: A Randomized Clinical Trial. *JAMA Intern Med*2021;181:32–40.

26. Ghosn L, Chaimani A, Evrenoglou T, Davidson M, Graña C, Schmucker C, Bollig C, Henschke N, Sguassero Y, Nejstgaard CH, Menon S, Nguyen TV, Ferrand G, Kapp P, Riveros C, Ávila C, Devane D, Meerpohl JJ, Rada G, Hróbjartsson A, Grasselli G, Tovey D, Ravaud P, Boutron I. Interleukin-6 blocking agents for treating COVID-19: a living systematic review.*Cochrane Database of Systematic Reviews*2021;doi:10.1002/14651858.CD013881.

27. Kyriakopoulos C, Ntritsos G, Gogali A, Milionis H, Evangelou E, Kostikas K. Tocilizumab administration for the treatment of hospitalized patients with COVID-19: A systematic review and meta-analysis.*Respirology* 2021;26:1027–1040.

28. RECOVERY Collaborative Group. Tocilizumab in patients admitted to hospital with COVID-19 (RECOVERY): a randomised, controlled, open-label, platform trial. *Lancet* 2021;397:1637–1645.

29. Marconi VC, Ramanan AV, de Bono S, Kartman CE, Krishnan V, Liao R, Piruzeli MLB, Goldman JD, Alatorre-Alexander J, de Cassia Pellegrini R, Estrada V, Som M, Cardoso A, Chakladar S, Crowe B, Reis P, Zhang X, Adams DH, Ely EW, COV-BARRIER Study Group. Efficacy and safety of baricitinib for the treatment of hospitalised adults with COVID-19 (COV-BARRIER): a randomised, double-blind, parallel-group, placebo-controlled phase 3 trial. *Lancet Respir Med*2021;9:1407–1418.

30. De Luca G, Cavalli G, Campochiaro C, Della-Torre E, Angelillo P, Tomelleri A, Boffini N, Tentori S, Mette F, Farina N, Rovere-Querini P, Ruggeri A, D'Aliberti T, Scarpellini P, Landoni G, De Cobelli F, Paolini JF, Zangrillo A, Tresoldi M, Trapnell BC, Ciceri F, Dagna L. GM-CSF blockade with mavrilimumab in severe COVID-19 pneumonia and systemic hyperinflammation: a single-centre, prospective cohort study. *The Lancet Rheumatology* 2020;2:e465–e473.

31. Kyriazopoulou E, Huet T, Cavalli G, Gori A, Kyprianou M, Pickkers P, Eugen-Olsen J, Clerici M, Veas F, Chatellier G, Kaplanski G, Netea MG, Pontali E, Gattorno M, Cauchois R, Kooistra E, Kox M, Bandera A, Beaussier H, Mangioni D, Dagna L, van der Meer JWM, Giamarellos-Bourboulis EJ, Hayem G, International Collaborative Group for Anakinra in COVID-19. Effect of anakinra on mortality in patients with COVID-19: a systematic review and patient-level meta-analysis.*Lancet Rheumatol* 2021;3:e690–e697.

32. Iwashyna TJ, Burke JF, Sussman JB, Prescott HC, Hayward RA, Angus DC. Implications of Heterogeneity of Treatment Effect for Reporting and Analysis of Randomized Trials in Critical Care. *Am J Respir Crit Care Med* 2015;192:1045–1051.

33. Rao SN, Manissero D, Steele VR, Pareja J. A Systematic Review of the Clinical Utility of Cycle Threshold Values in the Context of COVID-19.*Infect Dis Ther* 2020;9:573–586.

34. Jefferson T, Spencer EA, Brassey J, Heneghan C. Viral Cultures for Coronavirus Disease 2019 Infectivity Assessment: A Systematic Review.*Clin Infect Dis* 2021;73:e3884–e3899.

35. Gao CA, Cuttica MJ, Malsin ES, Argento AC, Wunderink RG, Smith SB, Argento AC, Wagh AA, McQuattie-Pimentel AC, Wolfe AR, Bharat A, Levenson AR, Joudi AM, Sinha A, Budd AN, Singer BD, Tran B, Gao CA, Pickens CO, Kurihara C, Soriano CJ, Schroedl CJ, Meza D, Kidd DA, Kamp DW, Malsin ES, Leibenguth EM, Cantey EP, Liu GY, *et al.* Comparing Nasopharyngeal and BAL SARS-CoV-2 Assays in Respiratory Failure.*Am J Respir Crit Care Med* 2021;203:127–129.

36. Geri P, Salton F, Zuccatosta L, Tamburrini M, Biolo M, Busca A, Santagiuliana M, Zuccon U, Confalonieri P, Ruaro B, D'Agaro P, Gasparini S, Confalonieri M. Limited role for bronchoalveolar lavage

to exclude COVID-19 after negative upper respiratory tract swabs: a multicentre study. *Eur Respir J* 2020;56:2001733.

37. van Smeden M, Harrell Jr FE, Dahly DE. Novel diabetes subgroups. Lancet Diabetes Endocrinol 2018 Jun;6(6):439-440.

38. Sinha P, Spicer A, Delucchi KL, McAuley DF, Calfee CS, Churpek MM. Comparison of machine learning clustering algorithms for detecting heterogeneity of treatment effect in acute respiratory distress syndrome: A secondary analysis of three randomised controlled trials. EBioMedicine 2021 Dec;74:103697. Epub 2021 Dec 1.

39. Gottlieb RL, Vaca CE, Paredes R, Mera J, Webb BJ, Perez G, Oguchi G, Ryan P, Nielsen BU, Brown M, Hidalgo A, Sachdeva Y, Mittal S, Osiyemi O, Skarbinski J, Juneja K, Hyland RH, Osinusi A, Chen S, Camus G, Abdelghany M, Davies S, Behenna-Renton N, Duff F, Marty FM, Katz MJ, Ginde AA, Brown SM, Schiffer JT, *et al.* Early Remdesivir to Prevent Progression to Severe Covid-19 in Outpatients. *N Engl J Med* 2022;386:305–315.

