

Biochemical, biophysical, and immunological characterization of respiratory secretions in severe SARS-CoV-2 infections

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Thick, viscous respiratory secretions are a major pathogenic feature of COVID-19, but the composition and physical properties of these secretions are poorly understood. We characterized the composition and rheological properties (i.e., resistance to flow) of respiratory secretions collected from intubated COVID-19 patients. We found the percentages of solids and protein content were greatly elevated in COVID-19 compared with healthy control samples and closely resembled levels seen in cystic fibrosis, a genetic disease known for thick, tenacious respiratory secretions. DNA and hyaluronan (HA) were major components of respiratory secretions in COVID-19 and were likewise abundant in cadaveric lung tissues from these patients. COVID-19 secretions exhibited heterogeneous rheological behaviors, with thicker samples showing increased sensitivity to DNase and hyaluronidase treatment. In histologic sections from these same patients, we observed increased accumulation of HA and the hyaladherin versican but reduced tumor necrosis factor-stimulated gene-6 staining, consistent with the inflammatory nature of these secretions. Finally, we observed diminished type I interferon and enhanced inflammatory cytokines in these secretions. Overall, our studies indicated that increases in HA and DNA in COVID-19 respiratory secretion samples correlated with enhanced inflammatory burden and suggested that DNA and HA may be viable therapeutic targets in COVID-19 infection.

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Introduction

Severe infections of SARS-CoV-2, the virus responsible for the COVID-19 pandemic, can result in acute respiratory distress syndrome (ARDS) (1), a condition marked by viscous respiratory secretions and respiratory distress (2). The compositional and rheological properties of these respiratory secretions impair their mucociliary clearance, resulting in a buildup of fluids in the lungs during ARDS (3). This buildup greatly inhibits oxygen exchange, often necessitating endotracheal intubation and mechanical ventilation (4). Treatments that target these respiratory secretions are desperately needed to improve clinical outcomes for patients with COVID-19 as well as for other patients with severe cases of ARDS. It is therefore important to understand the composition of these secretions to better guide treatment development efforts.

The typical composition of respiratory secretions consists of dilute mucins (5), which are long polymers that can form a network by entangling with one another within the aspirate fluid. Similar to mucin, other biopolymers, such as DNA and hyaluronan (HA), can form entanglements with themselves and with mucin, forming more entanglements with increasing polymer concentration and contributing to a greater modulus (i.e., a greater resistance to flow). Respiratory secretions with higher modulus are expected to be more challenging to clear from the airway and hence hinder oxygen exchange in the lungs (4, 6, 7).

Levels of HA, a linear glycosaminoglycan, are elevated in respiratory secretions in other forms of respiratory inflammation (8–12), including ARDS (13–15). HA is produced at the cell surface by a variety of cell types (16) in response to viral DNA and other factors (17). HA is present in the body at molecular weights ranging from low kilodaltons to megadaltons (16, 18) and is known to have major effects on the viscoelasticity of respiratory secretions and other materials (19, 20). Additionally, HA plays important roles in innate immunity and antigenic responses in the lungs (21–24).

DNA levels are also elevated in some forms of respiratory inflammation (25, 26). This increase likely originates from dead cells, infiltrating neutrophils (27, 28), and potentially microbial contaminants (7, 29). Relatively small increases in DNA concentrations can dramatically change the rheological properties of a solution, a phenomenon that has been leveraged both naturally in the production of bacterial biofilms (30) and synthetically in the development of DNA-based hydrogels (31). In the context of lung infections, extracellular DNA has been suggested to increase viscosity of mucosal fluid and provide colonization opportunities for bacterial infections (25).

We hypothesized that DNA and HA are major contributors to the tenacious behavior of respiratory secretions from patients with COVID-19. To evaluate this, we characterized the composition, rheological properties (e.g., viscosity and elasticity properties), and cytokine/chemokine profiles of respiratory secretions from patients under mechanical ventilation due to severe COVID-19, given the importance of these parameters in other respiratory diseases (32, 33). As controls for these studies, we have included sputum samples from both healthy individuals and patients with cystic fibrosis (CF) (34), a genetic disease associated with notoriously thick lung secretions (35). These studies provide biochemical, biophysical, and immunological assessment of respiratory secretions in COVID-19.

