Alveolar, Endothelial, and Organ Injury Marker Dynamics in Severe COVID-19

Daniel E. Leisman, MD, MSCR;1,2 Arnav Mehta, MD, PhD;3,4,5,6 B. Taylor Thompson, MD;2,3* Nicole C. Charland, BA;7,§ Anna L.K. Gonye, BS;4,8,§ Irena Gushterova, MS;4,8,§ Kyle R. Kays, BS;7,§ Hargun K. Khanna, BS;7,§ Thomas J. LaSalle, BS;4,8,§ Kendall M. Lavin-Parsons, BA;7,§ Brendan M. Lilley, BA;7,§ Carl L. Lodenstein, BS;7,§ Kasidet Manakongtreecheep, PhD;4,8,9,§ Justin D. Margolin, BS;7,§ Brenna N. McKaig, BS;7,§ Maricarmen Rojas-Lopez, PhD;3,10,11,§ Brian C. Russo, PhD;3,10,11,§ Nihaarika Sharma, BS;4,8,§ Jessica Tantivit, BSc;4,8,9,§ Molly F. Thomas, MD, PhD;4,8,9,§ Blair Alden Parry, BA;7 Alexandra-Chloé Villani, PhD;3,4,6 Moshe Sade-Feldman, PhD;3,4,6 Nir Hacohen, PhD;3,4,6 Michael R. Filbin, MD, MS;4,7,12 and Marcia B. Goldberg, MD.3,4,10,11

Author Affiliations:
1. Department of Anesthesiology, Critical Care, and Pain Medicine, Massachusetts General Hospital, Boston, MA
2. Department of Medicine, Massachusetts General Hospital, Boston, MA
3. Department of Medicine, Harvard Medical School, Boston, MA, USA
4. Broad Institute of Massachusetts Institute of Technology (MIT) and Harvard, Cambridge, MA, USA
5. Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA
6. Massachusetts General Hospital Cancer Center, Department of Medicine, Massachusetts General Hospital, Boston, MA, USA
7. Department of Emergency Medicine, Massachusetts General Hospital, Boston, MA, USA
8. Center for Cancer Research Department of Medicine, Massachusetts General Hospital, Boston, MA, USA
9. Center for Immunology and Inflammatory Diseases, Department of Medicine, Massachusetts General Hospital, Boston, MA, USA
10. Center for Bacterial Pathogenesis, Division of Infectious Diseases, Department of Medicine, Massachusetts General Hospital, Boston, MA, USA
Corresponding Author:
Daniel E. Leisman, MD, MSCR
Resident Physician (PGY-2)
Department of Anesthesiology, Critical Care, and Pain Medicine
Massachusetts General Hospital
55 Fruit Street
Boston, MA 02114
Gray Bigelow 7-730
Email: dleisman@mgh.harvard.edu

Author Contributions:

Conceptualization: DEL, AM, NH, MRF, MBG. Resources: MRF, NH, MBG, IG. Methodology: DEL, AM, NH, MRF, MBG, MS-F, A-CV, BAP, RPB, IG, BTT. Investigation: All investigators. Formal Analysis: DEL and AM. Writing – Original Draft: DEL. Writing – Review & Editing: DEL, AM, BTT, NH, MRF, MBG

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At a Glance

What is the current scientific knowledge on this subject?

Alveolar and endothelial injury have both been implicated in severe COVID-19 pneumonia and ARDS. How these disease processes evolve over time is not well described.

What does this study add to the field?

This observational study of hypoxemic COVID-19 patients found that alveolar injury markers peaked early whereas endothelial injury markers rose later and were associated with renin-angiotensin system activation, cardiorenovascular injury, and 28-day outcome. These results suggest alveolar and endothelial injury contribute at different times to disease progression in severe COVID-19.
ABSTRACT

**Rationale:** Alveolar and endothelial injury may be differentially associated with COVID-19 disease severity over time.

**Objectives:** To describe alveolar and endothelial injury dynamics and associations with COVID-19 severity, cardiorenovascular injury, and outcomes.

**Methods:** This single-center observational study enrolled COVID-19 patients requiring respiratory support at emergency department presentation. >40 markers of alveolar (including RAGE), endothelial (including angiopoietin-2), and cardiorenovascular injury (including renin, kidney injury molecule-1, troponin-I) were serially compared between invasively and spontaneously ventilated patients using mixed-effects repeated-measures models. Ventilatory ratios were calculated for intubated patients. Associations of biomarkers with modified World Health Organization scale at Day 28 were determined with multivariable proportional-odds regression.

**Results:** Of 225 patients, 74 (33%) received invasive ventilation at Day 0. RAGE was 1.80-fold higher in these patients at Day 0 (95%CI: 1.50-2.17) but decreased over time in all patients. Changes in alveolar markers did not correlate with changes in endothelial, cardiac, or renal injury markers. In contrast, endothelial markers were similar-to-lower for invasive ventilation at Day 0 but increased over time. In intubated patients, angiopoietin-2 was similar (fold-difference: 1.02, 95%CI: 0.89-1.17) to non-intubated patients at Day 0 but 1.80-fold higher (95%CI: 1.56-2.06) at Day 3; cardiorenovascular injury markers showed similar patterns. Endothelial markers were
not consistently associated with ventilatory ratios. Endothelial markers were more often significantly associated with 28-day outcomes than alveolar markers.

**Conclusions:** Alveolar injury markers rise early. Endothelial injury markers rise later and are associated with cardiorenovascular injury and 28-day outcome. Alveolar and endothelial injury likely contribute at different times to disease progression in severe COVID-19.
INTRODUCTION

Severe coronavirus disease-2019 (COVID-19) pneumonia frequently involves progression to hypoxemic respiratory failure and the acute respiratory distress syndrome (ARDS). ARDS is a heterogenous syndrome.\textsuperscript{1,2} In patients with a direct insult etiology (e.g., pneumonia, aspiration), ARDS is associated with higher plasma levels of pulmonary epithelial injury markers,\textsuperscript{3} whereas with indirect insults (e.g., sepsis, trauma, etc.), ARDS displays higher endothelial injury markers.\textsuperscript{3} Given that SARS-CoV-2 causes pneumonia, an alveolar injury predominant phenotype might be expected, yet the high prevalence of venous thromboembolism and shunt physiology\textsuperscript{4-7} and reported post-mortem pulmonary endothelialitis\textsuperscript{8} suggest significant endothelial dysfunction.

Whether epithelial and endothelial markers are indicative of clinical progression, extra-pulmonary organ dysfunction, and/or patient outcomes in SARS-CoV-2 infection is unknown. Direct comparisons of epithelial and endothelial injury patterns are limited and longitudinal analyses are lacking. Understanding their dynamics over time would facilitate biological interpretation of clinical trial results. We investigated the evolution of SARS-CoV-2 illness using serially measured pulmonary epithelial, endothelial, and organ injury markers in a cohort of prospectively enrolled COVID-19 patients presenting with respiratory distress. We hypothesized these markers would display distinct associations with clinical variables and would vary over time in ways that reflect the dynamics of underlying disease biology.

Some of the results have been previously reported in the form of an abstract.\textsuperscript{9}
METHODS

Overall Design and Aims:

We performed a secondary analysis of a prospectively enrolled observational cohort of patients with severe COVID-19, with three objectives:

1) Describe epithelial vs. endothelial markers over time by level of respiratory support.

2) Determine whether these markers correlate with markers of systemic inflammation, renin-angiotensin system (RAS) activation, and non-pulmonary organ dysfunction.

3) Determine whether epithelial and endothelial markers associate with 28-day patient outcomes.

The study was designed after enrollment had completed but without consideration of the results of the primary cohort analysis.

Patients:

Patients were enrolled in the Emergency Department of an urban, academic hospital in Boston during the peak of the initial COVID-19 surge (3/24-4/30/2020) as described. The institutional review board waived informed consent. Inclusion criteria were age ≥18 years, clinical concern for COVID-19 and ≥1 of the following: 1) respirations ≥22 breaths/minute, 2) SpO2 ≤92% on room air, 3) supplemental oxygen requirement, or 4) positive-pressure ventilation. For this study, we excluded enrolled patients without COVID-19 subsequently confirmed by polymerase chain reaction (PCR), or not requiring respiratory support, defined as supplemental oxygen or invasive mechanical ventilation.
Per hospital policy during the study period, no patients received non-invasive mechanical ventilation due to aerosolization concerns.

**Timeline and Clinical Data:**

Subjects contributed dedicated research blood samples with their initial clinical blood draw on Day 0, and if still hospitalized, at Day 3 and Day 7. Clinical course was followed for 28 days post-enrollment or until discharge, whichever occurred later. Recorded clinical data included demographics, comorbidities, home medications, presenting symptoms, serial vital signs, laboratory values, sequential organ failure assessment (SOFA) score, and World Health Organization (WHO) scale. Organ dysfunction criteria are described in Supplemental Methods.

Patients were categorized by respiratory support level based on WHO scale. Among intubated patients, dead space ventilation was estimated by ventilatory ratio,\textsuperscript{11} calculated using the formula:

\[
\text{Ventilatory Ratio} = \frac{(\text{Minute Ventilation} \times \text{PaCO}_2)}{(\text{Predicted Body Weight} \times 100 \times 37.5)}
\]

Ventilatory ratios ≥2.0 were considered “high”.\textsuperscript{11}

**Biomarker Assay:**

Specimen processing, banking, and assay were as described.\textsuperscript{10} Plasma biomarkers were measured using the Olink proximity extension assay, an oligonucleotide-labelled antibody assay for high-specificity high-dimensional multiplex that allows
measurement of low-abundance proteins. Because the signal is amplified by PCR, measurements are expressed in normalized protein expression (NPX) units, reflecting relative abundance on a log2 scale, rather than absolute concentration. This assay allows within-analyte comparisons between different samples but not between-analyte comparisons. For example, angiopoietin-2 levels between two different patients can be compared, but angiopoietin-2 level cannot be directly compared to interleukin-8 level in the same patient. Analytical performance validation for each protein assay is available (www.olink.com).

Biomarkers Selection:

We a priori selected markers from the Olink protein library based on prior literature and hypothesized importance. Markers were selected without consideration of the results of subsequently published analyses of this cohort. To facilitate interrogation of alveolar, club-cell, and endothelial injury, systemic inflammation, RAS activation, and non-pulmonary organ injury, the selected analytes included cell-type specific markers (Table E1). To confirm specificity, biomarkers were cross-referenced against the Human Protein Atlas: Tissue and Blood Atlases (www.proteinatlas.org).

Statistical Analysis:

Detailed methods are in Supplemental Methods. Briefly, to study how respiratory illness severity influenced each marker’s 7-day course, we constructed mixed-effects repeated measures models, adjusting for age, sex, and chronic heart, lung, and kidney disease. To account for within-subject correlation, models included a random effect for subject with study day treated categorically as a repeated measure.
Class variables for respiratory support level and interaction terms for day and support level were included.

To ensure results were robust and consistent, each biomarker was analyzed with three different approaches to specifying the respiratory support variable. First, a binary variable was used for whether the patient was invasively ventilated or deceased at that time point vs. not invasively ventilated and alive ("strategy-1"). Second, respiratory support was treated as a categorical variable with three levels: invasively ventilated or deceased, alive and requiring supplemental oxygen, or alive without respiratory support ("strategy-2"). Third, the respiratory status variable was kept fixed based on Day 0 status: invasively ventilated or requiring supplemental oxygen ("strategy-3"). As additional sensitivity analyses, we evaluated representative markers under complete-case analysis and when excluding patients who received steroids or tocilizumab.

C-statistics were calculated to measure marker discrimination for intubation or death at Days 0 and 3.

To determine the association of each marker at Days 0 and 3 with Day 28 clinical status, we used multivariable proportional-odds regression, with clinical status a three-level categorical variable: 1)died; 2)invasive ventilation; and 3)off invasive respiratory support. Covariates were age, sex, Day 0 SOFA, and chronic heart, lung, and kidney disease. Models assessing Day 3 biomarker levels’ association with clinical status included an adjustment for the Day 0 biomarker level. The proportional-odds assumption was assessed graphically in each case and found to be reasonable. We
report models in ascending format such that a higher odds-ratio indicates higher probability of worse outcome at Day 28.

Analyses were done in SAS (SAS Institute, Cary, NC). Figures were produced in SAS or Prism (GraphPad Inc., San Diego, CA).

RESULTS

Study Population and Clinical Course

Among 384 enrolled patients, 274 required respiratory support at Day 0. Forty-nine of these proved to not have COVID-19, yielding 225 for the current longitudinal analysis (Figure E1). At Day 0, 151 (67%) subjects received supplemental oxygen only and 74 (33%) invasive ventilation (Table 1). Attrition at Day 3 was minimal: 10 (4%) patients died and 10 (4%) were discharged (Figure 1A). By Day 28, 37 (16%) patients had died, 37 (16%) continued receiving invasive ventilation, and 148 (68%) had been discharged (Figure 1A). Severe hypoxemia was most prevalent on Day 0, whereas severe abnormalities of renal, coagulation, and circulatory function became more prevalent over time (Figure 1B-F; missing data prevalence in Tables E2 and E3).

Among patients intubated on Day 0, hypoxemia generally improved over time. Although ventilatory ratios also increased over time (Figure 2), suggesting increasing dead space ventilation, a minority of patients reached a “high” ventilatory ratio (≥ 2.0), increasing from n=7 (9%) on Day 0 to n=23 (31%) by Day 7.

Alveolar Injury Markers Peak Early and Decrease Over Time
Day 0 alveolar injury markers were generally higher in patients requiring invasive ventilation than those requiring only supplemental oxygen (Figures 3-4 and Figure E2-E3). Though remaining higher among invasively ventilated patients, these markers decreased over time in both groups (Figure 4; modeling “strategy-2’), and the differences between the two became less pronounced (Figure 3; modeling “strategy-1”). This pattern was consistent across the alveolar injury markers RAGE and surfactant proteins A1 and A2, but not surfactant protein D or LAMP3. Alveolar markers had moderate-to-poor discrimination for invasive ventilation at Day 0 (Table E4). C-statistics ranged between a maximum of 0.76 (95%CI: 0.69-0.83) for RAGE to a minimum of 0.60 (95%CI: 0.51-0.68) for LAMP3. At Day 3, the c-statistic was ≤0.72 for all alveolar injury markers.

Results were similar across all three modeling strategies (Figures 3, 4, and E4; “strategies” 1, 2, and 3, respectively.).