Table 1 . Comparison between the 3 bronchoalveolar lavage clusters and the total cohort.

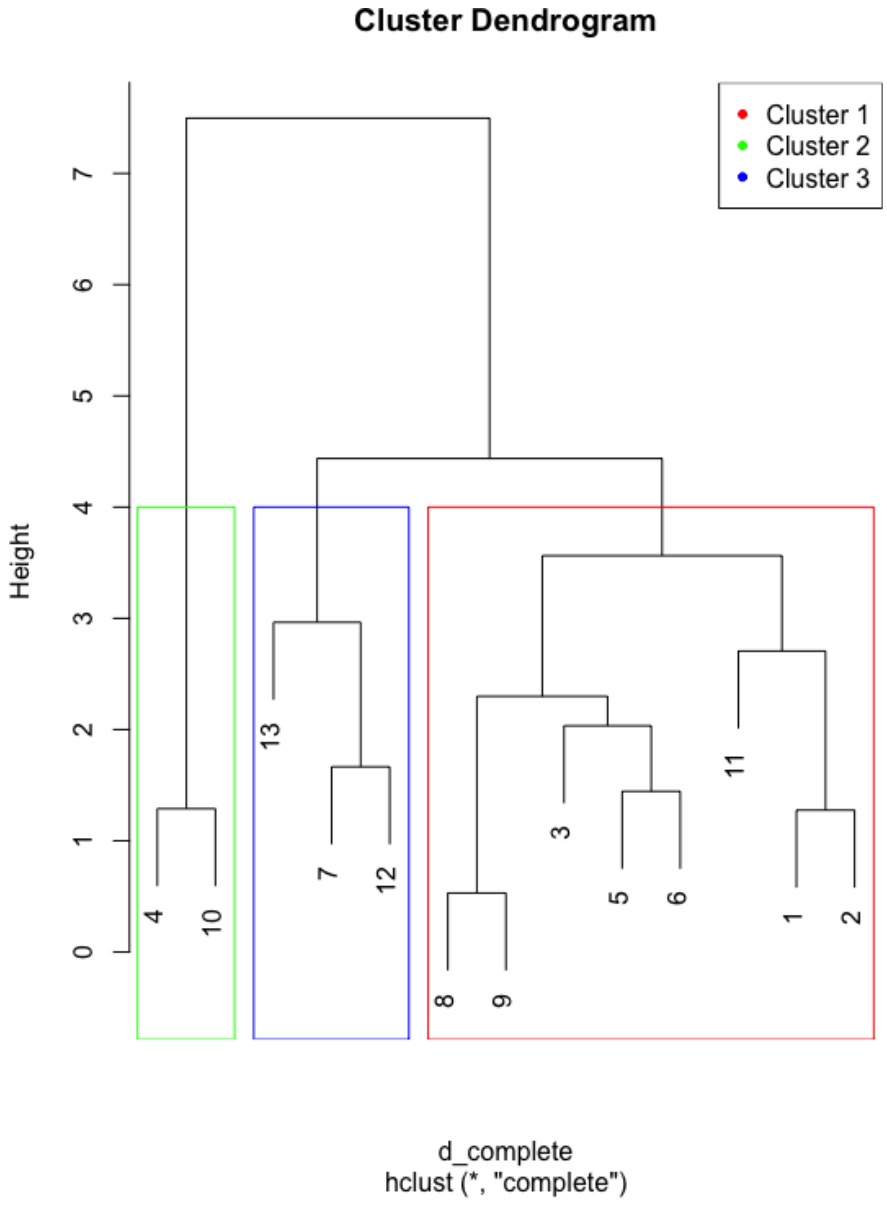
Variables	**Total cohort (n=18)**	**Hypoinflammatory low viral load (n=8, 61.5%)**	**Hyperinflammatory (n=2, 15.4%)**	**Hypoinflammatory high viral load (n=3, 23.1%)**
Age, mean ± SD, years	68 ± 8	69 ± 7.8	64 ± 15.6	67 ± 6
Male Gender	50% (9/18)	37.5% (3/8)	100% (2/2)	0 (0/3)
African American race	16.7% (3/18)	12.5% (1/8)	50% (1/2)	33.3% (1/3)
Immunosuppression	33.3% (6/18)	37.5% (3/8)	50% (1/2)	33.3% (1/3)
Body mass index	33.8 ± 12.3	28.9 ± 4.8	33.6 ± 16.5	44.6 ± 24.6
Diabetes mellitus	77.8% (14/18)	75% (6/8)	50% (1/2)	100% (3/3)
Days of intubation prior to BAL	3.6 ± 3.6	3.6 ± 2.1	4 ± 4.2	1.7 ± 1.1
Days from symptoms to BAL, days	13.6 ± 7.8	14.8 ± 7.6	12 ± 4.2	14 ±7
Dexamethasone prior to BAL	100%	100%	100%	100%
Tocilizumab prior to BAL	0	0	50% (1/1)	0
Remdesivir prior to BAL	55.6% (10/18)	62.5% (5/8)	50% (1/1)	33% (1/3)
Max temperature*, mean ± SD, ^{0}C	37.7 ±1.1	37.7 ±0.8	37.8 ±1.7	37.9 ±1.9
Vasopressors*	72.2% (13/18)	75% (6/8)	100% (2/2)	33% (1/3)
Min PaO2/FiO2*	161.4 ± 93.2	153.8 ± 121.1	102.5 ± 20.6	200.5 ± 110.5
CRP*, mean ±SD, mg/L	155.2 ± 117.1	105.6 ± 79	147.9 ± 81.7	151.3 ± 50.8
Max WBC*, mean ± SD, cells/µL	13.3 ± 6.3	12.5 ± 5.8	12.8 ±4.1	12.7 ± 6.1