Results

Solids and proteins are increased in COVID-19 respiratory secretions. We collected respiratory secretions from ventilated COVID-19 patients, ranging from 5 to 70 years of age (Table 1). These were collected via suction catheter, with only a single sample from each individual included in the data set. Respiratory secretion samples were collected from patients with CF via spontaneous expectoration and from healthy volunteers via sputum induction.

We observed that samples from healthy patients were typically clear and colorless, whereas samples from patients with COVID-19 were typically colored and opaque, similar to samples from patients with CF (Figure 1A). This suggested that the samples contained appreciable amounts of biopolymers and insoluble debris.

The percentage solids content of respiratory secretions, an index of hydration, affects the difficulty with which respiratory secretions can be cleared and correlates with clinical outcomes in CF and other settings (36–38). We found that COVID-19 samples had significantly higher percentage solids than healthy samples (Figure 1B). We further observed that protein concentrations in COVID-19 samples were nearly 5.5 times greater than those seen in healthy samples (Figure 1C, $P = 0.003$). COVID-19 and CF samples did not show statistically significant differences ($P = 0.983$). These data are consistent with infected and inflamed lungs being known to have protein deposits from increased mucin production (39), bacterial colonization (40), and infiltrating cells (28).

Of note, large variances in solids and protein were observed in the COVID-19 patient samples (Figure 1, B and C). Even though all the patient samples were collected from intubated patients with severe COVID-19 early during mechanical ventilation, this variance may reflect the differences in the individual response to the infection and the disease progression at the time of collection.

HA is increased in COVID-19 respiratory secretions and lung sections. We next evaluated HA content in the respiratory secretions. We observed a statistically significant, 10-fold increase in HA concentration in COVID-19 patient samples compared with samples from healthy individuals (Figure 2A, $P < 0.0001$). The average concentration of HA found in COVID-19 samples was comparable to that observed in CF samples ($P = 0.333$), a disease state associated with greatly increased sputum HA (41). Similar to our findings with percentage solids and protein, we observed larger variance in the amounts of HA in COVID-19 and CF samples than samples from healthy donors.

Table 1. Clinical characteristics of SARS-CoV-2* patients

Clinical characteristic	Adult patients (n = 22)	Pediatric patients (n = 2)
Age (y)	57 (41–69)	5, 14
Male sex	15 (68%)	2 (100%)
Female sex	7 (32%)	0 (0%)
Race and ethnicity	Non-Black Hispanic 13 (59%) White 3 (14%) Black 2 (9%) Asian 3 (14%) Pacific Islander 1 (5%)	Non-Black Hispanic 1 (50%) Pacific Islander 1 (50%)
Preexisting diabetes mellitus	12 (55%)	0
Preexisting pulmonary disease	4 (18%)	0
Preexisting cardiac disease	4 (18%)	1 (50%)
Hospital length of stay	34.5 (22–47)	113, 290+ ^A
In-hospital death	8 (36%)	0
Total ventilator days	19.6 (9–36)	9, 290+ ^A
In-hospital tracheostomy	7 (32%)	1 (50%)
Days from intubation to first collection	2 (2–4.6)	1, 16
ARDS severity at time of collection	Mild 1 (7%) Moderate 14 (64%) Severe 5 (23%) Recovered 2 (14%)	Moderate 1 (50%) Severe 1 (50%)
Antibiotics at time of collection	12 (55%)	0

For adult patients, values listed as *n* (%) or median (IQR). In pediatric patients, values listed as *n* (%) or given for both patients. ^APatient still admitted and mechanically ventilated at time of publication.

Given that the molecular weight of HA is known to influence both the immunogenic as well as the rheological properties of the resulting solution (42–44), we measured the molecular weight of the HA in the different samples (Figure 2, B and C, and Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.152629DS1>). HA size in samples from patients with COVID-19 skewed smaller than that seen in samples from donors with CF and healthy controls. Given that low molecular HA polymers promote inflammation in some systems (42, 43), our results are consistent with the highly inflammatory nature of respiratory disease in COVID-19 infection.