**Endothelial Markers Dynamics Are Distinct from Alveolar Marker Dynamics**

The dynamics of endothelial injury markers differed from those of alveolar markers (Figures 3, 4C-D, and E3-E4). At Day 0, endothelial injury markers were comparable for all respiratory support levels (Figures 3, 4, E4). Unlike alveolar markers, after Day 0, endothelial markers increased (Figure 4), and by Day 3, most were significantly higher in invasively ventilated patients than non-intubated patients (Figure 3-4 and E3). The endothelial markers tPA, PAI-1, tissue factor, and protein C were exceptions, demonstrating significantly more anomalous levels in intubated patients at all times. ADAMTS13, which is consumed during endothelial activation, showed an
inverse pattern, where levels were significantly lower in invasively ventilated patients ($p_{interaction}<0.0001$). All three methods of specifying respiratory severity, complete-case analysis, and analysis excluding patients who received steroids or tocilizumab all yielded similar results (Figures 3, 4, E4-E7).

For nearly all endothelial injury markers, discrimination for invasive ventilation was initially poor but markedly increased over time (Table E4). Day 0 C-statistics were <0.60 for angiopoietin-2 (0.58; 95%CI: 0.50-0.66), endocan (0.59; 95%CI: 0.51-0.67), and ICAM-1 (0.55; 95%CI: 0.47-0.63) whereas Day 3 C-statistics were 0.82 for angiopoietin-2 (95%CI: 0.76-0.88; $\Delta_c$:0.25), 0.78 for endocan (95%CI: 0.72-0.84; $\Delta_c$:0.19), 0.78 for ICAM-1 (95%CI: 0.71-0.84; $\Delta_c$:0.23), and >0.70 for all others.

Among intubated patients, higher endothelial injury marker levels were not reliably associated with higher ventilatory ratios (Figure E8).

**Club Cell Proteins Displayed Dynamics Similar to those of Endothelial Markers**

Club cell proteins followed the pattern of endothelial rather than alveolar markers (Figures 3, 4, E2, and E3). Club cell secretory protein (CC16) poorly discriminated respiratory severity at Day 0 (c-statistic: 0.59), but improved by Day 3 (c-statistic: 0.72, $\Delta_c$:0.13) (Table E4). Moreover, changes in club cell markers showed greater correlation with endothelial markers (Table E5).

**COVID-19 versus Non-COVID-19**

In multivariable regression, in invasively ventilated subjects, Day 0 alveolar injury markers were consistently higher in COVID-19 patients than non-COVID controls while
most endothelial injury markers were not (Figure E9). COVID-19-negative patients were not followed past Day 0.

RAS Activation and Cardiac and Renal Injury Markers Mirror the Endothelial Pattern

To explore whether endothelial abnormalities reflect transitions from predominantly pulmonary injury to dysregulated systemic responses, we examined markers of cardiac, renal, and vascular disturbances. Among these, markers of RAS activation, cardiac injury, and kidney injury showed dynamics similar to the endothelial pattern (Figures 3 and 5), with similarities between intubated and non-intubated patients at Day 0, yet at Days 3 and 7, significant and progressively higher levels in intubated versus non-intubated patients. Further suggestive of an increasingly systemic response, changes in cardiorenovascular injury markers were closely correlated with changes in endothelial, RAS activation, and club cell markers but not alveolar markers (Table E5-E6).

Some Inflammatory Markers Show Dynamics Similar to Endothelial Markers

Changes in sTNF-R1 ($p_{interaction}<0.0001$) and d-dimer ($p_{interaction}=0.0001$) levels mirrored the endothelial pattern and were more strongly correlated with cardiac and renal injury markers than interleukin-6, interleukin-8, or C-reactive protein. The latter, as described,\textsuperscript{14-17} were persistently higher among ventilated patients (Figures E10-E12 and Tables E7-E8).

Patients With Delayed Respiratory Failure Had Late Rises in Endothelial Markers
Among patients on supplemental oxygen at Day 0, 17 (11%) were intubated or deceased by Day 7. Their biomarkers course more closely resembled patients intubated on Day 0 than initially hypoxemic patients who were never intubated (Figure E13).

28-Day Outcomes were More Closely Associated with Endothelial Markers than Alveolar Markers

In multivariable proportional-odds analysis, worse 28-day outcome was significantly associated with Day 0 values of protein C, but not the other 11 endothelial markers, and none of the 7 epithelial markers (Figure 6 and Table E9). However, Day 3 values were significantly associated with 28-day WHO scale for 11 of 12 (92%) endothelial versus 3 of 7 (43%) epithelial markers. Endothelial effect sizes at Day 3 were significantly larger than epithelial effect sizes (median adjusted odds-ratio: 3.49 [interquartile-range: 2.41-5.46] vs. 1.57 [interquartile-range: 1.05-2.73], p=0.0297). Among endothelial markers, adjusted odds-ratios were uniformly larger for Day 3 than Day 0 values (median adjusted odds-ratio: 3.49 vs. 1.26 [interquartile-range: 1.22-1.40], p=0.0023).

RAS markers (renin, renin receptor, and ACE2) displayed an endothelial-like association with 28-day outcome. Whereas Day 0 levels were not associated with 28-day outcome, worse Day 28 clinical status was significantly associated with Day 3 renin (odds-ratio: 1.76, 95%CI: 1.08-2.88, p=0.0246), renin receptor (odds-ratio: 5.28, 95%CI: 1.64-17.04, p=0.0054), and ACE2 (odds-ratio: 1.85, 95%CI: 1.19-2.88, p=0.0065) (Table E10).
DISCUSSION

In this cohort of 225 COVID-19 patients requiring respiratory support at presentation, we observed distinct and consistent patterns in the dynamics of alveolar and endothelial injury markers: 1) elevations in alveolar injury markers diminish over time in both mechanically ventilated patients receiving lung protective ventilation and spontaneously breathing patients, 2) elevations in endothelial markers are delayed, are limited to invasively ventilated patients, and correlate with non-pulmonary organ injury, and 3) among intubated patients, despite severe hypoxemia, ventilatory ratios are not prominently elevated early.

First, the findings that RAGE and surfactant proteins peak at Day 0 suggests that alveolar injury represents an early insult in COVID-induced respiratory failure. This is consistent with our prior proteomic analyses showing strong association of Day 0 RAGE levels with disease severity. Other cohorts also report higher RAGE levels in COVID-19 vs. non-COVID ARDS. The similar decrease in alveolar markers between intubated and non-intubated patients over time could indicate that lung protective ventilation allows for alveolar recovery to a similar degree as spontaneous breathing. In the context of the controversy surrounding early intubation in COVID-19 and the opposing concerns of ventilator vs. self-inflicted lung injuries, we note institutional practice during the study period was early intubation; non-invasive mechanical ventilation was prohibited. However, decreasing alveolar injury markers clearly does not equate with clinical recovery, as many patients required prolonged intubation despite downward trajectories in alveolar markers.
Second, the delayed peak in endothelial markers among intubated patients suggests that unlike alveolar injury, endothelial activation and injury are prominent features of later severe disease. Consistent with our findings, COVID-19 patients display conspicuous pulmonary vasodilation,\(^5\) glycocalyx damage,\(^{20,21}\) post-mortem endothelialitis,\(^8\) and elevated von-Willebrand factor with low levels of ADAMTS13.\(^{22,23}\) Comparisons of COVID-19 and non-COVID-19 ARDS report comparable-to-lower initial levels of endothelial injury markers, such as angiopoietin-2, in COVID-19 ARDS.\(^{18,19}\)

The shift from alveolar- to endothelial-predominant injury may alternatively, or additionally, indicate that the “natural” course of COVID-19 respiratory failure involves a transition from a primarily lung-localized pathology to a systemic one. Herein, increased endothelial injury markers coincided with increased extra-pulmonary organ injury and dysfunction and were more strongly associated with 28-day outcome than alveolar markers. In ARDS, trajectories of endothelial markers vary,\(^{24,25}\) but higher levels consistently portend worse organ dysfunction and outcomes.\(^{25-29}\) In COVID-19, endothelial injury markers are associated with sustained elevations in viral mRNA.\(^{30}\) Therefore, the delayed rise in endothelial markers we observed might in part reflect increased systemic viral-induced injury.

Third, ventilatory ratios increased over time but were infrequently elevated and not consistently associated with endothelial injury. In contrast, SpO2/FiO2 was lowest at Day-0 and increased over time. A simple explanation might be imprecision in the ventilatory ratio as an estimate of dead space. However, intubated COVID-19 patients undergoing right-heart catheterization show increased cardiac output with elevated pulmonary capillary wedge-pressure but atypically low pulmonary vascular resistance.
and comparable intrapulmonary shunt versus non-COVID-19 ARDS controls.\textsuperscript{31} These observations suggest excess pulmonary vasodilation may contribute to severe COVID-19 hypoxemia, at least early on. This hypothesis is consistent with prominent early alveolar injury with minimal endothelial injury and does not necessarily invoke increased dead space ventilation. We note ventilatory ratios were infrequently elevated in our study but increased over time. This could simply reflect aggressive low-tidal volume ventilation and permissive hypercapnia. Alternatively, it could suggest intrapulmonary thrombosis accrues over time.

Together, these results may explain the seemingly contradictory findings of the ACTIV4a trial. In ACTIV4a, full-dose heparin improved mortality in hospitalized non-intubated patients,\textsuperscript{32} but for intubated patients was not beneficial and suggested harm.\textsuperscript{33} Heparin-treated critically-ill patients showed no mortality benefit despite a nearly two-fold reduction in major thrombotic events (5.7\% vs. 10.3\%) and similar frequency of (clinically detected) major bleeding (3.1\% vs. 2.4\%). Alveolar hemorrhage is common at autopsy in COVID-19.\textsuperscript{7} Therapeutic anticoagulation likely exacerbates risk of alveolar hemorrhage in patients with severe alveolar injury. We found alveolar injury markers were highest on Day 0 and among intubated patients. The delayed endothelial injury occurring among intubated patients could reflect alveolar bleeding and vessel injury, rather than thrombosis alone, an interpretation supported by the lack of association between endothelial markers and ventilatory ratios. Both intubated and non-intubated patients in ACTIV4a were enrolled at presentation (our study’s Day 0). Therefore, our results suggest anticoagulation of intubated patients in ACTIV4a was initiated when alveolar bleeding risk was high, and thrombosis was not a significant contributor to
hypoxemia. In contrast, at Day 0, non-intubated patients display less severe alveolar injury and similar levels of endothelial injury, such that these patients would be expected to have lower risk of alveolar bleeding and therefore be more likely to benefit from anticoagulation.

We included patients requiring both invasive and non-invasive respiratory support reasoning their common insult (i.e., COVID-19 pneumonia) made them an ideal biological comparator. Extending validity to this approach, “inflammatory” ARDS phenotypes are validated in patients at risk of ARDS, but who do not yet meet Berlin criteria. Some patients on supplemental oxygen might have met Berlin ARDS criteria had they received positive end-expiratory pressure; institutional policy prohibited non-invasive positive-pressure ventilation during the study period. Additionally, not all patients had an arterial blood-gas on Day 0. For this reason, we used modified Berlin criteria that did not consider PEEP and that used SpO$_2$/F$_{iO2}$ when P$_a$O$_2$ data were missing to designate ARDS.

The implications of the late rise in club cell markers are unclear. Injury of distal airways, the site of club cells, occurring after alveolar damage seems unlikely given that SARS-CoV-2 reaches alveoli via the airways. In vitro, club cells are particularly susceptible to SARS-CoV-2 infection, which induces inflammatory cytokine secretion. Whereas higher club cell markers were associated herein with greater disease severity, lower levels of club cell secretory protein have been previously associated with non-COVID-19 ARDS, albeit not with outcome.
RAS activation markers displayed marked elevation, with dynamics similar to endothelial injury. They correlated closely with cardiac and renal injury, albeit only in intubated patients, consistent with trials showing RAS antagonism does not modify disease course in moderate/mild COVID-19. Because our study did not measure angiotensin-II or ACE1, we cannot discriminate primary hyper-reninemia (i.e., true RAS activation) from a compensatory response to a relative hypo-RAS state, as described in distributive shock. Suggesting the latter are high levels of plasma ACE2, which suppresses RAS and is catalytically active in COVID-19, and decreases in angiotensin-II levels over time in COVID-19 ARDS. The close correlations we observed between renin and markers of renal and cardiac injury further support attenuated RAS effects, as described in sepsis, distributive shock, and following cardiac surgery. Alternatively, renin elevations could reflect responses to prolonged sedation-induced vasodilatation or hypovolemia following diuresis, representing the balance in ARDS management between protecting the lung and protecting the kidney. Regardless of mechanism, these findings indicate that renin’s utility as a marker of hypoperfusion and prognosticator of cardiovascular and renal injury in severe COVID-19 and ARDS should be explored.

This work differs from the previously published analysis of this cohort based on 1) pre-specified biomarker selection rather than ‘unbiased’ discovery analysis, 2) focus on patients presenting with hypoxemia rather than all-comers, and 3) specific interrogation of lung epithelial, endothelial, and non-pulmonary organ injury markers. These tissue-specific injury markers may inform disease biology more than characterizations of non-specific inflammatory markers alone.
Important limitations of this study include its single-center observational design, which facilitates thorough description but precludes causal evaluation of any markers under investigation. Specifically, we cannot definitively discern whether chronological differences between intubated and non-intubated patients results from COVID-19 severity or mechanical ventilation itself. Additionally, because we enrolled subjects before the approval of remdesivir and dexamethasone treatment for COVID-19, these drugs were not administered to most subjects. Whereas this may limit extrapolation to current practice, it presents a valuable opportunity to observe biology unconfounded by the pleiotropic effects of steroids. Further, while proximity extension assays increase assay sensitivity and enable detection of plasma proteins that cannot be detected by other methods, NPX units cannot be translated to ELISA-based readouts, which precluded applying validated models for ARDS phenotype identification.27 Multiple comparison adjustments were applied only to biomarker associations with patient outcomes. Finally, as with all biomarker studies, protein levels in plasma variably reflect those in tissue compartments.

CONCLUSION

Alveolar injury markers peak early in severe COVID-19 and decrease among both spontaneously breathing and invasively ventilated patients. Endothelial injury markers increase with delayed kinetics and are significantly associated with evidence of systemic inflammation, renin-angiotensin system activation, extrapulmonary organ injury, and 28-day outcome. In severe COVID-19, alveolar and endothelial injury likely contribute at different times to disease progression.
FIGURE LEGENDS

Figure 1. Longitudinal Clinical Status and Organ Dysfunctions

A) Longitudinal distribution of patients by modified WHO scale. Lines reflect the temporal redistribution or maintenance of patients between WHO scale levels. B) SpO2/FiO2 ratio for each patient at Day 0, 3, and 7 by level of respiratory support. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles; dots, individual patients. C-F) Severity of hypoxemia, presence of circulatory dysfunction, degree of d-dimer elevation, and presence of Stage-2 or higher acute kidney injury, respectively. Odds ratios display the change in odds of most severe dysfunction per day in a simple mixed effects logistic model with subject as a random effect and day as a fixed effect. Plotted as in panel A. WHO = World Health Organization; IMV = invasive mechanical ventilation; MAP = mean arterial pressure; KDIGO = Kidney Disease: Improving Global Outcomes.