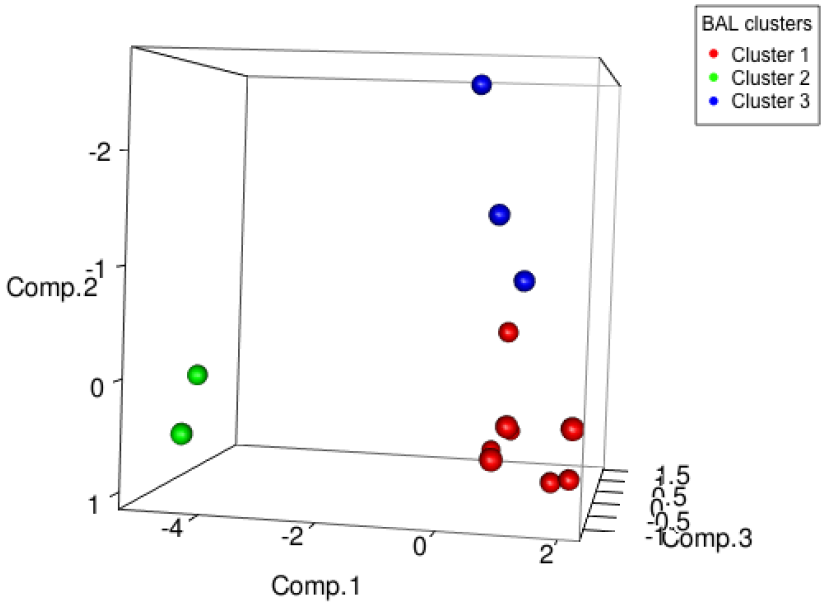
Variables	Total cohort (n=18)	Hypoinflammatory low viral load (n=8, 61.5%)	Hyperinflammatory (n=2, 15.4%)	Hypoinflammatory high viral load (n=3, 23.1%)
Min lymphocyte count*, mean ± SD cells/μL	0.7 ± 0.4	0.6 ± 0.3	0.9 ± 1.1	0.5 ± 0.1
BAL neutrophil %	38.5 ± 29.8	22.8 ± 23.7	43 ± 41	38
BAL lymphocyte %	8.4 ± 8.7	9.2 ± 7.7	3.5 ± 2.1	28
Ct value, mean ± SD	22 ± 6.5	26.6 ± 2.6	20 ± 1.1	14.4 ± 1.2
Ventilator- free days, mean ±SD, days	1.8 ± 2.9	1.6 ± 3.1	1.5 ± 2.1	1.3 ± 2.3
Length of stay, mean ± SD, days	31.1 ± 15.6	28 ± 11.4	51.5 ± 3.5	18.7 ± 14.4
Mortality	66.7% (12/18)	75% (6/8)	50% (1/2)	100% (3/3)

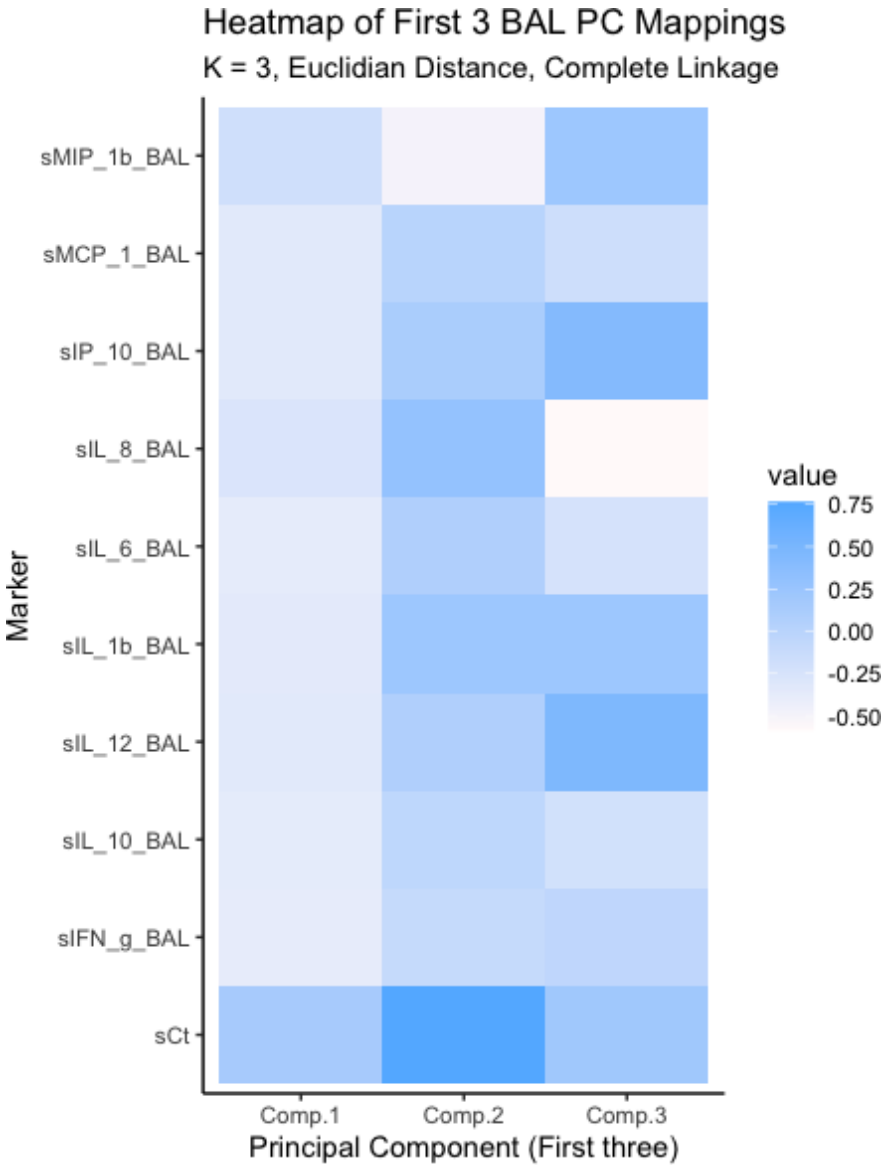
13 patients had complete immune-inflammatory panels and SARS-CoV-2 RT-PCRs obtained from the BAL