HA is increased in COVID-19 cadaveric lung sections. We next examined cadaveric lung sections from patients with COVID-19, patients with CF, and patients with healthy lungs (i.e., without a diagnosed pulmonary disease) for HA deposits by staining with HA binding protein (HABP). We observed very little HABP staining in sections treated with hyaluronidase (HADase) (Figure 2, D–F), suggesting very low nonspecific background staining. However, we observed a substantial increase in HA staining in lung sections from both COVID-19 and CF donors compared with healthy samples when no prior HADase treatment was used (Figure 2, G–I). We observed strong, diffuse staining in areas of necrosis and inflammation. Higher magnification images demonstrated the accumulation of HA within alveolar spaces (Figure 2, J–L). These data, together with the aforementioned respiratory secretion studies, indicated that patients with severe COVID-19 have elevated levels of HA in their lungs.

HA and hyaladherins are increased in blood vessels of COVID-19 lung sections. We next determined whether changes in HA accumulation corresponded to changes in hyaladherins, the extracellular matrix molecules that bind and interact with HA, including versican and tumor necrosis factor–stimulated gene-6 (TSG-6). The donor profiles for these histologic tissue samples are included in Table 2.

The expression and accumulation of versican and TSG-6 increase significantly during inflammation in many diseases (45). We evaluated HABP, versican, and TSG-6 staining patterns (localization and intensity) in lung sections from patients with COVID-19 ARDS, non-COVID-19 ARDS, CF, and healthy lungs (patients who died without known lung disease). Compared with healthy control samples, both COVID-19 ARDS and non-COVID-19 ARDS lung sections demonstrated intense HABP staining of the distorted alveolar-capillary barrier, including HABP staining in the alveolar spaces (Figure 3, A–C, and Supplemental Figure 2). We observed diffuse alveolar hemorrhage, distended alveolar-capillary membrane with strong heterogeneous staining, and diffuse bronchial and vascular staining in the COVID-19 lung sections. We did not observe