Figure 2. SpO2/FiO2 and Ventilatory Ratios Over Time Among Patients Who Were Invasively Ventilated at Day 0

Grey dotted lines show individual patients' trajectories. Red dots are individual patient data points. Δ95% indicates the 95% confidence interval for average slope between the two corresponding time points (Day 0, Day 3, or Day 7). Blue line and shaded area indicate mean slope between time points and 95% confidence interval for the mean, respectively. The dotted horizontal line on the ventilatory ratio graph demarcates ventilatory ratios > 2.0, which are considered “high”.
Figure 3. Differences in Alveolar, Endothelial, Club Cell, and Cardiovascular Injury Markers Among Intubated vs. Non-Intubated COVID-19 Patients at Day 0, 3, and 7.

Multivariable estimates expressed as fold-difference in biomarker levels between patients who were invasively ventilated or who died (n=74) vs. patients who were alive and ventilating spontaneously (n=151), by study day. The y-axis shows the fold-difference in biomarker level and the x-axis the p-value. Error bars indicate the 95% confidence interval for the fold-difference. The horizontal dotted lines indicate a fold-difference of 1.0 (no difference). The vertical dotted lines indicate p = 0.05.

Figure 4. Plasma Levels of Alveolar Injury, Endothelial Activation, and Endothelial Injury Markers Show Distinct Patterns Over Time

Representative markers of alveolar (A), club cell (B), and endothelial injury and activation (C-D) over the study period by level of respiratory support. p-values indicate the statistical hypothesis tests from the multivariable mixed-effects repeated measures models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles. Dotted lines connect the means at each time point. NPX units are on a log2 scale, i.e., a 1-unit increase corresponds to a doubling in level. NPX = normalized protein expression units.
Figure 5. Plasma Levels of Renin-Angiotensin System Activation, Cardiac Injury, and Renal Injury Markers Over Time Show Patterns Similar to Endothelial Markers

Representative markers of renin-angiotensin system activation (A) and cardiorenal injury and dysfunction (B-C) over the study period by level of respiratory support. p-values indicate the results of the statistical hypothesis tests from the multivariable mixed-effects repeated measures models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box, 25<sup>th</sup> to 75<sup>th</sup> percentiles; whiskers, 5<sup>th</sup> to 95<sup>th</sup> percentiles. Dotted lines connect the means at each time-point. NPX units are on a log2 scale, i.e., a 1-unit increase corresponds to a doubling in level. NPX = normalized protein expression units; ACE-2 = angiotensin converting enzyme-2; NT-proBNP = N-terminal pro-brain natriuretic peptide.

Figure 6. Association of Initial Levels and Changes in Level of Plasma Epithelial and Endothelial Markers with 28-Day Clinical Outcome

Adjusted odds ratios of twelve endothelial and seven pulmonary epithelial markers based on multivariable proportional odds models for modified WHO status at Day 28 for Day 0 levels (A) and changes in marker level from Day 0 to Day 3 (B). Response levels were died (n=37, 16%), invasive mechanical ventilation (n=37, 16%), or off mechanical ventilation (n=151 (67%), 148 (98%) of whom were discharged alive from the hospital at Day 28). The response is coded in ascending order such that a higher odds-ratio indicates worse clinical status at Day 28. All models adjusted for age, sex, body mass index, initial SOFA score, heart failure, chronic kidney disease, and chronic obstructive
pulmonary disease. Error bars, 95% confidence intervals. The right-hand columns display the model estimated p-value (p) and the p-value after a false-discovery rate correction (p_{adj}). ICAM-1 = intercellular adhesion molecule 1; VCAM-1 = vascular cell adhesion protein 1; sTM = soluble thrombomodulin; tPA = tissue-type plasminogen activator; PAI-1 = plasminogen activator inhibitor 1; vWF = von Willebrand factor; ADAMTS13 = a disintegrin and metalloproteinase with thrombospondin motifs 13; SP-A1 = pulmonary surfactant-associated protein A1; SP-A2 = pulmonary surfactant-associated protein A2; SP-D = pulmonary surfactant-associated protein D; LAMP3 = lysosome-associated membrane glycoprotein 3; CC-16 = club cell secretory protein; PnSP1 = pneumocyte secretory protein 1.
**Table 1. Cohort Characteristics at Day 0**

| Variable                                      | All Patients | Supplemental O₂ | Invasive Ventilation |
|------------------------------------------------|--------------|------------------|----------------------|
| N                                             | 225          | 151              | 74                   |
| Age (years)                                   | 61 (18)      | 60 (19)          | 64 (16)              |
| Female – n (%)                                | 103 (46%)    | 74 (49%)         | 29 (39%)             |
| Body Mass Index                               | 31.0 (7.6)   | 31.3 (7.5)       | 30.4 (8.0)           |
| Comorbidities – n (%)                         |              |                  |                      |
| Heart Failure                                 | 27 (12%)     | 20 (13%)         | 7 (9%)               |
| Coronary Disease                              | 21 (9%)      | 17 (11%)         | 4 (5%)               |
| Hypertension                                  | 112 (50%)    | 74 (49%)         | 38 (51%)             |
| COPD without home O₂                          | 16 (7%)      | 14 (9%)          | 2 (3%)               |
| COPD with home O₂                             | 6 (3%)       | 4 (3%)           | 2 (3%)               |
| Current Smoker                                | 63 (28%)     | 43 (19%)         | 20 (27%)             |
| Pre-hospital Baseline Creatinine (mg/dL)      | 1.2 (1.1)    | 1.1 (0.5)        | 1.6 (1.8)            |
| Chronic Kidney Disease without Hemodialysis   | 31 (14%)     | 19 (13%)         | 12 (16%)             |
| End-stage Renal Disease                       | 3 (1%)       | 0 (0%)           | 3 (4%)               |
| Non-insulin Dependent Diabetes                | 56 (25%)     | 29 (19%)         | 27 (36%)             |
| Insulin Dependent Diabetes                    | 16 (7%)      | 12 (8%)          | 4 (5%)               |
| Immunosuppression                             | 20 (9%)      | 10 (7%)          | 10 (14%)             |
| Active Malignancy                             | 12 (5%)      | 7 (5%)           | 5 (7%)               |
| Home ACE-inhibitor                            | 20 (9%)      | 11 (7%)          | 9 (12%)              |
| Presenting Characteristics                    |              |                  |                      |
| Symptom Duration (days)                       | 7 [4-11]     | 7 [4-11]         | 7 [3-10]             |
| Bilateral Radiographic Opacities – n(%)       | 188 (84%)    | 122 (81%)        | 66 (89%)             |
| S/F                                           | 210 (105)    | 264 (87)         | 101 (24)             |
| P/F                                           | -            | (n=0)            | 194 (52) [n=59]      |
| Hypoxemia Severity*                           |              |                  |                      |
| P:F > 300 / S:F > 315                         | 54 (24%)     | 54 (36%)         | 0 (0%)               |
| P:F 200-300 or S:F 235-315                    | 46 (20%)     | 46 (30%)         | 0 (0%)               |
| P:F < 200 or S:F < 235                        | 125 (56%)    | 51 (34%)         | 74(100%)             |
| ARDS on Day 0†                                 | 145 (64%)    | 79 (52%)         | 66 (89%)             |
| Initial SOFA score                            | 4.4 (3.8)    | 2.4 (2.6)        | 8.5 (2.5)            |
| Mean Arterial Pressure (mmHg)                 | 72 (13)      | 77 (11)          | 63 (11)              |
| Lactate (mmol/L)                              | 2.0 (1.3)    | 1.7 (0.8)        | 2.3 (1.6)            |
| High-sensitivity Troponin-T (ng/L)            | 11 [6-31]    | 8 (6-24)         | 17 [7-47]            |
| Creatinine (mg/dL)                            | 1.3 (1.3)    | 1.1 (0.9)        | 1.6 (1.8)            |
| Fold-change in Creatinine from Pre-hospital Baseline | 1.11 (0.7)  | 1.06 (1.06)      | 1.21 (0.87)          |
| Bicarbonate                                   | 23.1 (3.7)   | 23.5 (3.7)       | 22.2 (3.5)           |
| C-Reactive Protein (mg/dL)                    | 137.8 [69.1-189.3] | 104.2 [58.4-168.3] | 149.5 [124.7-225.0] |
| D-Dimer (ng/mL)                               | 1,201 [760-1,985] | 1,082 [710-1,784] | 1,533 [951-2,301]   |
| Absolute Lymphocyte Count                     | 0.94 [0.62-1.27] | 1.01 [0.71-1.36] | 0.78 [0.49-1.06]    |
| Absolute Neutrophil Count                     | 5.70 [4.22-7.96] | 5.27 [3.92-7.54] | 6.57 [4.93-8.23]    |
| Platelet Count                                | 209 [160-281] | 208 [160-280]    | 209 [158-287]       |
| In-Hospital Treatments                         |              |                  |                      |
| Steroids                                      | 23 (10%)     | 14 (9%)          | 9 (12%)              |
| Remdesivir                                    | 6 (3%)       | 4 (3%)           | 2 (3%)               |
| Tocilizumab                                   | 16 (7%)      | 15 (10%)         | 1 (1%)               |
| Inhaled Nitric Oxide                          | 12 (5%)      | 4 (3%)           | 8 (11%)              |

Continuous variables presented as mean (standard deviation) or as median [interquartile range].
* Hypoxemia severity based hierarchically on P:F, or when P:F unavailable, the S:F.
† Indicates ARDS by modified Berlin criteria, i.e., both bilateral radiographic opacities and either P:F < 300 or S:F < 315 felt unlikely to be in the setting of cardiac failure or volume overload. Hypoxemia despite > 5cm H₂O of PEEP could not be assessed among patients who received only supplemental oxygen.

COPD = chronic obstructive pulmonary disease; SOFA = sequential organ failure assessment; S:F = SpO₂/FiO₂ ratio; P:F = PaO₂/FiO₂ ratio.
REFERENCES

1. Matthay MA, Zemans RL, Zimmerman GA, et al. Acute respiratory distress syndrome. *Nat Rev Dis Primers*. 03 2019;5(1):18. doi:10.1038/s41572-019-0069-0

2. Wilson JG, Calfee CS. ARDS Subphenotypes: Understanding a Heterogeneous Syndrome. *Critical Care*. 2020/03/24 2020;24(1):102. doi:10.1186/s13054-020-2778-x

3. Calfee CS, Janz DR, Bernard GR, et al. Distinct molecular phenotypes of direct vs indirect ARDS in single-center and multicenter studies. *Chest*. Jun 2015;147(6):1539-1548. doi:10.1378/chest.14-2454

4. Iba T, Levy JH, Levi M, Connors JM, Thachil J. Coagulopathy of Coronavirus Disease 2019. *Crit Care Med*. May 2020;doi:10.1097/CCM.0000000000004458

5. Reynolds AS, Lee AG, Renz J, et al. Pulmonary Vascular Dilatation Detected by Automated Transcranial Doppler in COVID-19 Pneumonia. *Am J Respir Crit Care Med*. 10 2020;202(7):1037-1039. doi:10.1164/rccm.202006-2219LE

6. Mirsadraee S, Gorog DA, Mahon CF, et al. Prevalence of Thrombotic Complications in ICU-Treated Patients With Coronavirus Disease 2019 Detected With Systematic CT Scanning. *Crit Care Med*. Jan 2021;doi:10.1097/CCM.0000000000004890

7. Wichmann D, Sperhake JP, Lütgehetmann M, et al. Autopsy Findings and Venous Thromboembolism in Patients With COVID-19: A Prospective Cohort Study. *Ann Intern Med*. 08 2020;173(4):268-277. doi:10.7326/M20-2003
8. Ackermann M, Verleden SE, Kuehnel M, et al. Pulmonary Vascular Endothelialitis, Thrombosis, and Angiogenesis in Covid-19. *N Engl J Med*. May 2020;doi:10.1056/NEJMoa2015432

9. DE L, A M, Team MC-CP, N H, MR F, MB G. Trajectories of Pulmonary Epithelial and Endothelial Injury Markers in COVID-19 Patients Requiring Respiratory Support at Presentation. *A13 A013 ARDS IN THE TIME OF COVID-19*. A1061-A1061.

10. Filbin MR, Mehta A, Schneider AM, et al. Longitudinal proteomic analysis of severe COVID-19 reveals survival-associated signatures, tissue-specific cell death, and cell-cell interactions. *Cell Rep Med*. May 18 2021;2(5):100287. doi:10.1016/j.xcrm.2021.100287

11. Sinha P, Calfee CS, Beitler JR, et al. Physiologic Analysis and Clinical Performance of the Ventilatory Ratio in Acute Respiratory Distress Syndrome. *Am J Respir Crit Care Med*. 02 2019;199(3):333-341. doi:10.1164/rccm.201804-0692OC

12. Ware LB, Calfee CS. Biomarkers of ARDS: what's new? *Intensive Care Med*. May 2016;42(5):797-799. doi:10.1007/s00134-015-3973-0

13. Uhlén M, Fagerberg L, Hallström BM, et al. Proteomics. Tissue-based map of the human proteome. *Science*. Jan 2015;347(6220):1260419. doi:10.1126/science.1260419

14. Leisman DE, Ronner L, Pinotti R, et al. Cytokine elevation in severe and critical COVID-19: a rapid systematic review, meta-analysis, and comparison with other inflammatory syndromes. *Lancet Respir Med*. Oct 2020;doi:10.1016/S2213-2600(20)30404-5
15. Sinha P, Calfee CS, Cherian S, et al. Prevalence of phenotypes of acute respiratory distress syndrome in critically ill patients with COVID-19: a prospective observational study. *Lancet Respir Med*. 12 2020;8(12):1209-1218. doi:10.1016/S2213-2600(20)30366-0

16. England JT, Abdulla A, Biggs CM, et al. Weathering the COVID-19 storm: Lessons from hematologic cytokine syndromes. *Blood Reviews*. 2020/05/15/2020:100707. doi:https://doi.org/10.1016/j.blre.2020.100707

17. Arabi YM, Jawdat D, Hajeer AH, et al. Inflammatory Response and Phenotyping in Severe Acute Respiratory Infection From the Middle East Respiratory Syndrome Coronavirus and Other Etiologies. *Crit Care Med*. 02 2021;49(2):228-239. doi:10.1097/CCM.0000000000004724

18. Spadaro S, Fogagnolo A, Campo G, et al. Markers of endothelial and epithelial pulmonary injury in mechanically ventilated COVID-19 ICU patients. *Crit Care*. 02 2021;25(1):74. doi:10.1186/s13054-021-03499-4

19. Bhatraju PK, Morrell ED, Zelnick L, et al. Comparison of host endothelial, epithelial and inflammatory response in ICU patients with and without COVID-19: a prospective observational cohort study. *Crit Care*. 04 2021;25(1):148. doi:10.1186/s13054-021-03547-z