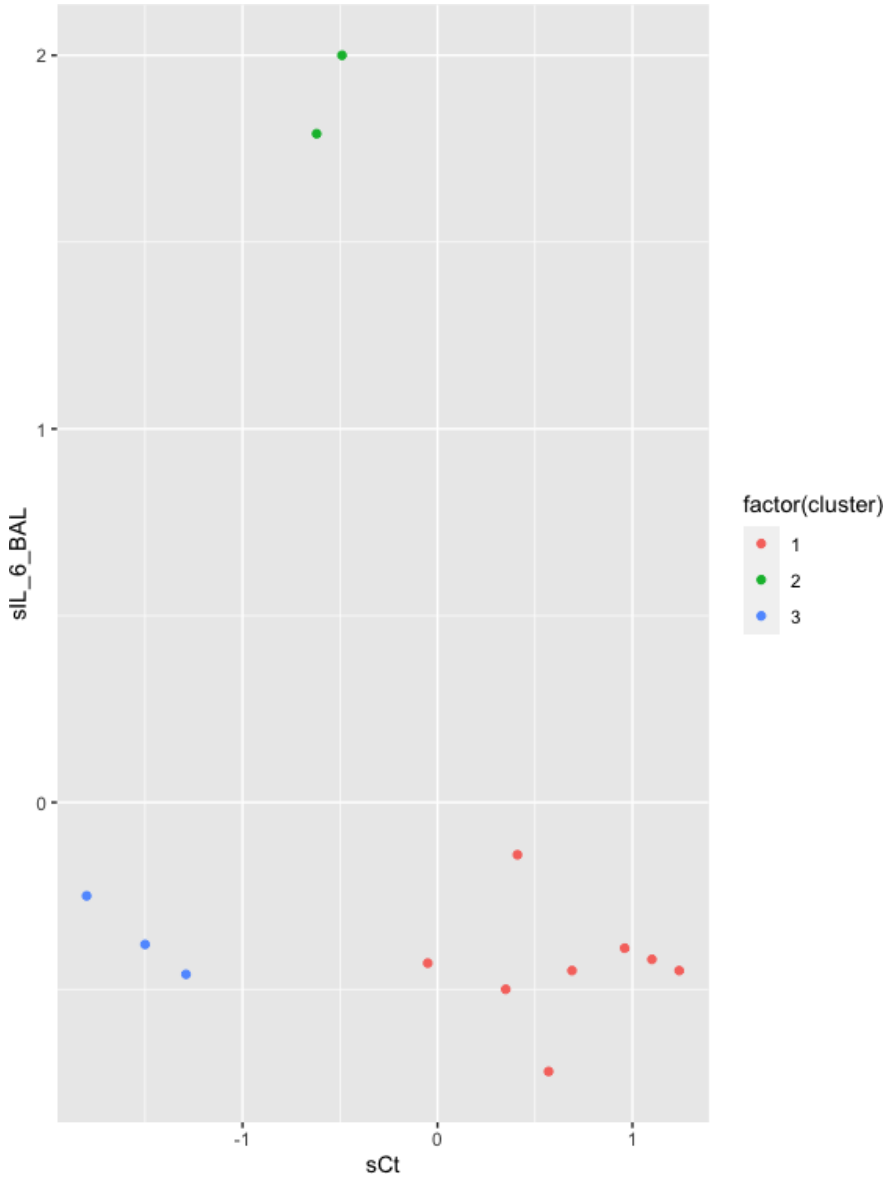
*During the week of the bronchoalveolar lavage week

Abbreviations: BAL= bronchoalveolar lavage; CRP= C-reactive protein; Ct= Cycle threshold; PaO2/FiO2 = Oxygen arterial pressure/ fraction of inspired oxygen; SD = standard deviation; WBC=white blood cell count