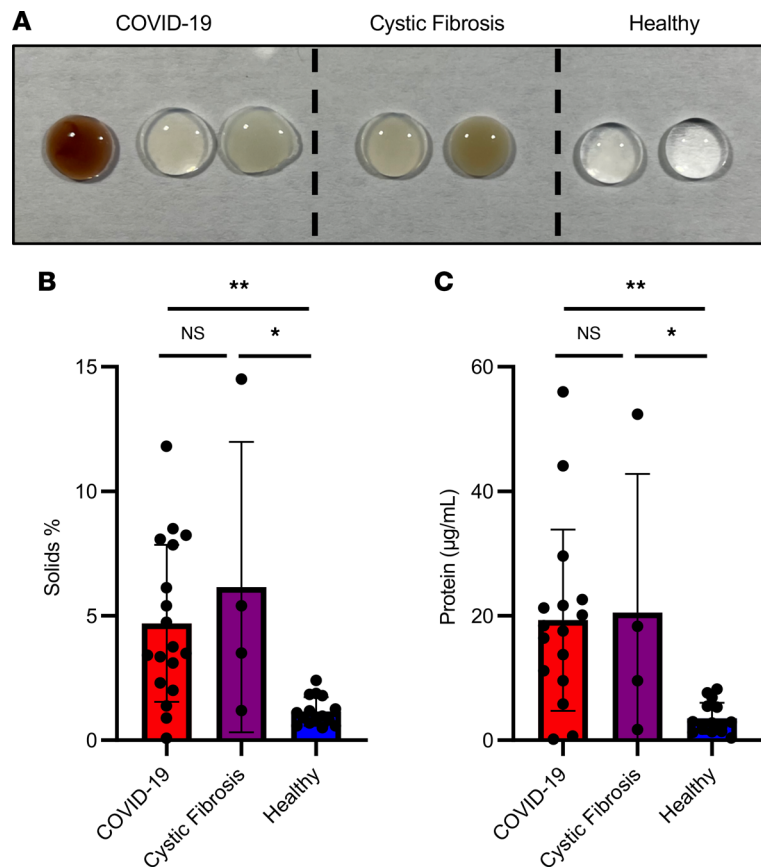


Figure 1. Respiratory secretions from patients with COVID-19 are high in solids and protein compared with healthy controls. (A) Representative images of respiratory secretions collected from ventilated patients with COVID-19 are viscous and tenacious. Similar to CF samples, COVID-19 samples are often colored and opaque, whereas healthy samples are clear and colorless. (B) Quantification of solids found in COVID-19 ($n = 18$), CF ($n = 4$), and healthy ($n = 15$) respiratory secretions. (C) Quantification of protein concentration in COVID-19 ($n = 16$), CF ($n = 4$), and healthy ($n = 15$) respiratory secretions. One-way ANOVA with Tukey's multiple comparisons tests. * $P < 0.05$, ** $P < 0.01$.

major differences in HABP staining between these 2 patient groups. For the CF samples, HABP staining was mostly seen in inflammatory cells, mucus, and the medial layer of bronchi and vessels, in addition to strong diffuse staining in the alveolar spaces. Far more HA staining was observed in all these conditions than in tissues from individuals who died without lung disease (Figure 3, D and M, and Supplemental Figure 2).

In healthy lung tissues, versican staining was strong in alveolar macrophages, bronchial epithelia, and the peribronchial layer, with some staining in the medial layer of blood vessels. In contrast, both COVID-19 ARDS and non-COVID-19 ARDS samples displayed much stronger and prominent versican staining in the medial layer of blood vessels and the alveolar-capillary membrane, compared with healthy controls. Similar findings occurred in CF except that strong staining was also seen in the bronchial epithelium (Figure 3, E–H, and Supplemental Figure 3). Although the total accumulation of versican was not significantly different (trending higher) in COVID-19 ARDS, non-COVID-19 ARDS, and CF lung sections, compared with healthy controls (Figure 3N), the tissue distribution and localization were altered as described above and in Supplemental Table 2.

For TSG-6, we observed strong staining of bronchial cells and immune cells in lung sections from healthy, CF, and non-COVID-19 ARDS. However, interestingly, in COVID-19 ARDS samples, the stain was much fainter in alveolar macrophages and other immune cells (Figure 3, I–L, and Supplemental Figure 4). We also quantified the total TSG-6⁺ staining in all the lung sections and observed no significant change in the overall accumulation of TSG-6 (Figure 3O). The full review of each stained histology slide is included in Supplemental Table 2.

Overall, for both COVID-19 ARDS and non-COVID-19 ARDS, there was a shift toward stronger staining of versican in the blood vessels, which correlated with the severity of tissue damage.