20. Stahl K, Gronski PA, Kiyan Y, et al. Injury to the Endothelial Glycocalyx in Critically Ill Patients with COVID-19. *Am J Respir Crit Care Med*. 10 2020;202(8):1178-1181. doi:10.1164/rccm.202007-2676LE
21. Fraser DD, Patterson EK, Slessarev M, et al. Endothelial Injury and Glycocalyx Degradation in Critically Ill Coronavirus Disease 2019 Patients: Implications for Microvascular Platelet Aggregation. *Crit Care Explor.* Sep 2020;2(9):e0194. doi:10.1097/CCE.0000000000000194

22. Delrue M, Siguret V, Neuwirth M, et al. von Willebrand factor/ADAMTS13 axis and venous thromboembolism in moderate-to-severe COVID-19 patients. *Br J Haematol.* Dec 2020;doi:10.1111/bjh.17216

23. Doevelaar AAN, Bachmann M, Hölzer B, et al. von Willebrand Factor Multimer Formation Contributes to Immunothrombosis in Coronavirus Disease 2019. *Critical Care Medicine.* 2021;Online Firstdoi:10.1097/ccm.0000000000004918

24. Calfee CS, Gallagher D, Abbott J, Thompson BT, Matthay MA, Network NA. Plasma angiopoietin-2 in clinical acute lung injury: prognostic and pathogenetic significance. *Crit Care Med.* Jun 2012;40(6):1731-7. doi:10.1097/CCM.0b013e3182451c87

25. Kitsios GD, Yang L, Manatakis DV, et al. Host-Response Subphenotypes Offer Prognostic Enrichment in Patients With or at Risk for Acute Respiratory Distress Syndrome. *Crit Care Med.* 12 2019;47(12):1724-1734. doi:10.1097/CCM.0000000000004018

26. Calfee CS, Delucchi K, Parsons PE, et al. Subphenotypes in acute respiratory distress syndrome: latent class analysis of data from two randomised controlled trials. *Lancet Respir Med.* Aug 2014;2(8):611-20. doi:10.1016/S2213-2600(14)70097-9
27. Sinha P, Delucchi KL, McAuley DF, O’Kane CM, Matthay MA, Calfee CS. Development and validation of parsimonious algorithms to classify acute respiratory distress syndrome phenotypes: a secondary analysis of randomised controlled trials. *Lancet Respir Med*. Mar 2020;8(3):247-257. doi:10.1016/S2213-2600(19)30369-8

28. Calfee CS, Delucchi KL, Sinha P, et al. Acute respiratory distress syndrome subphenotypes and differential response to simvastatin: secondary analysis of a randomised controlled trial. *Lancet Respir Med*. 09 2018;6(9):691-698. doi:10.1016/S2213-2600(18)30177-2

29. Short SAP, Gupta S, Brenner SK, et al. D-dimer and Death in Critically Ill Patients With Coronavirus Disease 2019. *Critical Care Medicine*. 2021;Online Firstdoi:10.1097/ccm.0000000000004917

30. Li Y, Schneider AM, Mehta A, et al. SARS-CoV-2 Viremia is Associated with Distinct Proteomic Pathways and Predicts COVID-19 Outcomes. *medRxiv*. Feb 2021;doi:10.1101/2021.02.24.21252357

31. Caravita S, Baratto C, Di Marco F, et al. Haemodynamic characteristics of COVID-19 patients with acute respiratory distress syndrome requiring mechanical ventilation. An invasive assessment using right heart catheterization. *Eur J Heart Fail.* 12 2020;22(12):2228-2237. doi:10.1002/ejhf.2058

32. Lawler PR, Goligher EC, Berger JS, et al. Therapeutic Anticoagulation with Heparin in Noncritically Ill Patients with Covid-19. *N Engl J Med*. Aug 26 2021;385(9):790-802. doi:10.1056/NEJMoa2105911
33. Goligher EC, Bradbury CA, McVerry BJ, et al. Therapeutic Anticoagulation with Heparin in Critically Ill Patients with Covid-19. *N Engl J Med.* Aug 26 2021;385(9):777-789. doi:10.1056/NEJMoa2103417

34. Ranieri VM, Rubenfeld GD, Thompson BT, et al. Acute respiratory distress syndrome: the Berlin Definition. *JAMA.* Jun 2012;307(23):2526-33. doi:10.1001/jama.2012.5669

35. Fiege JK, Thiede JM, Nanda HA, et al. Single cell resolution of SARS-CoV-2 tropism, antiviral responses, and susceptibility to therapies in primary human airway epithelium. *PLoS Pathog.* Jan 2021;17(1):e1009292. doi:10.1371/journal.ppat.1009292

36. Kropski JA, Fremont RD, Calfee CS, Ware LB. Clara cell protein (CC16), a marker of lung epithelial injury, is decreased in plasma and pulmonary edema fluid from patients with acute lung injury. *Chest.* Jun 2009;135(6):1440-1447. doi:10.1378/chest.08-2465

37. Ware LB, Koyama T, Zhao Z, et al. Biomarkers of lung epithelial injury and inflammation distinguish severe sepsis patients with acute respiratory distress syndrome. *Crit Care.* Oct 2013;17(5):R253. doi:10.1186/cc13080

38. Cohen JB, Hanff TC, William P, et al. Continuation versus discontinuation of renin–angiotensin system inhibitors in patients admitted to hospital with COVID-19: a prospective, randomised, open-label trial. *The Lancet Respiratory Medicine.* doi:10.1016/S2213-2600(20)30558-0
39. Bellomo R, Forni LG, Busse LW, et al. Renin and Survival in Patients Given Angiotensin II for Catecholamine-Resistant Vasodilatory Shock. A Clinical Trial. *Am J Respir Crit Care Med*. 11 2020;202(9):1253-1261. doi:10.1164/rccm.201911-2172OC

40. Patel SK, Juno JA, Lee WS, et al. Plasma ACE2 activity is persistently elevated following SARS-CoV-2 infection: implications for COVID-19 pathogenesis and consequences. *medRxiv*. 2020:2020.10.06.20207514. doi:10.1101/2020.10.06.20207514

41. Kragstrup TW, Singh HS, Grundberg I, et al. Plasma ACE2 predicts outcome of COVID-19 in hospitalized patients. *PLoS One*. 2021;16(6):e0252799. doi:10.1371/journal.pone.0252799

42. Ozkan S, Cakmak F, Konukoglu D, et al. Efficacy of Serum Angiotensin II Levels in Prognosis of Patients With Coronavirus Disease 2019. *Crit Care Med*. 06 2021;49(6):e613-e623. doi:10.1097/CCM.0000000000004967

43. du Cheyron D, Lesage A, Daubin C, Ramakers M, Charbonneau P. Hyperreninemic hypoaldosteronism: a possible etiological factor of septic shock-induced acute renal failure. journal article. *Intensive Care Medicine*. October 01 2003;29(10):1703-1709. doi:10.1007/s00134-003-1986-6

44. Findling JW, Waters VO, Raff H. The dissociation of renin and aldosterone during critical illness. *J Clin Endocrinol Metab*. Mar 1987;64(3):592-5. doi:10.1210/jcem-64-3-592
45. Tumlin JA, Murugan R, Deane AM, et al. Outcomes in Patients with Vasodilatory Shock and Renal Replacement Therapy Treated with Intravenous Angiotensin II. *Crit Care Med.* 06 2018;46(6):949-957. doi:10.1097/CCM.0000000000003092

46. Leisman DE, Fernandes TD, Bijol V, et al. Impaired angiotensin II type 1 receptor signaling contributes to sepsis induced acute kidney injury. *Kidney Int.* Aug 2020;doi:10.1016/j.kint.2020.07.047

47. Leisman DE, Mastroianni F, Fisler G, et al. Physiologic Response to Angiotensin II Treatment for Coronavirus Disease 2019-Induced Vasodilatory Shock: A Retrospective Matched Cohort Study. *Crit Care Explor.* Oct 2020;2(10):e0230. doi:10.1097/CCE.0000000000000230

48. Küllmar M, Saadat-Gilani K, Weiss R, et al. Kinetic Changes of Plasma Renin Levels Predict Acute Kidney Injury in Cardiac Surgery Patients. *Am J Respir Crit Care Med.* Dec 2020;doi:10.1164/rccm.202005-2050OC

49. Gleeson PJ, Crippa IA, Mongkolpun W, et al. Renin as a Marker of Tissue-Perfusion and Prognosis in Critically Ill Patients. *Crit Care Med.* Feb 2019;47(2):152-158. doi:10.1097/CCM.0000000000003544
Figure 1. Longitudinal Clinical Status and Organ Dysfunctions

A) Longitudinal distribution of patients by modified WHO scale. Lines reflect the temporal redistribution or maintenance of patients between WHO scale levels. B) SpO2/FiO2 ratio for each patient at Day 0, 3, and 7 by level of respiratory support. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles; dots, individual patients. C-F) Severity of hypoxemia, presence of circulatory dysfunction, degree of d-dimer elevation, and presence of Stage-2 or higher acute kidney injury, respectively. Odds ratios display the change in odds of most severe dysfunction per day in a simple mixed effects logistic model with subject as a random effect and day as a fixed effect. Plotted as in panel A. WHO = World Health Organization; IMV = invasive mechanical ventilation; MAP = mean arterial pressure; KDIGO = Kidney Disease: Improving Global Outcomes.

OR=0.80 (95%CI: 0.73-0.87)

OR=1.20 (95%CI: 1.12-1.28)

OR=1.28 (95%CI: 1.15-1.42)

OR=0.90 (95%CI: 0.79-1.03)
Figure 2. SpO2/FiO2 and Ventilatory Ratios Over Time Among Patients Who Were Invasively Ventilated at Day 0
Grey dotted lines show individual patients’ trajectories. Red dots are individual patient data points. \( \Delta95\% \) indicates the 95% confidence interval for average slope between the two corresponding time points (Day 0, Day 3, or Day 7). Blue line and shaded area indicate mean slope between time points and 95% confidence interval for the mean, respectively. The dotted horizontal line on the ventilatory ratio graph demarcates ventilatory ratios > 2.0, which are considered “high”.

152x107mm (300 x 300 DPI)
Figure 3. Differences in Alveolar, Endothelial, Club Cell, and Cardiovascular Injury Markers Among Intubated vs. Non-Intubated COVID-19 Patients at Day 0, 3, and 7.

Multivariable estimates expressed as fold-difference in biomarker levels between patients who were invasively ventilated or who died (n=74) vs. patients who were alive and ventilating spontaneously (n=151), by study day. The y-axis shows the fold-difference in biomarker level and the x-axis the p-value. Error bars indicate the 95% confidence interval for the fold-difference. The horizontal dotted lines indicate a fold-difference of 1.0 (no difference). The vertical dotted lines indicate p = 0.05.

178x93mm (300 x 300 DPI)
Figure 4. Plasma Levels of Alveolar Injury, Endothelial Activation, and Endothelial Injury Markers Show Distinct Patterns Over Time

Representative markers of alveolar (A), club cell (B), and endothelial injury and activation (C-D) over the study period by level of respiratory support. p-values indicate the statistical hypothesis tests from the multivariable mixed-effects repeated measures models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles. Dotted lines connect the means at each time point. NPX units are on a log2 scale, i.e., a 1-unit increase corresponds to a doubling in level. NPX = normalized protein expression units.

178x216mm (300 x 300 DPI)
Figure 5. Plasma Levels of Renin-Angiotensin System Activation, Cardiac Injury, and Renal Injury Markers Over Time Show Patterns Similar to Endothelial Markers

Representative markers of renin-angiotensin system activation (A) and cardiorenal injury and dysfunction (B-C) over the study period by level of respiratory support. p-values indicate the results of the statistical hypothesis tests from the multivariable mixed-effects repeated measures models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles. Dotted lines connect the means at each time-point. NPX units are on a log2 scale, i.e., a 1-unit increase corresponds to a doubling in level. NPX = normalized protein expression units; ACE-2 = angiotensin converting enzyme-2; NT-proBNP = N-terminal pro-brain natriuretic peptide.

178x264mm (300 x 300 DPI)
Figure 6. Association of Initial Levels and Changes in Level of Plasma Epithelial and Endothelial Markers with 28-Day Clinical Outcome

Adjusted odds ratios of twelve endothelial and seven pulmonary epithelial markers based on multivariable proportional odds models for modified WHO status at Day 28 for Day 0 levels (A) and changes in marker level from Day 0 to Day 3 (B). Response levels were died (n=37, 16%), invasive mechanical ventilation (n=37, 16%), or off mechanical ventilation (n=151 (67%), 148 (98%) of whom were discharged alive from the hospital at Day 28). The response is coded in ascending order such that a higher odds-ratio indicates worse clinical status at Day 28. All models adjusted for age, sex, body mass index, initial SOFA score, heart failure, chronic kidney disease, and chronic obstructive pulmonary disease. Error bars, 95% confidence intervals. The right-hand columns display the model estimated p-value (p) and the p-value after a false-discovery rate correction (padj). ICAM-1 = intercellular adhesion molecule 1; VCAM-1 = vascular cell adhesion protein 1; sTM = soluble thrombomodulin; tPA = tissue-type plasminogen activator; PAI-1 = plasminogen activator inhibitor 1; vWF = von Willebrand factor; ADAMTS13 = a disintegrin and metalloproteinase with thrombospondin motifs 13; SP-A1 = pulmonary surfactant-associated protein A1; SP-A2 = pulmonary surfactant-associated protein A2; SP-D = pulmonary surfactant-associated protein D; LAMP3 = lysosome-associated membrane glycoprotein 3; CC-16 = club cell secretory protein; PnSP1 = pneumocyte secretory protein 1.

178x92mm (300 x 300 DPI)
Alveolar, Endothelial, and Organ Injury Marker Dynamics in Severe COVID-19

Daniel E. Leisman; Arnav Mehta; B. Taylor Thompson; Nicole C. Charland; Anna L.K. Gonye; Irena Gushterova; Kyle R. Kays; Hargun K. Khanna; Thomas J. LaSalle; Kendall M. Lavin-Parsons; Brendan M. Lilley; Carl L. Lodenstein; Kasidet Manakongtreecheep; Justin D. Margolin; Brenna N. McKaig; Maricarmen Rojas-Lopez; Brian C. Russo; Nihaarika Sharma; Jessica Tantivit; Molly F. Thomas; Blair Alden Parry; Alexandra-Chloé Villani; Moshe Sade-Feldman; Nir Hacohen; Michael R. Filbin; and Marcia B. Goldberg.

ONLINE DATA SUPPLEMENT
SUPPLEMENTAL METHODS

Data Collection and Management – Detailed Methods

Ethics Statement

Samples were collected at the peak of the initial surge of the COVID-19 pandemic. The study was approved by the Mass General Brigham (previously Partners) Healthcare System Institutional Review Board (IRB) (Protocol Number: 2017P001681). A waiver of informed consent was granted by the institutional IRB in the context and because the study was deemed no more than minimal risk as dedicated research blood samples were collected only at the time of clinically indicated blood draws.