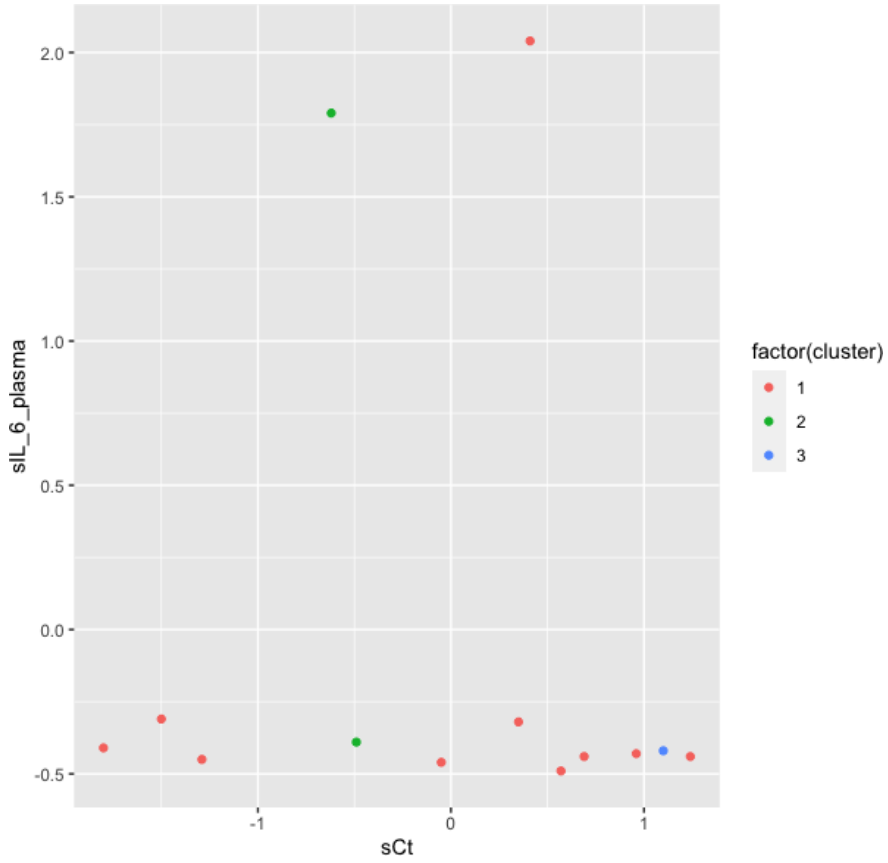


Table E1: Comparison between the general cohort and the three bronchoalveolar lavage clusters

Immune marker in BAL*	**Total cohort (n=18)**	**Hypoinflammatory low viral load (n=8, 61.5%)**	**Hyperinflammatory (n=2, 15.4%)**	**Hypoinflammatory high viral load (n=3, 23.1%)**
IL-6	997.4 ± 2916.2	102.3 ± 81.1	5897.6 ± 6725.4^{+}	117.6 ± 100.5
IFN-γ	3.1 ± 4.3	1 ± 1.1	11.9 ± 4.7	2.8 ± 0.9
IL-1β	5.8 ± 16.2	1.4 ± 0.7	30.9 ± 41	1.1 ± 0.2
IL-8	2201 ± 3908	1638.8 ± 2862.6	6981.4 ± 8302.9	514.3 ± 354.3
IL-10	10.7 ± 16	2.7 ± 1.2	37.7 ± 20.3	14 ± 18.2
IL-12	17.1 ± 10.7	13.1 ± 6.5	35.8 ± 15.1	15.4 ± 1.5
MIP-1β	108 ± 118.1	71.3 ± 94.3	193.3 ± 111.1	153.2 ± 175.2
MCP-1	1293 ± 1267	937.6 ± 915.9	3516.6 ± 1153.4	757.9 ± 398.8
IP-10	444.6 ± 698	211.8 ± 181.5	1643.3 ± 1487.5	266.2 ± 51.2

13 pts had complete sets of BAL cytokines and SARS-CoV-2 RT PCR

*levels reported as mean ± SD in pg/ml

$^{+}$ when excluding patient s/p treatment with tocilizumab, mean IL-6 level was 1142

Abbreviations: BAL=bronchoalveolar lavage; IFN=interferon; IL=interleukin; IP = interferon gamma

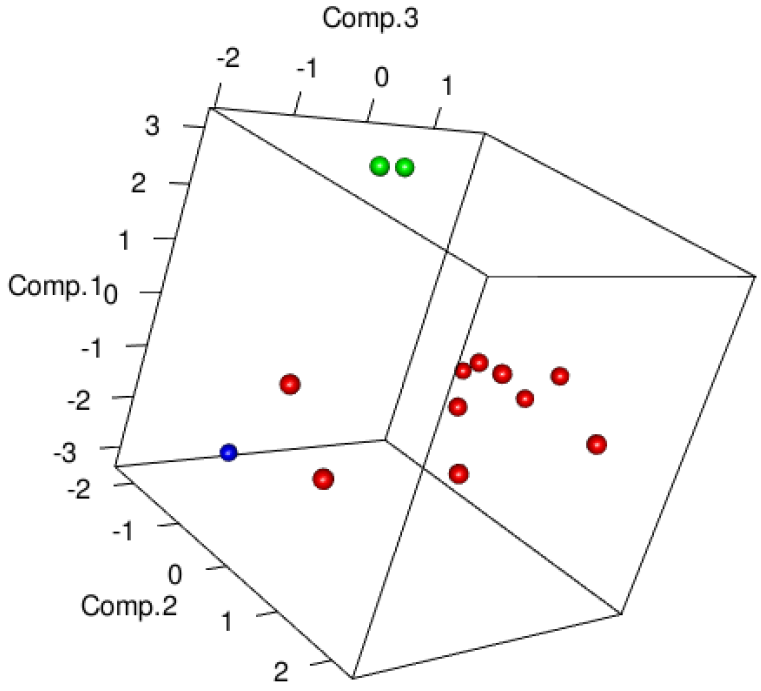
inducible protein-10; MIP -1= macrophage inflammatory protein-1; MCP-1= monocyte chemoattractant protein-1; SD=standard deviation

Table E2: Principal component analysis loadings for the bronchoalveolar lavage clusters

Variables*	Component 1	Component 2	Component 3
Ct	0.15	0.76	0.20
IL-1β	-0.35	0.22	0.23
IL-10	-0.36	-0.04	-0.20
IL-6	-0.36	0.07	-0.23
IL-12	-0.34	0.07	0.47
MIP-1β	-0.19	-0.49	0.23
MCP-1	-0.34	0.00	-0.17
IFN-γ	-0.37	-0.10	-0.04
IL-8	-0.28	0.30	-0.58
IP-10	-0.34	0.12	0.42

*All variables were standardized per week and rescaled between 0 and 1.