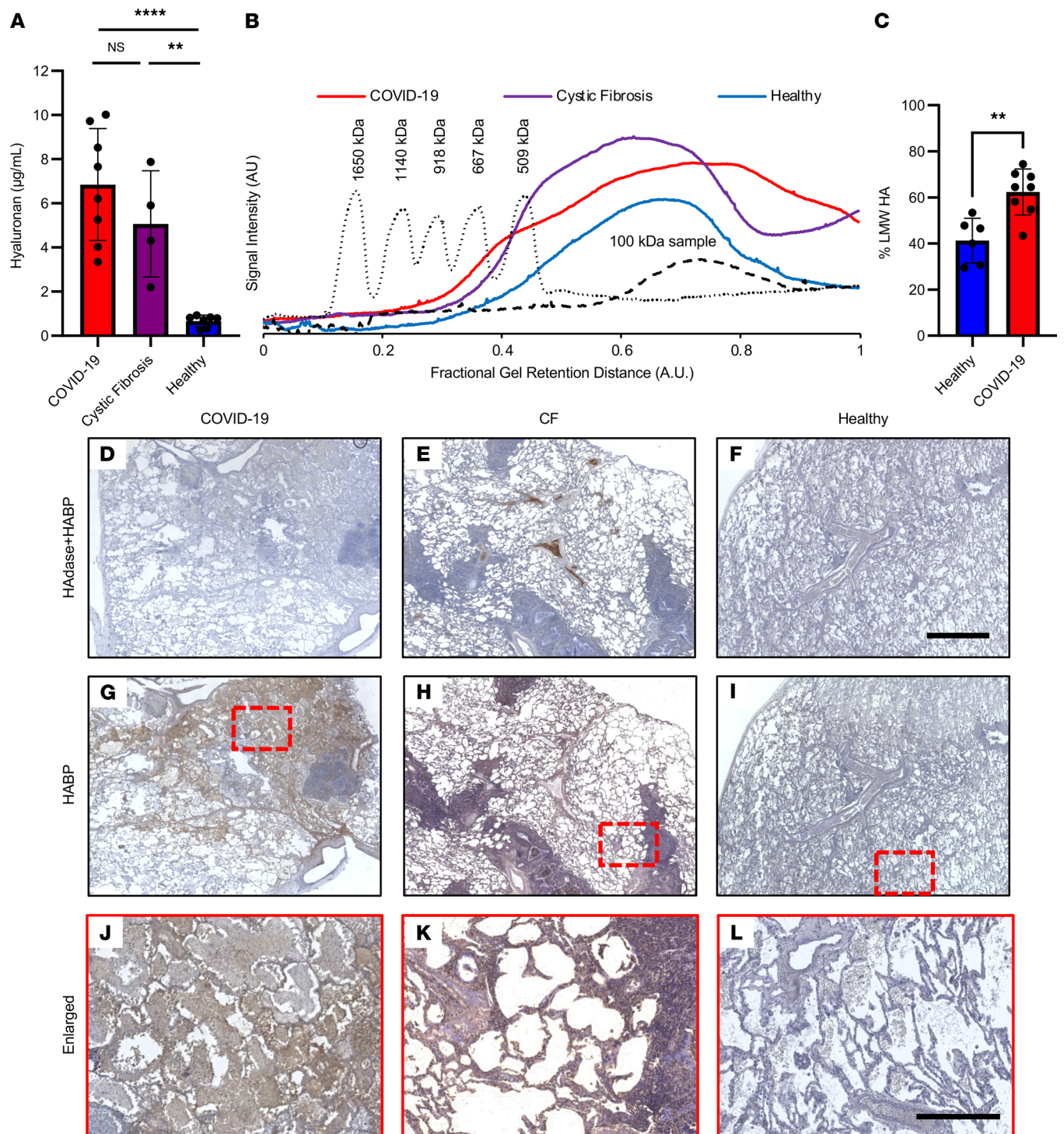


Figure 2. COVID-19 human lung sections have high levels of HA. (A) Quantification of HA in respiratory secretion samples. COVID-19 ($n = 8$), CF ($n = 4$), and healthy ($n = 7$) respiratory secretion samples. One-way ANOVA with Tukey's multiple comparisons tests; $**P < 0.01$, $****P < 0.0001$. (B) Representative chromatogram of HA molecular weight (MW). Solid traces are the averages of COVID-19, CF, and healthy respiratory secretion samples. The dotted trace is the chromatogram of standard loaded with HA of known MWs, as indicated on graph. The dashed trace is representative of a commercially available 100 kDa MW HA. (C) The bar graph represents the percentage low-molecular weight HA in respiratory secretion samples: healthy ($n = 6$) and COVID-19 ($n = 8$). We chose 250 kDa as the cutoff to define low-molecular weight HA. Unpaired t test with Welch's correction; $**P < 0.005$. (D–I) Representative histologic cadaveric lung sections from donors with COVID-19, donors with CF, and healthy donors, both with (D–F) and without (G–I) HADase treatment. Nuclei are stained in blue, and HA binding proteins (HABPs) are stained in brown. (J–L) Enlarged sections from G–I, respectively. Scale bars: 800 μm (D–I), 400 μm (J–L).