Clinical Organ Dysfunction Variables

Organ dysfunction criteria are described in the supplement. Briefly, we categorized patients by respiratory support level based on their WHO scale. Non-pulmonary organ dysfunctions of interest included acute kidney injury (AKI) and circulatory dysfunction. AKI was operationalized as renal impairment \( \geq \text{Stage-2} \) by Kidney Disease-Improving Global Outcomes (KDIGO) creatinine criteria.\(^1\) When pre-hospital baseline creatinine was unavailable, it was calculated using Modified Diet in Renal Disease equations as recommended by KDIGO guidelines.\(^1\) Because many patients required vasopressors solely due to sedation, we felt typical criteria for circulatory dysfunction were too sensitive. Therefore, we used conservative criteria for circulatory dysfunction: mean arterial pressure \( \leq 60\text{mmHg} \) or lactate \( \geq 2.1\text{mmol/L} \). Among intubated patients we calculated ventilatory ratios as an estimate of dead space ventilation. Ventilatory ratio was calculated using the formula derived and validated by Sinha et al.\(^2\) That is,
Ventilatory Ratio = \((\text{Minute Ventilation} \times \text{PaCO2}) / (\text{Predicted Body Weight} \times 100 \times 37.5)\)

Ventilatory ratios \(\geq 2.0\) were considered “high”.\(^2\)

**Biomarker Assays**

The collection and processing procedures in this study have been previously described. For convenience, they are outlined below.

**Plasma collection and processing**

Blood samples were collected in EDTA tubes and processed no more than 3 hours post blood draw in a Biosafety Level 2+ laboratory on site. Whole blood was diluted with room temperature RPMI medium in a 1:2 ratio to facilitate cell separation for other analyses using the SepMate PBMC isolation tubes (STEMCELL) containing 16 mL Ficoll (GE Healthcare). Diluted whole blood was centrifuged at 1200 g for 20 minutes at 20 °C. After centrifugation, plasma (5 mL) was pipetted into 15 mL conical tubes and placed on ice during PBMC separation procedures, centrifuged at 1000 g for 5 min at 4°C, aliquoted into cryovials, and stored at −80°C. Study samples (45 μL) were randomly allocated onto 96-well plates based on disease outcome grouping and were treated with 1% Triton X-100 for virus inactivation at room temperature for 2 hrs.
**Olink plasma proteomic assays**

The Olink Proximity Extension Assay (PEA) is a technology developed for high-multiplex analysis of proteins using 1 μL of sample. In PEA, oligonucleotide-labelled monoclonal or polyclonal antibodies (PEA probes) are used to bind target proteins in a pair-wise manner thereby preventing all cross-reactive events. Upon binding, the oligonucleotides come in close proximity and hybridize followed by extension generating a unique sequence used for digital identification of the specific protein assay. With recent developments, PEA enables an increased number of 384 multiplex assays and higher throughput using next-generation sequencing (NGS) as a readout method. PEA probe design is based on addition of Illumina adapter sequences, unique barcodes for protein identification and indexes to distinguish samples in multiplex sequencing. The protocol has also been miniaturized and automated using liquid handlers to further improve robustness and maximize output.

The full library (Olink® Explore 1536) consists of 1472 proteins and 48 controls assays divided into four 384-plex panels focused on inflammation, oncology, cardiometabolic and neurology proteins. In each of the four 384-plex panels, overlapping assays of IL-6, IL-8 (CXCL8), and TNF are included for quality control (QC) purposes. Library content is based on target selection of low-abundant inflammation proteins, actively secreted proteins, organ-specific proteins leaked into circulation, drug targets (established and from ongoing clinical trials), and proteins detected in blood by mass spectrometry. Selection, classification, and categorization of proteins were based on using various databases (e.g. Gene Ontology), the Blood Atlas – the human secretome (www.proteinatlas.org), a collaboration with the Institute of Systems Biology, Seattle WA, for tissue-specific proteins, www.clinicaltrials.gov for mapping of drug targets,
detection of proteins in blood measured by mass spectrometry and finally, various text-mining approaches identifying protein biomarkers described in the literature. The analytical performance of PEA is carefully validated for each protein assay; performance data are available at www.olink.com. Technical criteria include assessing sensitivity, dynamic range, specificity, precision, scalability, endogenous interference, and detectability in healthy and pathological plasma and serum samples.

In the immune reaction, 2.8 μL of sample is mixed with PEA probes and incubated overnight at 4 °C. Then, a combined extension and pre-amplification mix is added to the incubated samples at room temperature for PCR. The PCR products are pooled before a second PCR step following addition of individual sample index sequences. All samples are thereafter pooled, followed by bead purification and QC of the generated libraries on a Bioanalyzer. Finally, sequencing is performed on a NovaSeq 6000 system using two S1 flow cells with 2 × 50 base read lengths. Counts of known sequences are thereafter translated into normalized protein expression (NPX) units through a QC and normalization process developed and provided by Olink.

**Quality control for Olink plasma proteomics**

The Olink PEA QC process consists of specifically engineered controls to monitor the performance of the main steps of the assays (immunoreaction, extension and amplification/detection) as well as the individual samples. Internal controls are spiked into each sample and represent a control using a non-human assay, an extension control composed of an antibody coupled to a unique DNA-pair always in proximity and, finally, a detection control based on a double stranded DNA amplicon. In addition, each plate run with Olink includes a control strip with sample controls used to estimate precision (intra- and inter-coefficient of variation). A negative control (buffer) run in
triplicate is utilized to set background levels and calculate limit of detection (LOD), a plate control (plasma pool) is run in triplicate to adjust levels between plates, and a sample control (reference plasma) is included in duplicate to estimate CV between runs.

NPX is Olink’s relative protein quantification unit on a log2 scale and values are calculated from the number of matched counts on the NovaSeq run. Data generation of NPX consists of normalization to the extension control (known standard), log2-transformation, and level adjustment using the plate control (plasma sample).

**Statistical Analysis – Detailed Methods**

**Missing Data**

All patients had blood collected and clinical data recorded at the day of enrollment (Day 0). Tables E1 and E2 show the completeness of the data for the overall cohort, and among the patients who were intubated at Day 0, respectively. Clinical variables with < 10% missingness at Day 0 were imputed by maximum likelihood estimation multiple imputation (PROC MI in SAS). Variables with >10% missingness were not used in analysis. No patients were enrolled who did not have the Day 0 blood draw for biomarker measurement obtained. To reduce the effect of survival bias – i.e., for a subject to have data available they must have been both sick enough to remain in the hospital and healthy enough to survive until the time of measurement – we addressed missing data on Day 3 and 7 in two ways. For analysis of biomarkers over time, a mixed effects modeling approach was used as described below. For the analysis of biomarker associations with Day 28 clinical status (detailed below), missing values at Day 3 were imputed as above. Complete case analyses is reported as a sensitivity analysis for key results.
Biomarker data (measured on the O-link platform) were most often missing because a patient had either died, or had been discharged from the hospital. This attrition was minimal (8% of the total cohort) at Day 3, but was significant (35% of the total cohort) at Day 7. Because of the potential for survival bias with this pattern, we chose to focus our analysis on the early period (Day 0 and 3) where these missing data issues were considerably less prominent.

**Respiratory Status Variables**

Respiratory status is represented in three contexts. For the purposes of data-collection, respiratory status was recorded on Day 0, 3, 7, and 28 based on the World Health Organization (WHO) scale. This was a categorical variable with the following levels:

1 = Dead

2 = Invasive Mechanical Ventilation

3 = Noninvasive Mechanical Ventilation

4 = Supplemental Oxygen without Mechanical Ventilation

5 = Hospitalized but Not Receiving Supplemental Oxygen

6 = Alive and Discharged From the Hospital

No patients in this study were at level 3 because during the study period, it was against institutional policy to administer noninvasive mechanical ventilation due to aerosolization concerns.

This variable was used in different ways depending on the analysis at hand.
When assessing biomarker levels over the study period, we performed each analysis using three approaches to variable specification, as outlined below. The rationale for these differing analyses is described in the statistical methods section of the supplement.

**Biomarkers Over Time – Analysis Set 1:**

In the first set of models, respiratory status was categorized as whether the patient, on that study day was either:

1. Dead or invasively ventilated (WHO scale 1-2)
2. Alive and free from invasive mechanical ventilation (WHO scale ≥4)

**Biomarkers Over Time – Analysis Set 2:**

In the second set of models, respiratory status was categorized as whether the patient, on that study day was either:

1. Dead or invasively ventilated (WHO scale ≤2)
2. Receiving supplemental oxygen (WHO scale = 4)
3. Alive and free from respiratory support (WHO scale ≥5)

**Biomarkers Over Time – Analysis Set 3:**

In the third set of models, respiratory status was categorized as whether the patient, on the Day of hospital presentation (i.e., study enrollment) was:

1. Invasively ventilated (WHO scale = 2)
2. Receiving supplemental oxygen (WHO scale = 4)

Note there are only two WHO scale levels for this analysis as all subjects included in this study were either on supplemental oxygen or intubated on Day 0.

Clinical Status at Day 28:

At Day 28, all but n=3 patients (98%) had one of three statuses: death, alive and receiving invasive mechanical ventilation, or alive and discharged from the hospital. Two patients were alive and receiving supplemental oxygen and 1 was alive and hospitalized without respiratory support. Notably, all 3 of these patients survived to discharge. Therefore, to facilitate analysis of 28-day clinical status, (i.e., the patient outcome), we included the n=3 patients who remained hospitalized at Day 28 with the discharged patients and used the following levels:

1. Dead (WHO scale =1)
2. Alive and Requiring Invasive Mechanical Ventilation (WHO Scale =2)
3. Alive and Not Requiring Invasive Respiratory Support (WHO scale ≥4)

Plasma Biomarker Levels Over the First 7 Study Days by Respiratory Support Level

We constructed mixed-effects repeated measures generalized linear models to describe how severity of respiratory illness influenced each marker over the 7 from admission. To account for within-subject correlation, models included a random-effect for subject and treated day as a repeated measure. Models included age as a covariate, and fixed-effect binary variables for sex, and chronic heart, lung, and kidney disease. Class
variables for day, respiratory support level, and interaction-terms for the two were included. To account for diverging within-class trajectories, subjects were permitted to transition between class levels – for example, if they were receiving supplemental oxygen at Day 0 but invasive ventilation by Day 3.

Each analysis was performed with three different ways of specifying the respiratory support variable.

**Analysis Set 1:**

In the first set of models, respiratory support level was a binary variable: intubated or deceased vs. alive and free from intubation. This approach was used to maximize interpretability.

**Analysis Set 2:**

In the second set of models, respiratory support was categorical variable with three levels: 1) receiving invasive ventilation or deceased, 2) supplemental oxygen only, or 3) alive with no respiratory support. This specification allowed distinction between patients who were recovering from their initial hypoxemia and those with persistent hypoxemia but the overall interaction coefficients and p-values are less intuitively interpretable.

**Analysis Set 3:**

Because of the inclusion criteria, no patients were “off respiratory support” at Day 0. To further ensure this was not distorting results of analysis set 2, we built models as above, but instead entered respiratory support level as a binary fixed-effect based on the patient’s respiratory status at Day 0. This variable therefore had only two levels: 1) intubated at Day 0 or 2) on supplemental oxygen at Day 0.
Plasma Biomarker Levels’ Gross Discrimination for Respiratory Support Level in Early Disease Course

We calculated C-statistics (i.e., the area under a receiver operating characteristic curve) to measure biomarkers’ gross discrimination for invasive ventilation or death Day 0 and Day 3.

C-statistics (i.e., the area under a receiver operating characteristic curve) were calculated to measure marker discrimination for invasive ventilation or death at Day 0 and Day 3. As sensitivity analyses, models were also constructed with group membership fixed by respiratory support at Day 0.

Correlations Between Biomarkers

To determine the correlations between various biomarkers, we computed age-adjusted partial correlation coefficients for levels at Day 0, Day 3, and for the change from Day 0 to Day 3. We purposely did not adjust for severity of illness factors, as many of these markers are well established predictors of illness severity in critically ill patients, and hypothesized to be on the same causal pathway (i.e., either as mediators or colliders). Including these adjustments would likely have inappropriately penalized the correlation between markers, particularly given that were also interested in their correlations with markers of specific organ dysfunctions. However, many biomarkers of interest are well known to increase with age in the absence of clinically significant acute disease or increased severity of illness. Therefore, we considered the effect of normal aging to be a confounder, (age is correlated with both normal biomarker levels and acute disease severity) and adjusted for it accordingly.

Time of Biomarker Peak
We report the proportion of patients who had their “peak” biomarker measurement on each of the three study days for representative biomarkers. This analysis groups patients based on whether they were intubated vs. receiving supplemental oxygen on Day 0. We report frequencies rather than probability densities or more serialized time approaches because the latter would imply a fully continuous time variable, which would be misleading given that blood was only collected on Day 0, 3, and 7.

**Biomarker Associations with Ventilatory Ratios**

In similar fashion to the biomarker models above, mixed-effects repeated measures models were constructed to determine whether alveolar or endothelial injury biomarkers were more strongly associated with the ventilatory ratio (as a surrogate for dead space ventilation). Because ventilatory ratios could only be calculated among patients who were intubated, we limited this analysis to patient who were intubated on Day 0.

**Non-COVID Controls**

Of the 274 patients who had a requirement for supplemental oxygen or invasive mechanical ventilation on Day 0, there were 49 who were ultimately found to be COVID-19 negative by PCR test. These patients were not followed longitudinal for proteomic analysis. However, to explore differences in baseline levels of plasma proteins, these patients were compared to the COVID-19 patients using multivariable linear regression, stratified by invasive ventilation status and adjusted for age and baseline SOFA score.

**Clinical Trajectory Groups**
As an exploratory analysis, we also plotted the levels of representative biomarkers overtime for patients categorized into one of five groups based on their early clinical trajectories. These groups were:

1. Intubated on Day 0 and remained Intubated or died by Day 7
2. Intubated on Day 0 and was extubated and alive by Day 7
3. Supplemental O2 on Day 0 and intubated or died by Day 7
4. Supplemental O2 on Day 0 and remained alive and on supplemental oxygen without invasive ventilation by Day 7.
5. Supplemental O2 on Day 0 no longer on respiratory support and alive by Day 7.

More detailed modeling was not undertaken for these analyses due to sample size limitations.

**Day 28 Patient Outcomes**

We used multivariable proportional-odds regression to determine the association of each marker at Day 0 and at Day 3 with Day 28 clinical status. Clinical status was categorical variable with three levels: 1=died, 2=invasive ventilation, 3=off invasive respiratory support. Models for Day 3 biomarker levels included an adjustment for the Day 0 biomarker level. Covariates were age, sex, chronic heart, lung, and kidney disease, and Day 0 SOFA. The proportional-odds assumption was assessed graphically in each case and found to be reasonable. We report models in ascending format such that a higher odds-ratio indicates higher probability of worse outcome at Day 28.
As a sensitivity measure, we applied a false-discovery rate correction for multiple comparisons to the p-values associated with model estimates, using the method described by Benjamani and Hochberg.\textsuperscript{3}
SUPPLEMENTAL FIGURES

Figure E1. Enrollment and Inclusion Diagram

N=384 Patients Enrolled
(all included in Filbin et al.)