Abbreviations: Ct=cycle threshold; IFN=interferon; IL=interleukin; IP = interferon gamma inducible protein-10; MIP -1= macrophage inflammatory protein-1; MCP-1= monocyte chemoattractant protein-1; SD=standard deviation



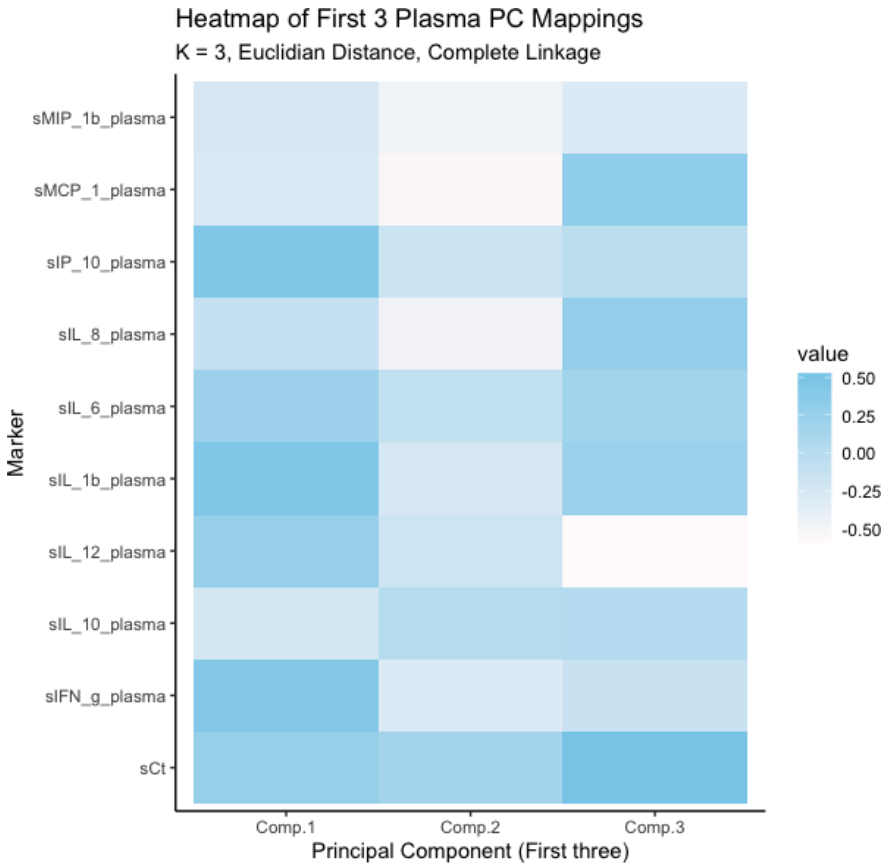


Table E3: Comparison between the plasma clusters and the total cohort

Variables	Total cohort (n=18)	Hypoinflammatory low viral load (n=10)	Hyperinflammatory (n=2)	Hypoinflammatory low viral load (n=1)
Age, mean ± SD, years	68 ± 8	65 ±7.6	77± 2.8	73
Male Gender	50% (9/18)	30%	100%	0
African American race	16.7% (3/18)	20%	0	0
Immunosuppression	33.3% (6/18)	30%	50%	100%
Body mass index	33.8 ± 12.3	34.1±2.6	23.8±2.6	43.7
Diabetes mellitus	77.8% (14/18)	70% (7/10)	100% (2/2)	100% (1/1)
Days of intubation prior to BAL, mean ±SD	3.6 ± 3.6	3.3±2.1	4±4.2	1
Days from symptoms to BAL	13.6 ± 7.8	13.8±7.5	13.5±2.1	19
Dexamethasone prior to BAL	100% (18/18)	100% (10/10)	100% (2/2)	100% (1/1)

Variables	Total cohort (n=18)	Hypoinflammatory low viral load (n=10)	Hyperinflammatory (n=2)	Hypoinflammatory low viral load (n=1)
Tocilizumab prior to BAL	0	0	50% (1/2)	0
Remdesivir prior to BAL	55.6% (10/18)	50% (5/10)	50% (1/2)	100% (1/1)
Max temperature*, mean ± SD, ^{0}C	37.7 ±1.1	37.4 ±0.8	38.3 ±1.1	40.1
Vasopressors*	72.2% (13/18)	70% (7/10)	100% (2/2)	0 (0/1)
Min PaO2/FiO2*	161.4 ± 93.2	168.7±120.7	101.2±18.6	148
CRP*, mean±SD, mg/L	155.2 ± 117.1	117.2±72.8	141.4±90.8	Not available
Max WBC*, mean ± SD, cells/μL	13.3 ± 6.3	12.9±5.6	13.4±3.5	7.7
Min lymphocyte count*, mean ± SD cells/μL	0.7 ± 0.4	0.5±0.3	1.2±0.6	0.5
BAL neutrophil %	38.5 ± 29.8	31±29.2	14	38
BAL lymphocyte %	8.4 ± 8.7	8±7.5	5	28
Ct value, mean ± SD	22 ± 6.5	23.4±5.6	23.6±6.2	14.4
IL-6 plasma, mean ± SD, pg/ml	1630.7±5383	160.4±306.6	9778.1±13782.8$^+$	38.5
IFN-γ plasma, mean ± SD, pg/ml	0.7±0.2	0.6±0	1±0.3	0.6
IL-1β plasma, mean ± SD, pg/ml	0.87±0.8	0.7±0.5	1.9±1.2	0.2
IL-8 plasma, mean ± SD, pg/ml	48.4±42.9	40.4±39.2	44.9±6.6	135.1
IL-10 plasma, mean ± SD, pg/ml	75±178.3	93.3±201.9	14.7±20.1	12.7
IL-12 plasma, mean ± SD, pg/ml	42.5±29.1	37.9±30	71.3±8.1	31.6
MIP-1β plasma, mean ± SD, pg/ml	223.7±470.7	83.3±62.7	151±1208.5	1773.2
MCP-1 plasma, mean ± SD, pg/ml	994.5±759.7	978.2±848.9	955±613.3	1237.5

Variables	Total cohort (n=18)	Hypoinflammatory low viral load (n=10)	Hyperinflammatory (n=2)	Hypoinflammatory low viral load (n=1)
IP-10 plasma, mean ± SD, pg/ml	183.3±229.6	111.9±67.3	613.5±386.4	37.8
Ventilator- free days, mean ± SD	1.8 +/- 2.9	1.7±2.9	1.5±2.1	0
Length of stay, mean ± SD, days	31.1 ± 15.6	29.9±14.2	38±15.6	8
Mortality	66.7% (12/18)	80% (8/10)	50% (1/2)	100% (1/1)

13 patients had complete immune-inflammatory panels in plasma and SARS-CoV-2 RT-PCRs obtained from the BAL

*During the week of the bronchoalveolar lavage week

$^{+}$when excluding the patient s/p tocilizumab, the mean level was 32

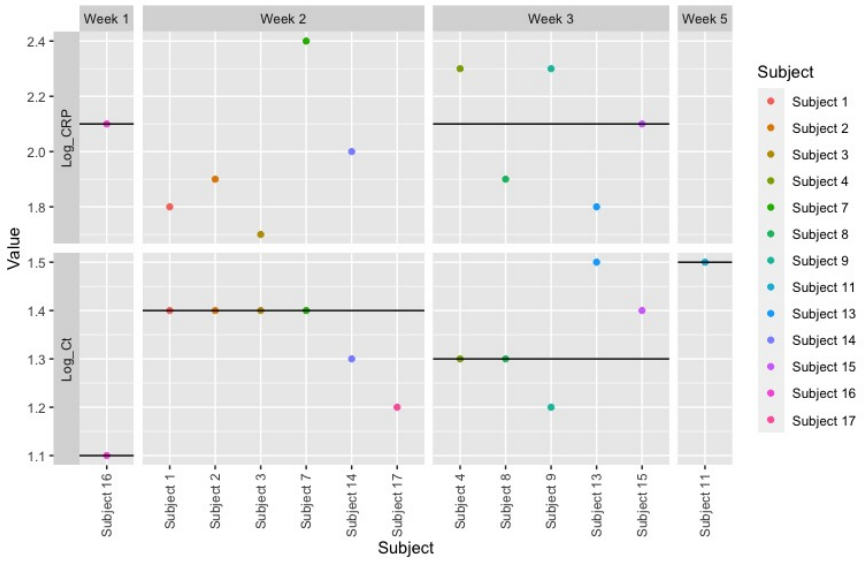
Abbreviations: BAL= bronchoalveolar lavage; CRP= C-reactive protein; Ct= Cycle threshold; IFN=interferon; IL=interleukin; IP = interferon gamma inducible protein-10; MIP -1= macrophage inflammatory protein-1; MCP-1= monocyte chemoattractant protein-1; PaO2/FiO2 = Oxygen arterial pressure/ fraction of inspired oxygen; SD = standard deviation; WBC=white blood cell count

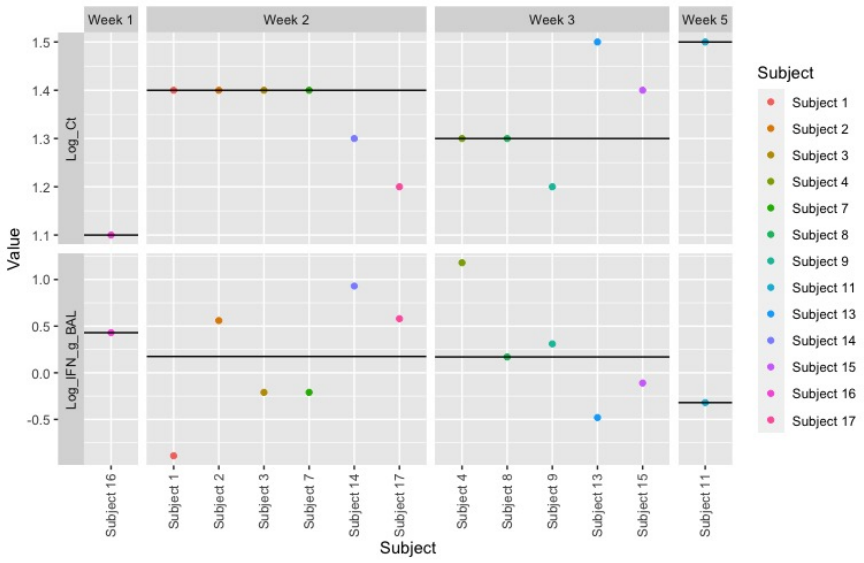
Table E4: Principal component analysis loadings for the plasma clusters

Variables*	Component 1	Component 2	Component 3
Ct	0.28	0.17	0.53
IL-1β	0.46	-0.24	0.23
IL-10	-0.23	0.02	0.03
IL-6	0.21	-0.08	0.18
IL-12	0.27	-0.18	-0.59
MIP-1β	-0.26	-0.48	-0.29
MCP-1	-0.28	-0.55	0.32
IFN-γ	0.43	-0.30	-0.14
IL-8	-0.12	-0.47	-0.29
IP-10	0.45	-0.16	-0.02

*All variables were standardized per week and rescaled between 0 and 1.