N=110 Patients Excluded
- No respiratory support at Day 0

N=274 on Supplemental O₂ or Positive-Pressure Ventilation at Day 0

N=49 Patients Not COVID-19 Positive
- Included in Day 0 comparison of COVID-19 vs. non-COVID-19 patients
- Excluded from remaining analyses

N=225 Patients Included For Analysis

LEGEND
Diagram of cohort enrollment and inclusion in the present study.
Figure E2. All Pulmonary Epithelial Markers Over Time by Level of Respiratory Support

**A) AT1 Cell Markers**

| Marker          | Status | P_{Day}  | P_{Status} | P_{Interaction} |
|-----------------|--------|----------|------------|-----------------|
| RAGE            |        | <0.0001  | <0.0001    | 0.0006          |

**B) AT2 Cell Markers**

| Marker          | Status | P_{Day}  | P_{Status} | P_{Interaction} |
|-----------------|--------|----------|------------|-----------------|
| Surfactant Protein A1 |        | <0.0001  | <0.0001    | 0.16            |
| Surfactant Protein A2 |        | <0.0001  | 0.027      | 0.0687          |

**C) Club Cell Markers**

| Marker          | Status | P_{Day}  | P_{Status} | P_{Interaction} |
|-----------------|--------|----------|------------|-----------------|
| Club Cell Sectary Protein |        | <0.0001  | <0.0001    | 0.0001          |

**LEGEND**

Markers associated with A) alveolar type-1 cells, B) alveolar type-3 cells, and C) club cells. p-values indicate the results of the statistical hypothesis tests from the multivariable mixed-effects models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles. Dotted lines connect the means at each time-point. NPX units are on a log2 scale. AT1 = alveolar type 1; AT2 = alveolar type 2; RAGE = receptor for advanced glycosylation end-products; LAMP3 = lysosome-associated membrane protein 3; NPX = normalized protein expression units.
Figure E3. Day of Highest Measurement for Representative Biomarkers in Intubated and Non-Intubated Patients

**Alveolar Markers**

| Biomarker | Day | 0% | 20% | 40% | 60% | 80% |
|-----------|-----|----|-----|-----|-----|-----|
| RAGE      |     |    | 70% | 20% | 9%  | 4%  |
| SPA1      |     |    |     | 62% | 16% | 4%  |
| CC16      |     |    |     | 62% | 16% | 4%  |

**Club Cell Markers**

| Biomarker | Day | 0% | 20% | 40% | 60% | 80% |
|-----------|-----|----|-----|-----|-----|-----|
| RAGE      |     |    | 70% | 20% | 9%  | 4%  |
| SPA1      |     |    |     | 62% | 16% | 4%  |
| CC16      |     |    |     | 62% | 16% | 4%  |

**Endothelial Markers**

| Biomarker | Day | 0% | 20% | 40% | 60% | 80% |
|-----------|-----|----|-----|-----|-----|-----|
| ANGPT2    |     |    | 38% | 51% | 54% | 28% |
| ICAM-1    |     |    | 66% | 22% | 23% | 20% |
| sTM       |     |    | 58% | 27% | 23% | 21% |

**Respiratory Status on Day 0**

| Day | Intubated or Died | Supplemental O₂ |
|-----|-------------------|-----------------|
| 0   | 74                | 151             |
| 3   | 68                | 137             |
| 7   | 66                | 81              |

**LEGEND**

The proportion of patients who had their highest biomarker measurement on the indicated study day. Patients are grouped by whether, at Day 0, they were intubated (blue) or receiving supplemental oxygen only (pink). Error bars indicate 95% confidence intervals for the proportion. RAGE = receptor for advanced glycation end-products; SPA1 = surfactant protein A-1; CC16 = club cell secretory protein; ANGPT2 =
angiopoietin-2; ICAM-1 = intracellular adhesion molecule-1; sTM = soluble thrombomodulin.
Figure E4. Sensitivity Analysis for Plasma Levels of Pulmonary Epithelial and Endothelial Markers with Respiratory Group Fixed at Day 0 Status

A) Alveolar Injury Markers
B) Club Cell Markers
C) Endothelial Injury Markers
D) Endothelial Activation Markers

LEGEND

Respiratory Status:  
- Intubated or Died  
- Supplemental O₂
Results of sensitivity analysis where patient respiratory status group is held fixed in mixed-effects models based on their status over Day 0 (modeling “strategy” 3). Representative markers of A) alveolar, B) club cell, and C-D) endothelial injury. The p-values indicate the results of the statistical hypothesis tests from the multivariable mixed-effects models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles. Dotted lines connect the means at each time-point. NPX units are on a log2 scale. RAGE = receptor for advanced glycosylation end-products; ICAM-1 = intercellular adhesion molecule 1; ADAMTS13 = a disintegrin and metalloprotease with thrombospondin motifs 13; NPX = normalized protein expression units.
Figure E5. All Endothelial Markers Over Time by Respiratory Support Level

LEGEND

Markers of endothelial injury and activation by level of respiratory support. p-values indicate the results of the statistical hypothesis tests from the multivariable mixed-effects models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles. Dotted lines connect the means at each time-point. NPX units are on a log2 scale. ICAM-1 = intercellular adhesion molecule 1; VCAM-1 = vascular adhesion molecule 1; ADAMTS13 = a disintegrin and metalloprotease with thrombospondin motifs 13; NPX = normalized protein expression units.
**Endothelial Injury and Activation Markers**

A) Angiopoietin-2

B) Soluble Thrombomodulin

C) ICAM-1

D) VCAM-1

E) Endocan

F) Syndecan-1

G) von Willebrand Factor

H) Plasminogen Activator Inhibitor-1

I) Tissue Plasminogen Activator

J) ADAMTS13

K) Protein C

L) Tissue Factor

**Respiratory Status:**
- 🟢 Intubated or Died
- 🔵 Supplemental O₂
- 🔹 No Respiratory Support
Figure E6. Representative Markers Under Complete-Case Analysis

**LEGEND**
Results of sensitivity analysis using complete case analysis for representative markers of alveolar and endothelial injury. The p-values indicate the results of the statistical hypothesis tests from the multivariable mixed-effects models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box height, 25th to 75th percentiles; whiskers, 5th to 95th percentiles.
Dotted lines connect the means at each time-point. NPX units are on a log2 scale.

*RAGE* = receptor for advanced glycosylation end-products; *NPX* = normalized protein expression units.
Figure E7. Sensitivity Analysis for Representative Markers Excluding Patients Who Received Steroids or Tocilizumab during their Hospitalization

**LEGEND**

Results of sensitivity analysis excluding patients who received steroids or tocilizumab during their hospitalization for representative markers of alveolar and endothelial injury. The p-values indicate the results of the statistical hypothesis tests from the multivariable mixed-effects models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles. Dotted lines connect the means at each time-point.
NPX units are on a log2 scale. RAGE = receptor for advanced glycosylation end-products

NPX = normalized protein expression units.
**Figure E8. Endothelial Injury Markers Are Not Consistently Associated with Ventilatory Ratios Among COVID-19 Patients Intubated on Day 0**

LEGEND
Repeated measure model estimates expressed as fold-difference in biomarker levels on the indicated day per 1-unit change in ventilatory ratio. The x-axis shows the fold-difference in biomarker level and the y-axis shows the p-value. Error bars indicate the 95% confidence interval for the fold-difference. The vertical dotted lines indicate a fold-difference of 1.0 (no difference). The horizontal dotted lines indicate p = 0.05.
Figure E9. Day 0 Alveolar and Endothelial Injury Markers In Patients With COVID-19 vs. Non-COVID-19 Hypoxemia

**LEGEND**
Multivariable linear regression estimates expressed as fold-difference in Day 0 biomarker levels for COVID-19 patients vs. non-COVID-19 controls. At Day 0, n=74 COVID-19 were invasively ventilated and n=151 were received only supplemental oxygen while n=14 non-COVID-19 patients were invasively ventilated and n=35 received only supplemental oxygen. The y-axis shows the fold-difference in biomarker level and the x-axis shows the p-value. Error bars indicate the 95% confidence interval for the fold-difference. The horizontal dotted lines indicate a fold-difference of 1.0 (no difference). The vertical dotted lines indicate p = 0.05.
Figure E10. Cytokines Over Time by Respiratory Support Level

Inflammatory Cytokines

Interleukin-6

sTNF-R1

Tumor Necrosis Factor

Neutrophil Chemokines

Interleukin-8

CXCL1

CXCL6

Clinical Markers

C Reactive Protein

D-Dimer

Absolute Neutrophil Count

Respiratory Status: • Intubated or Died •• Supplemental O₂ ••• No Respiratory Support
**LEGEND**

Representative inflammatory cytokines (A), neutrophil chemokines (B), and clinical inflammatory markers (C) by level of respiratory support over the study period. p-values indicate the results of the statistical hypothesis tests from the multivariable mixed-effects models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles. Dotted lines connect the means at each time-point. NPX units are on a log2 scale, i.e., a 1-unit increase corresponds to a doubling in level. NPX = normalized protein expression units; sTNF-R1 = soluble tumor necrosis factor receptor-1.
Figure E11. Cytokines Over Time by Respiratory Support Level

LEGEND
Cytokines by level of respiratory support over the study period. p-values indicate the results of the statistical hypothesis tests from the multivariable mixed-effects models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles. Dotted lines connect the means at each time-point. NPX units are on a log2 scale. sTNFR-1 = soluble tumor necrosis factor receptor 1; NPX = normalized protein expression units.
Figure E12. Chemokines Over Time by Respiratory Support Level

Neutrophil Chemokines

**Legend**

Neutrophil chemokines by level of respiratory support over the study period. p-values indicate the results of the statistical hypothesis tests from the multivariable mixed-effects models for class differences by time in study (Day), level of respiratory support (Status), and whether respiratory support level is an effect-modifier for time (interaction). Each dot represents an individual patient. Box, 25th to 75th percentiles; whiskers, 5th to 95th percentiles. Dotted lines connect the means at each time-point. NPX units are on a log2 scale. NPX = normalized protein expression units.
Figure E13. Representative Alveolar, Endothelial, and Club Cell Markers in Patients with Different Clinical Trajectories

Alveolar

Endothelial

Club Cell

Resolving O₂ Requirement (n=86)
Persistent O₂ Requirement (n=46)
Early, Persistent Intubation (n=69)
Early Intubation with Early Resolution (n=5)

Delayed Intubation (n=17)
**LEGEND**

Courses of representative biomarkers among patients with five different clinical trajectories: 1) patients on supplemental oxygen on Day 0 who were alive and off oxygen by Day 7 (brown, n=86); 2) patients on supplemental oxygen on Day 0 and who remained alive and on supplemental oxygen by Day 7 without having been intubated (blue, n=47); 3) patients who were on supplemental oxygen on Day 0 and subsequently were intubated or died by Day 7 (red n=18); 4) patients intubated on Day 0 who remained alive and were extubated by Day 7 (pink, n=5); and 5) patients intubated on Day 0 who remained intubated or were deceased by Day 7 (green, n=69). Dots are individual patients. Box and whiskers show interquartile and overall ranges.
### Table E1. Plasma Markers Included in this Study

| Protein                                                                 | Comments                                                                                                                                                                                                 |
|-------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| **Pulmonary Epithelial Injury**                                         |                                                                                                                                                                                                       |
| RAGE                                                                    | • Pro-inflammatory mediator associated with type-I alveolar cell injury
|                                                                         | • Prognostic and potentially causal marker in ARDS.                                                                                                                                                     |
| Surfactant Proteins A1, A2, and D, and LAMP3                           | • Specific to Type-II Alveolar Cells                                                                                                                                                                    |
| CC16 and PnSP1                                                          |                                                                                                                                                                                                       |
| **Endothelial Injury and Activation**                                   |                                                                                                                                                                                                       |
| Angiopoietin-2                                                          | • Endothelial injury and dysfunction marker.
|                                                                         | • Associated with systemic inflammation and capillary leak.                                                                                                                                              |
| Endocan, Syndecan-1                                                     | • Glycocalyx damage markers                                                                                                                                                                             |
| ICAM-1, VCAM-1, Soluble Thrombomodulin                                 | • Endothelial cell injury markers                                                                                                                                                                        |
| Tissue Plasminogen Activator                                          | • Endothelial activation marker
|                                                                         | • Promotes fibrinolysis                                                                                                                                                                                  |
| von-Willebrand Factor                                                   | • Mostly endothelial derived, small fraction from platelets
|                                                                         | • Released during endothelial activation, pro-thrombotic                                                                                                                                                 |
| ADAMTS13                                                                | • Binds von-Willebrand Factor to inhibit platelet aggregation
|                                                                         | • Consumed during endothelial activation                                                                                                                                                                 |
| Plasminogen Activator Inhibitor-1                                      | • Endothelial activation marker
|                                                                         | • Inhibits fibrinolysis
|                                                                         | • Upregulated by angiotensin-II                                                                                                                                                                           |
| Protein C                                                               | • Anticoagulant and anti-inflammatory endothelial protein.
|                                                                         | • High discrimination for ARDS phenotypes.                                                                                                                                                                |
| Tissue Factor                                                           | • Endothelial activation and injury marker, involved in regeneration and fibrosis                                                                                                                      |
| **RAS Activation**                                                      |                                                                                                                                                                                                       |
| Renin                                                                   | • Cleaves angiotensinogen to angiotensin-I.
|                                                                         | • Pro-inflammatory.
|                                                                         | • High levels indicate intrinsic RAS activation – either primary (e.g., hypovolemia) or secondary (e.g., ACE-1 inhibition).                                                                           |
| Renin Receptor                                                          | • Marker of RAS activation.
|                                                                         | • Renin-independent pro-inflammatory effects                                                                                                                                                              |
| ACE-2                                                                   | • Converts angiotensin-II to angiotensin(1-7).
|                                                                         | • Downregulated by angiotensin-II
|                                                                         | • Receptor for SARS-CoV-2 Cell Entry                                                                                                                                                                     |
| **Cardiac and Renal Dysfunction Markers**                              |                                                                                                                                                                                                       |
| Cystatin C                                                              | • Freely filtered plasma protein and (probably) more reliable surrogate for glomerular filtration than creatinine.                                                                                      |
| Kidney Injury Molecule-1                                               | • Highly specific marker of renal proximal tubular cell injury                                                                                                                                          |
| Tropin-I                                                               | • Validated as an acute kidney injury biomarker                                                                                                                                                         |
| Natriuretic Peptide B                                                  | • Well-established specific marker of myocardial injury.
|                                                                         | • Can also be elevated with severe renal impairment                                                                                                                                                     |
| **Inflammatory Cytokines**                                             |                                                                                                                                                                                                       |
| Interleukin-6, sTNF-R1, Tumor Necrosis Factor                          | • Non-specific pro-inflammatory cytokines
|                                                                         | • High discrimination for ARDS phenotypes                                                                                                     |
| Interleukin-8                                                           | • Neutrophil chemokine                                                                                                                                                                                 |
| CXCL1, CXCL3, CXCL5, CXCL6                                             | • High discrimination for ARDS phenotypes                                                                                                     |
| Interleukin-1β, Interferon-γ                                           | • Neutrophil chemokines                                                                                                                                                                                 |
| Interleukin-10                                                          | • Non-specific pro-inflammatory cytokines                                                                                                                                                               |
|                                                                         | • Non-specific anti-inflammatory cytokine                                                                                                                                                                 |