Abbreviations: Ct= Cycle threshold; IFN=interferon; IL=interleukin; IP = interferon gamma inducible protein-10; MIP -1= macrophage inflammatory protein-1; MCP-1= monocyte chemoattractant protein-1; PaO2/FiO2 = Oxygen arterial pressure/ fraction of inspired oxygen;





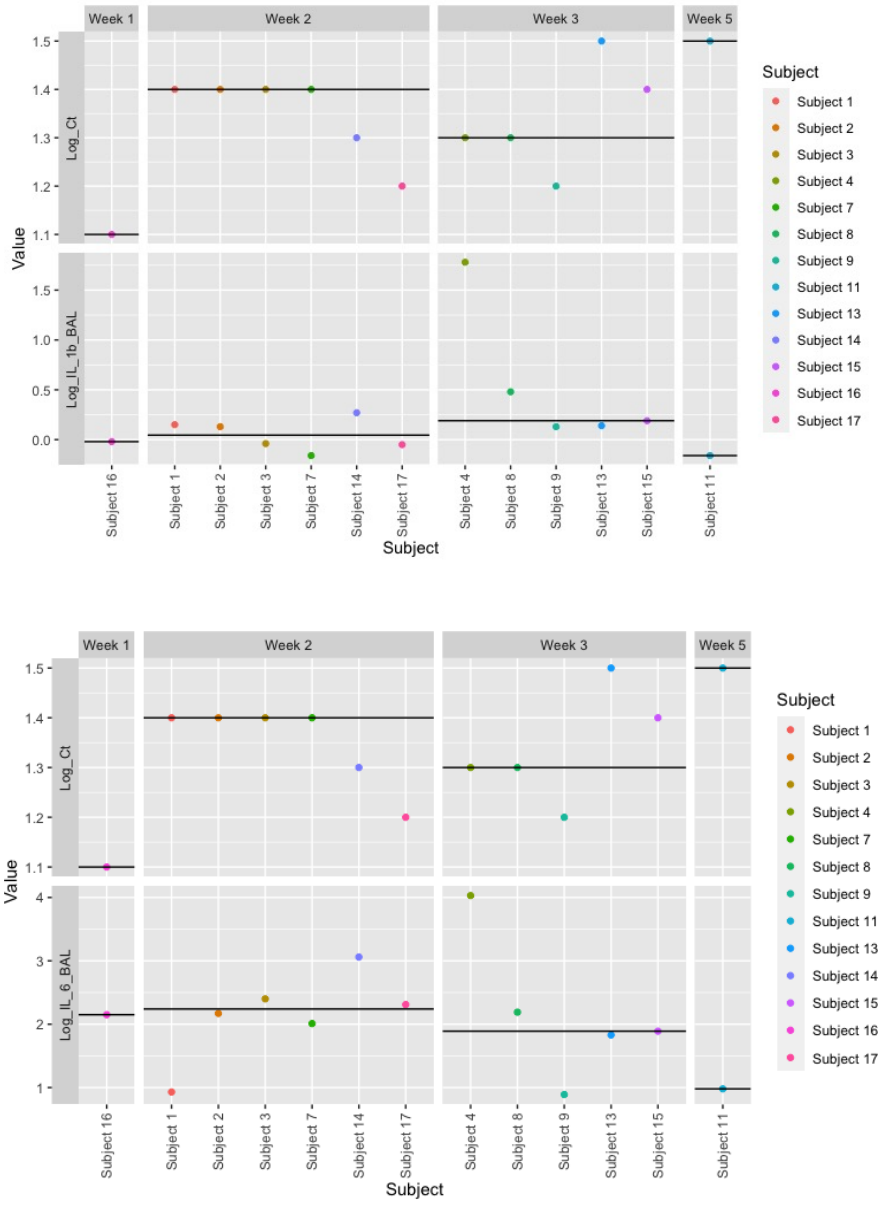


Fig.E3: Immune inflammatory markers vs cycle threshold values per week.

Panel A: CRP vs Ct; **Panel B:** IFN- γ vs Ct;**Panel C:** IL-1β vs Ct values; **Panel D:** IL-6 and Ct values. Bars represent median values. CRP was measured in the serum. Cytokine measurements and SARS-CoV-2 RT-PCR Ct values were obtained from the bronchoalveolar lavage and transformed logarithmically.

Abbreviations: BAL=bronchoalveolar lavage; Ct=cycle threshold; IFN=interferon; IL=interleukin

Online supplemental material

Changing from 2 to 3 clusters in the plasma, replicated the path of BAL clusters: cluster 1 divided into clusters 1 and 3. Cluster 1 (hypoinflammatory low viral load) remained the most populated with 10 patients (76.9%), cluster 2 (hyperinflammatory) with 2 patients (15.4%) and cluster 3 (hypoinflammatory high viral load) with 1 patient (7.7%). Overall 4 patients (30.8%) changed cluster assignments between the BAL and

plasma clusters and 2 of these belonged to the hypoinflammatory low viral load group. The PCs explained 64.6% of the variation, less than in the BAL clusters. Il-1β, IFN-γ and IP-10 had the highest loading on PC1 which accounted for 31.9% of the variance. As opposed to the BAL, not all immune markers were concordant in plasma (IL-10, MIP-1β and MCP-1 had negative loadings). IL-6 carried less significance than the other cytokines in defining the plasma clusters. Cluster 2 (hyperinflammatory) had higher values along the PC1. The immune markers of interest had in general negative loadings on PC2 which explained 18% of the variance. SARS-CoV-2 RT-PCR Ct value was less relevant than in the BAL clusters and it represented the main loading on PC3 which accounted for 14.7% of the variance. Cluster 3 was characterized by lower values on PC3 (higher viral loads). Table 2 shows the clinical characteristics, laboratory results, cytokine concentrations in plasma and outcomes between the 3 plasma clusters and the total cohort. While the hyperinflammatory cluster is relatively similar to the BAL cluster 2, no comparisons can be made regarding the hypoinflammatory high viral load clusters between plasma and BAL.