*Measured by proximity extension assay. RAGE = receptor for advanced glycosylation end-products; LAMP3 = lysosome-associated membrane protein 3; CC16 = club cell secretory protein; PnSP1 = pneumocyte secretory protein 1; ADAMTS13 = a disintegrin metalloproteinase with thrombospondin motifs 13; VCAM-1 = vascular cell adhesion molecule 1; ICAM-1 = intercellular adhesion molecule 1; RAS = renin-Angiotensin system; ACE = angiotensin converting enzyme; sTNF-R1 = soluble tumor necrosis factor alpha receptor 1.
| Variable                                                                 | N     | Day 0  | Day 3  | Day 7  |
|-------------------------------------------------------------------------|-------|--------|--------|--------|
| **Enrollment Clinical Variables**                                        | 225   | 225    | 225    |
| Age                                                                     | 223 (99.1%) | --      | --      |
| Sex                                                                     | 225 (100%) | --      | --      |
| Body Mass Index                                                         | 219 (97.3%) | --      | --      |
| Pre-hospital Baseline Creatinine Documented                            | 113 (50.2%) | --      | --      |
| Pre-hospital Baseline Creatinine Documented or MDRD-Equation Estimated  | 223 (99.1%) | --      | --      |
| Symptom Duration                                                        | 225 (100%) | --      | --      |
| Bilateral Radiographic Opacities                                       | 225 (100%) | --      | --      |
| **Serial Clinical Variables**                                           |       |        |        |        |
| Proximity Extension Assay Analytes                                      | 225 (100%) | 188 (83.6%) | 122 (54.2%) |
| S/F                                                                     | 223 (99.1%) | 186 (82.7%) | 121 (53.8%) |
| P/F                                                                     | 59 (26.2%) | 74 (32.9%) | 69 (30.67%) |
| Mean Arterial Pressure                                                 | 223 (99.1%) | 186 (82.7%) | 121 (53.8%) |
| Lactate                                                                 | 174 (77.3%) | 39 (17.3%) | 33 (14.7%) |
| High-sensitivity Troponin-T                                             | 219 (97.3%) | 50 (22.2%) | 44 (19.6%) |
| Creatinine                                                             | 225 (100%) | 188 (83.6%) | 122 (54.2%) |
| Fold-change in Creatinine from Pre-hospital Baseline                   | 223 (99.1%) | 186 (82.7%) | 121 (53.8%) |
| Bicarbonate                                                            | 214 (95.1%) | 0 (0%) | 0 (0%) |
| C-Reactive Protein                                                     | 223 (99.1%) | 188 (83.6%) | 122 (54.2%) |
| D-Dimer                                                                | 219 (97.3%) | 187 (83.1%) | 121 (53.8%) |
| Absolute Lymphocyte Count                                              | 222 (98.7%) | 188 (83.6%) | 122 (54.2%) |
| Absolute Neutrophil Count                                             | 223 (99.1%) | 188 (83.6%) | 122 (54.2%) |
| Platelet Count                                                         | 223 (99.1%) | 188 (83.6%) | 122 (54.2%) |

Indicates the frequency and percentage of each data field that was not missing at the indicated time point.

* All biomarkers measured by Proximity Extension Assay had the same distribution of missingness and are therefore reported as a single row in this table.

S:F – $SpO_2/F_O_2 \text{ Ratio}$; P:F – $P_aO_2/F_O_2 \text{ Ratio}$. 
Table E3. Proportion of Data Available for Analysis Over Time Among Patients Receiving Invasive Mechanical Ventilation at Day 0

| Variable                                                        | Day 0 | Day 3 | Day 7 |
|-----------------------------------------------------------------|-------|-------|-------|
| **N**                                                          | 74    | 74    | 74    |
| **Enrollment Clinical Variables**                               |       |       |       |
| Age                                                             | 74 (100%) | --    | --    |
| Sex                                                             | 74 (100%) | --    | --    |
| Body Mass Index                                                 | 72 (97.3%) | --    | --    |
| Pre-hospital Baseline Creatinine Documented                     | 50 (67.6%) | --    | --    |
| Pre-hospital Baseline Creatinine Documented or                  | 74 (100%) | --    | --    |
| MDRD-Equation Estimated                                          |       |       |       |
| Symptom Duration                                                | 74 (100%) | --    | --    |
| Bilateral Radiographic Opacities                                | 74 (100%) | --    | --    |
| **Serial Clinical Variables**                                   |       |       |       |
| O-link Proximity Extension Assay Analytes*                      | 74 (100%) | 68 (91.9%) | 61 (82.4%) |
| S:F                                                             | 74 (100%) | 68 (91.9%) | 61 (82.4%) |
| P:F                                                             | 59 (79.7%) | 66 (89.2%) | 60 (81.1%) |
| Mean Arterial Pressure                                          | 74 (100%) | 68 (91.9%) | 61 (82.4%) |
| Lactate                                                         | 72 (97.3%) | 29 (39.2%) | 26 (35.1%) |
| High-sensitivity Troponin-T                                     | 72 (97.3%) | 21 (28.4%) | 30 (40.5%) |
| Creatinine                                                      | 74 (100%) | 68 (91.9%) | 61 (82.4%) |
| Fold-change in Creatinine from Pre-hospital Baseline            | 74 (100%) | 68 (91.9%) | 61 (82.4%) |
| Bicarbonate                                                     | 69 (93.2%) | 0 (0%) | 0 (0%) |
| C-Reactive Protein                                              | 73 (98.7%) | 68 (91.9%) | 61 (82.4%) |
| D-Dimer                                                         | 72 (97.3%) | 67 (90.5%) | 61 (82.4%) |
| Absolute Lymphocyte Count                                       | 72 (97.3%) | 68 (91.9%) | 61 (82.4%) |
| Absolute Neutrophil Count                                       | 73 (98.7%) | 68 (91.9%) | 61 (82.4%) |
| Platelet Count                                                  | 73 (98.7%) | 68 (91.9%) | 61 (82.4%) |

Indicates the frequency and percentage of each data field that was available for analysis at the indicated time point.

* All biomarkers measured by Proximity Extension Assay had the same distribution of missingness and are therefore reported as a single row in this table.

S:F – $\text{SpO}_2/F_i\text{O}_2$ Ratio; P:F – $P_a\text{O}_2/F_i\text{O}_2$ Ratio.
| Marker                  | $C_{\text{Day 0}}$ | 95%CI         | $C_{\text{Day 3}}$ | 95%CI         | $\Delta C$ |
|------------------------|---------------------|--------------|---------------------|--------------|------------|
| **Alveolar Injury**    |                     |              |                     |              |            |
| RAGE                   | 0.76                | 0.69-0.83    | 0.72                | 0.65-0.80    | -0.04      |
| Surfactant Protein-A1  | 0.68                | 0.60-0.75    | 0.70                | 0.63-0.77    | 0.02       |
| Surfactant Protein-A2  | 0.66                | 0.58-0.73    | 0.64                | 0.57-0.72    | -0.02      |
| Surfactant Protein-D   | 0.62                | 0.54-0.70    | 0.59                | 0.51-0.67    | -0.03      |
| LAMP3                  | 0.60                | 0.51-0.68    | 0.60                | 0.52-0.68    | 0.00       |
| **Club Cell Markers**  |                     |              |                     |              |            |
| CC16                   | 0.59                | 0.51-0.68    | 0.72                | 0.65-0.79    | 0.13       |
| PnSP-1                 | 0.61                | 0.53-0.69    | 0.69                | 0.62-0.76    | 0.08       |
| **Endothelial Injury** |                     |              |                     |              |            |
| Angiopoietin-2         | 0.58                | 0.50-0.66    | 0.82                | 0.76-0.88    | 0.24       |
| Endocan                | 0.59                | 0.51-0.67    | 0.78                | 0.72-0.84    | 0.19       |
| Syndecan-1             | 0.68                | 0.60-0.75    | 0.84                | 0.78-0.89    | 0.16       |
| ICAM-1                 | 0.55                | 0.47-0.63    | 0.78                | 0.71-0.84    | 0.23       |
| VCAM1                  | 0.57                | 0.49-0.65    | 0.74                | 0.67-0.80    | 0.17       |
| Thrombomodulin         | 0.62                | 0.54-0.70    | 0.76                | 0.69-0.82    | 0.14       |
| **Endothelial Activation** |                 |              |                     |              |            |
| tPA                    | 0.67                | 0.60-0.75    | 0.78                | 0.71-0.85    | 0.11       |
| von-Willebrand Factor  | 0.64                | 0.56-0.72    | 0.68                | 0.61-0.75    | 0.04       |
| ADAMTS13               | 0.57                | 0.49-0.65    | 0.70                | 0.62-0.77    | 0.13       |
| PAI-1                  | 0.68                | 0.61-0.76    | 0.77                | 0.70-0.85    | 0.09       |
| Protein C              | 0.65                | 0.57-0.73    | 0.72                | 0.65-0.79    | 0.07       |
| Tissue Factor          | 0.73                | 0.66-0.81    | 0.73                | 0.66-0.81    | 0.00       |

$C$ = C-statistic (i.e., the area under the receiver operating characteristic curve); RAGE = receptor for advanced glycosylation end-products; LAMP3 = lysosome-associated membrane protein 3; CC16 = club cell secretory protein; PnSP-1 = pneumocyte secretory protein 1; ICAM-1 = intercellular adhesion molecule 1; VCAM-1 = vascular adhesion molecule 1; tPA = tissue plasminogen activator; PAI-1 = plasminogen activator inhibitor 1
### Table E5. Kinetic Correlations for Pulmonary Epithelial, Endothelial, and Renin-Angiotensin Markers with Markers of Cardiac and Renal Injury

| Marker                      | CARDIAC INJURY | RENAL INJURY |
|-----------------------------|----------------|--------------|
|                             | Troponin-I     | BNP          | KIM-1 | Cystatin C | Creatinine |
| Alveolar                    |                |              |       |            |            |
| RAGE                        | 0.09           | 0.11         | 0.02  | 0.17       | 0.15       |
|                             | p=0.22         | p=0.13       | p=0.84| p=0.0232   | p=0.0443   |
| Surfactant Protein-A1       | 0.07           | 0.13         | 0.08  | 0.20       | 0.21       |
|                             | p=0.31         | p=0.0762     | p=0.30| p=0.0052   | p=0.0040   |
| Surfactant Protein-A2       | 0.00           | 0.20         | 0.03  | 0.03       | -0.02      |
|                             | p=0.96         | p=0.0069     | p=0.66| p=0.63     | p=0.77     |
| LAMP3                       | 0.00           | -0.15        | -0.02 | 0.23       | 0.14       |
|                             | p=0.95         | p=0.0407     | p=0.78| p=0.0014   | p=0.0523   |
| Surfactant Protein-D        | -0.01          | 0.16         | 0.17  | -0.02      | -0.19      |
|                             | p=0.85         | p=0.0295     | p=0.0196| p=0.78   | p=0.0110   |
| Club Cell                   |                |              |       |            |            |
| Club Cell Secretory Protein | 0.24           | -0.05        | 0.36  | 0.78       | 0.70       |
|                             | p=0.0012       | p=0.49       | p<0.001| p<0.001   | p<0.001    |
| Pneumocyte Secretory Protein| 0.17           | 0.06         | 0.27  | 0.44       | 0.36       |
|                             | p=0.0204       | p=0.45       | p=0.0002| p<0.001   | p<0.001    |
| Endothelial                 |                |              |       |            |            |
| Angiopoietin-2              | 0.31           | 0.13         | 0.52  | 0.47       | 0.39       |
|                             | p<0.0001       | p=0.0725     | p<0.001| p<0.001   | p<0.001    |
| ICAM-1                      | 0.28           | -0.02        | 0.55  | 0.48       | 0.32       |
|                             | p<0.0001       | p=0.82       | p<0.001| p<0.001   | p<0.001    |
| VCAM-1                      | 0.17           | 0.07         | 0.53  | 0.55       | 0.39       |
|                             | p<0.0001       | p=0.37       | p<0.001| p<0.001   | p=0.0001   |
| Soluble Thrombomodulin      | 0.33           | -0.02        | 0.51  | 0.60       | 0.50       |
|                             | p<0.0001       | p=0.77       | p<0.001| p<0.001   | p<0.001    |
| Endocan                     | 0.06           | -0.05        | 0.44  | 0.21       | 0.15       |
|                             | p=0.45         | p=0.46       | p<0.001| p=0.0014  | p=0.0469   |
| Syndecan-1                  | 0.18           | -0.01        | 0.58  | 0.34       | 0.23       |
|                             | p<0.0001       | p=0.88       | p<0.001| p<0.001   | p=0.0015   |
| Tissue Plasminogen Activator| 0.13           | 0.04         | 0.30  | 0.19       | 0.13       |
|                             | p=0.0798       | p=0.63       | p<0.001| p=0.0083  | p=0.0487   |
| von-Willebrand Factor       | 0.17           | 0.02         | 0.19  | 0.34       | 0.28       |
|                             | p=0.0254       | p=0.82       | p=0.0096| p<0.001  | p<0.001    |
| ADAMTS13                    | -0.05          | -0.05        | -0.25 | 0.12       | -1.00      |
|                             | p=0.46         | p=0.49       | p=0.0006| p=0.10    | p=0.16     |
| Plasminogen Activator       | 0.13           | 0.07         | 0.15  | 0.06       | 0.02       |
|                             | p=0.0789       | p=0.37       | p=0.0416| p=0.40    | p=0.77     |
| Protein C                   | -0.08          | -0.09        | 0.06  | 0.32       | 0.01       |
|                             | p=0.25         | p=0.21       | p=0.42| p<0.001   | p=0.87     |
| Tissue Factor               | 0.16           | 0.09         | 0.30  | 0.34       | 0.15       |
|                             | p=0.0313       | p=0.21       | p<0.001| p<0.001   | p=0.0443   |
| Renin-Angiotensin System    |                |              |       |            |            |
| Renin                       | 0.36           | -0.10        | 0.48  | 0.60       | 0.61       |
|                             | p<0.0001       | p=0.18       | p<0.001| p<0.001   | p<0.001    |
| ACE-2                       | 0.16           | 0.08         | 0.43  | 0.32       | 0.22       |
|                             | p=0.0347       | p=0.31       | p<0.001| p<0.001   | p=0.028    |
| Renin Receptor              | 0.28           | -0.08        | 0.43  | 0.37       | 0.30       |
|                             | p=0.0004       | p=0.26       | p<0.001| p<0.001   | p=0.0003   |

Data are Pearson age-adjusted partial correlation coefficients (R) for changes in biomarker values from Day 0 to Day 3 with changes in markers of cardiac and kidney injury over time. The associated p-value is shown below. Statistically significant correlations are in boldface, although results should be interpreted in context of both strength of correlation (R) and statistical significance (p). BNP = natriuretic peptide B; KIM-1 = kidney injury molecule-1; RAGE = receptor for advanced glycosylation end-products; LAMP3 = lysosome-associated membrane protein 3; VCAM-1 = vascular cell adhesion molecule 1; ICAM-1 = intercellular adhesion molecule 1; ADAMTS13 = a disintegrin metalloproteinase with thrombospondin motifs 13; ACE-2 = angiotensin-converting enzyme 2.
| Marker                      | C<sub>Day 0</sub> ± 95%CI | C<sub>Day 3</sub> ± 95%CI | ΔC   |
|----------------------------|---------------------------|---------------------------|------|
| **RAS Markers**            |                           |                           |      |
| Renin                      | 0.58 ± 0.50-0.67          | 0.81 ± 0.75-0.87          | 0.23 |
| Renin Receptor             | 0.60 ± 0.52-0.68          | 0.72 ± 0.65-0.79          | 0.12 |
| ACE2                       |                           |                           |      |
| **Cardiac Injury Markers** |                           |                           |      |
| Troponin I                 | 0.68 ± 0.61-0.75          | 0.75 ± 0.69-0.82          | 0.07 |
| NT-proBNP                  | 0.62 ± 0.54-0.69          | 0.57 ± 0.49-0.65          | -0.05|
| **Renal Injury Markers**   |                           |                           |      |
| KIM-1                      | 0.59 ± 0.51-0.66          | 0.82 ± 0.76-0.87          | 0.23 |
| Cystatin C                 | 0.52 ± 0.44-0.60          | 0.65 ± 0.57-0.72          | 0.13 |
| Creatinine (Log Fold-Change from Baseline) | 0.53 ± 0.45-0.62 | 0.64 ± 0.55-0.72 | 0.11 |

*C = C-statistic (i.e., the area under the receiver operating characteristic curve); ACE2 = Angiotensin Converting Enzyme-2; NT-proBNP = N-terminal pro-Brain Natriuretic Peptide; KIM-1 = Kidney Injury Molecule-1.*
Table E7. Kinetic Correlations of Inflammatory Markers with Markers of Cardiac and Renal Injury

| Marker                | CARDIAC INJURY | RENAL INJURY |
|-----------------------|----------------|--------------|
|                       | Troponin-I     | BNP          | KIM-1 | Cystatin C | Creatinine |
| **Cytokines**         |                |              |       |            |            |
| sTNF-R1               | 0.36           | -0.03        | 0.51  | 0.75       | 0.61       |
|                       | <.0001         | 0.68         | <.0001| <.0001     | <.0001     |
| Tumor Necrosis Factor | 0.16           | -0.13        | 0.37  | 0.55       | 0.46       |
|                       | 0.0175         | 0.0573       | <.0001| <.0001     | <.0001     |
| Interleukin-6         | 0.18           | -0.03        | 0.24  | 0.21       | 0.24       |
|                       | 0.0166         | 0.66         | 0.0011| 0.0044     | 0.0010     |
| Interleukin-8         | 0.11           | -0.10        | 0.09  | 0.07       | 0.17       |
|                       | 0.15           | 0.16         | 0.21  | 0.34       | 0.0217     |
| Interleukin-1β        | -0.01          | -0.07        | -0.03 | -0.01      | 0.03       |
|                       | 0.90           | 0.34         | 0.61  | 0.92       | 0.65       |
| Interleukin-10        | 0.13           | 0.02         | 0.06  | 0.06       | 0.10       |
|                       | 0.0914         | 0.79         | 0.39  | 0.49       | 0.19       |
| Interferon-γ          | -0.02          | -0.13        | 0.13  | 0.05       | 0.10       |
|                       | 0.82           | 0.0716       | 0.0715| 0.53       | 0.19       |
| **Clinical Markers**  |                |              |       |            |            |
| D-dimer               | 0.10           | -0.05        | 0.12  | 0.19       | 0.24       |
|                       | 0.18           | 0.52         | 0.0941| 0.0114     | p=0.0009    |
| C-Reactive Protein    | 0.07           | 0.14         | 0.33  | -0.03      | 0.08       |
|                       | 0.36           | 0.0640       | <.0001| 0.71       | 0.28       |
| Absolute Neutrophil Count | 0.23          | 0.14         | 0.14  | 0.16       | 0.26       |
|                       | 0.0021         | 0.0515       | 0.0681| 0.0256     | 0.0004     |
| Absolute Lymphocyte Count | 0.13          | -0.05        | 0.02  | 0.15       | 0.11       |
|                       | 0.0774         | 0.49         | 0.82  | 0.0438     | 0.14       |

Data are Pearson age-adjusted partial correlation coefficients (R) for changes in biomarker values from Day 0 to Day 3 with changes in markers of cardiac and kidney injury over time. The associated p-value is shown below. Statistically significant correlations are in bold-face, although results should be interpreted in context of both strength of correlation (R) and statistical significance (p). BNP = natriuretic peptide B; KIM-1 = kidney injury molecule 1; sTNF-R1 = soluble tumor necrosis factor receptor 1.
| Marker                     | C<sub>Day 0</sub> | 95%CI      | C<sub>Day 3</sub> | 95%CI      | ΔC      |
|---------------------------|-------------------|------------|-------------------|------------|---------|
| **Cytokines**             |                   |            |                   |            |         |
| Interleukin-6             | 0.78              | 0.72-0.85  | 0.82              | 0.76-0.88  | 0.04    |
| sTNF-R1                   | 0.66              | 0.58-0.73  | 0.79              | 0.73-0.85  | 0.13    |
| Tumor Necrosis Factor     | 0.55              | 0.47-0.64  | 0.75              | 0.68-0.83  | 0.20    |
| Interleukin-1β            | 0.60              | 0.52-0.67  | 0.59              | 0.51-0.69  | -0.01   |
| Interferon-γ              | 0.56              | 0.48-0.64  | 0.62              | 0.54-0.70  | 0.02    |
| Interleukin-10            | 0.58              | 0.51-0.66  | 0.52              | 0.43-0.60  | -0.01   |
| **Chemokines**            |                   |            |                   |            |         |
| Interleukin-8             | 0.67              | 0.59-0.74  | 0.79              | 0.72-0.86  | 0.12    |
| CXCL1                     | 0.53              | 0.44-0.61  | 0.67              | 0.59-0.75  | 0.14    |
| CXCL6                     | 0.51              | 0.43-0.59  | 0.64              | 0.56-0.72  | 0.15    |
| CXCL3                     | 0.55              | 0.47-0.63  | 0.62              | 0.54-0.71  | 0.06    |
| CXCL5                     | 0.56              | 0.48-0.63  | 0.56              | 0.47-0.64  | 0.0     |
| **Clinical Markers**      |                   |            |                   |            |         |
| Log(C-Reactive Protein)   | 0.67              | 0.59-0.74  | 0.78              | 0.71-0.84  | 0.12    |
| Log(D-Dimer)              | 0.64              | 0.57-0.72  | 0.74              | 0.67-0.80  | 0.10    |
| Log(ANC)                  | 0.63              | 0.55-0.70  | 0.70              | 0.63-0.78  | 0.07    |

C = C-statistic (i.e., the area under the receiver operating characteristic curve); sTNF-R1 = soluble tumor necrosis factor receptor-1; ANC = absolute neutrophil count.
| Marker                      | Day 0 OR (95% CI) | p  | Day 3 Adjusted for Day 0 OR (95% CI) | p    |
|----------------------------|-------------------|----|------------------------------------|------|
| **Alveolar Markers**       |                   |    |                                    |      |
| RAGE                       | 1.21 (0.89 to 1.63) | p=0.22 | 1.57 (1.02 to 2.41) | p=0.0417 |
| Surfactant Protein A1      | 1.01 (0.60 to 1.72) | p=0.96 | 2.80 (1.42 to 5.52) | p=0.0029 |
| Surfactant Protein A2      | 1.15 (0.78 to 1.68) | p=0.49 | 1.41 (0.80 to 2.48) | p=0.24 |
| Surfactant Protein D       | 1.17 (0.84 to 1.63) | p=0.34 | 1.16 (0.75 to 1.79) | p=0.51 |
| LAMP3                      | 1.03 (0.71 to 1.49) | p=0.90 | 1.40 (0.67 to 2.92) | p=0.37 |
| **Club Cell Markers**      |                   |    |                                    |      |
| CC16                       | 0.84 (0.61 to 1.14) | p=0.26 | 1.64 (1.03 to 2.63) | p=0.0383 |
| PnSP-1                     | 1.05 (0.78 to 1.43) | p=0.75 | 1.58 (0.87 to 2.90) | p=0.15 |
| **Endothelial Markers**    |                   |    |                                    |      |
| Angiopoietin-2             | 1.51 (0.95 to 2.41) | p=0.0843 | 2.79 (1.46 to 5.36) | p=0.0020 |
| Endocan                    | 1.23 (0.80 to 1.89) | p=0.36 | 2.45 (1.56 to 3.85) | p<0.0001 |
| Syndecan-1                 | 1.22 (0.85 to 1.76) | p=0.28 | 2.19 (1.42 to 3.37) | p=0.0004 |
| ICAM-1                     | 1.22 (0.59 to 2.52) | p=0.58 | 5.23 (1.88 to 14.56) | p=0.0016 |
| VCAM-1                     | 1.34 (0.71 to 2.56) | p=0.37 | 10.99 (2.98 to 40.55) | p=0.0003 |
| Thrombomodulin             | 1.04 (0.58 to 1.88) | p=0.89 | 6.15 (2.10 to 17.98) | p=0.0009 |
| Tissue Plasminogen Activator | 1.40 (0.87 to 2.25) | p=0.16 | 2.28 (1.39 to 3.74) | p=0.0011 |
| Plasminogen Activator      | 0.78 (0.55 to 1.11) | p=0.17 | 1.45 (0.95 to 2.21) | p=0.0903 |
| von-Willebrand Factor      | 1.22 (0.66 to 2.26) | p=0.53 | 2.82 (1.23 to 6.50) | p=0.0148 |
| ADAMTS13                   | 1.05 (0.39 to 2.85) | p=0.93 | 0.09 (0.01 to 0.55) | p=0.0099 |
| Protein C                  | 0.42 (0.20 to 0.90) | p=0.0245 | 0.24 (0.08 to 0.71) | p=0.0100 |
| Tissue Factor              | 1.41 (0.85 to 2.34) | p=0.18 | 4.19 (1.76 to 9.98) | p=0.0012 |

Data are adjusted ORs from multivariable proportional hazard models for 28-day status. The response levels were deceased, alive on invasive mechanical ventilation, and alive without invasive mechanical ventilation (98% of whom were alive and discharged from the hospital). A higher OR indicates higher odds of worse outcome. All models adjusted for age, sex, initial SOFA score, heart failure, chronic kidney disease, and chronic obstructive pulmonary disease. Models for Day 3 values were adjusted for Day 0 values. RAGE = Receptor for advanced glycosylation end-products; LAMP3 = lysosome associated membrane protein 3; CC16 = club cell secretory protein; PnSP1 = pneumocyte secretory protein 1; ICAM-1 = intercellular adhesion molecule 1; VCAM-1 = vascular cell adhesion molecule 1; ADAMTS13 = a disintegrin metalloproteinase with thrombospondin motifs 13; SOFA = sequential organ failure assessment.
### TABLE E10 – Adjusted Odds Ratios for 28-Day Clinical Status for Renin-Angiotensin System Markers

| Marker            | Day 0 OR   | 95%CI       | p  | Day 3 Adjusted for Day 0 OR | 95%CI       | p   |
|-------------------|------------|-------------|----|-----------------------------|-------------|-----|
| Renin             | 0.91       | 0.65 to 1.28 | p=0.59 | 1.76                       | 1.08 to 2.88 | p=0.0246 |
| ACE-2             | 1.35       | 0.89 to 2.05 | p=0.16 | 1.85                       | 1.19 to 2.88 | p=0.0065 |
| Renin Receptor    | 1.29       | 0.52 to 3.18 | p=0.59 | 5.28                       | 1.64 to 17.04 | p=0.0054 |

Data are adjusted ORs from multivariable proportional hazard models for 28-day status. The response levels were deceased, alive on invasive mechanical ventilation, and alive without invasive mechanical ventilation (98% of whom were alive and discharged from the hospital). A higher OR indicates higher odds of worse outcome. All models adjusted for age, sex, initial SOFA score, chronic ACE inhibitor use, heart failure, chronic kidney disease, and chronic obstructive pulmonary disease. Models for Day 3 values were adjusted for Day 0 values. ACE-2 = angiotensin-converting enzyme 2. SOFA = sequential organ failure assessment.
REFERENCES

1. Kellum JA, Lameire N, Group KAGW. Diagnosis, evaluation, and management of acute kidney injury: a KDIGO summary (Part 1). Crit Care. 2013;17(1):204. doi:10.1186/cc11454

2. Sinha P, Calfee CS, Beitler JR, et al. Physiologic Analysis and Clinical Performance of the Ventilatory Ratio in Acute Respiratory Distress Syndrome. Am J Respir Crit Care Med. 02 2019;199(3):333-341. doi:10.1164/rccm.201804-0692OC

3. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological). 1995;57(1):289-300.

4. Uhlén M, Fagerberg L, Hallström BM, et al. Proteomics. Tissue-based map of the human proteome. Science. Jan 2015;347(6220):1260419. doi:10.1126/science.1260419

5. Jones TK, Feng R, Kerchberger VE, et al. Plasma sRAGE Acts as a Genetically Regulated Causal Intermediate in Sepsis-associated Acute Respiratory Distress Syndrome. Am J Respir Crit Care Med. 01 2020;201(1):47-56. doi:10.1164/rccm.201810-2033OC

6. Jabaudon M, Blondonnet R, Pereira B, et al. Plasma sRAGE is independently associated with increased mortality in ARDS: a meta-analysis of individual patient data. Intensive Care Med. Sep 2018;44(9):1388-1399. doi:10.1007/s00134-018-5327-1

7. Sinha P, Delucchi KL, McAuley DF, O'Kane CM, Matthay MA, Calfee CS. Development and validation of parsimonious algorithms to classify acute respiratory
distress syndrome phenotypes: a secondary analysis of randomised controlled trials.

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