Table 2. Demographic details and clinical summary for histology studies

Sample ID	Age	Sex	Group	COD/diagnosis for Tx
1012A	37	F	Healthy	Died with malignant intracranial HTN due to SAH and intraventricular hemorrhage with severe vasospasm of cerebral arteries. This was thought to be due to ruptured cerebral aneurysm involving left internal carotid artery at area of posterior communicating artery branch.
1051	62	M	Healthy	Died from anoxia secondary to a cardiovascular event. PMH/PSH significant for HTN (>10 years, noncompliant with meds), CAD, and aortic stenosis s/p TAVR. He was a former smoker, known heavy alcohol use. CT had shown bilateral lower lobe atelectasis.
1007A	63	M	Healthy	Died from a CVA secondary to ICH. His PMH/PSH included HTN diagnosed 10 years prior and cholecystectomy. Cigarettes/ETOH/drugs all unknown amount/frequency but stopped 20 years prior to event. No medications taken at home.
1041D	56	M	Healthy	Died from a cerebrovascular event/stroke.
87-H	55	M	Healthy	Died from ruptured aneurysm.
HA20-44	55	F	COVID-19 ARDS	PMH of HTN and diabetes, was admitted to the hospital due to worsening hypoxic respiratory failure secondary to COVID-19 pneumonia.
HA20-36	53	F	COVID-19 ARDS	PMH of HTN and diabetes mellitus; developed an increasing cough, exertional intolerance, fever, nausea, vomiting, and diarrhea for 1 week. She was subsequently diagnosed with COVID-19 infection with a positive nasopharyngeal swab.
HA20-39	59	M	COVID-19 ARDS	Nonsmoker with history of diabetes mellitus, HTN, CAD.
HA20-55	63	M	COVID-19 ARDS	History of obesity, chronic kidney disease (unknown stage), diabetes mellitus type 2, HTN, and hyperlipidemia who presented with dyspnea, cough, and subjective fever. The onset of symptoms occurred approximately 3–4 days after contact with a client who would later test positive for COVID-19.
603	75	M	COVID-19 ARDS	Multiple medical issues, including diabetes; died of ARDS; found to be COVID-19*.
HA20-16	21	M	Non-COVID-19 ARDS	No significant PMH, presented to the emergency department with complaints of fever and shortness of breath.
HA19-113	27	M	Non-COVID-19 ARDS	No significant PMH. He has a history of marijuana, tobacco, and vaping use.
HA20-30	44	F	Non-COVID-19 ARDS	PMH of HTN, treatment for which was started on hydrochlorothiazide, and a recent diagnosis of oral herpes.
HA20-98	38	M	Non-COVID-19 ARDS	Presented at the emergency room after being found down in a house fire for an unknown length of time.
38-A	35	F	CF	Died from pulmonary embolism and hemoptysis.
46-O	17	F	CF	Died from pneumonia leading to sepsis.
51-L	75	F	CF	Died from coronary disease.

COD; cause of death; Tx, treatment; SAH, subarachnoid hemorrhage; PMH/PSH, past medical/surgical history; HTN, hypertension; CAD, coronary artery disease; s/p, status/post; TAVR, transcatheter aortic valve replacement; CVA, cerebrovascular accident; ICH, intracerebral brain hemorrhage.

There also seemed to be more evidence of alveolar hemorrhage associated with strong and diffuse HA staining in alveolar spaces of COVID-19 ARDS lung sections.

DNA is increased in COVID-19 respiratory secretions. We next examined the double-stranded DNA (dsDNA) content in these respiratory secretion samples. The respiratory secretions collected from patients with COVID-19 had increased dsDNA content compared with healthy controls (Figure 4A, $P = 0.032$). The average dsDNA content in the COVID-19 samples was 14 times greater than that in the healthy samples, with 8 of the observed samples having over 10 times more dsDNA. CF sputum, by comparison, had an average dsDNA concentration roughly comparable to COVID-19 samples ($P = 0.999$). Sizing the dsDNA in the samples suggested that the dsDNA was very large (greater than 10 kb, i.e., >6,000 kDa) (Figure 4B and Supplemental Figure 5). The variance observed in samples from patients with COVID-19 and CF was again very large in comparison with that observed in healthy donor samples.

High-modulus secretions are more susceptible to enzymatic treatment. We next evaluated the rheological properties of COVID-19 respiratory secretions and the contribution of HA and dsDNA to the physical flow properties of the secretions. These flow properties can be expected to greatly affect the ability of patients to clear secretions from the lungs. Dynamic light scattering microrheology, a noninvasive rheology technique, was used to evaluate the rheological properties of the sample due to the small sample volume required and the ability of the technique to not alter the sample properties during measurement (46, 47). The samples were measured both before and following enzymatic treatment (microrheology protocol further described

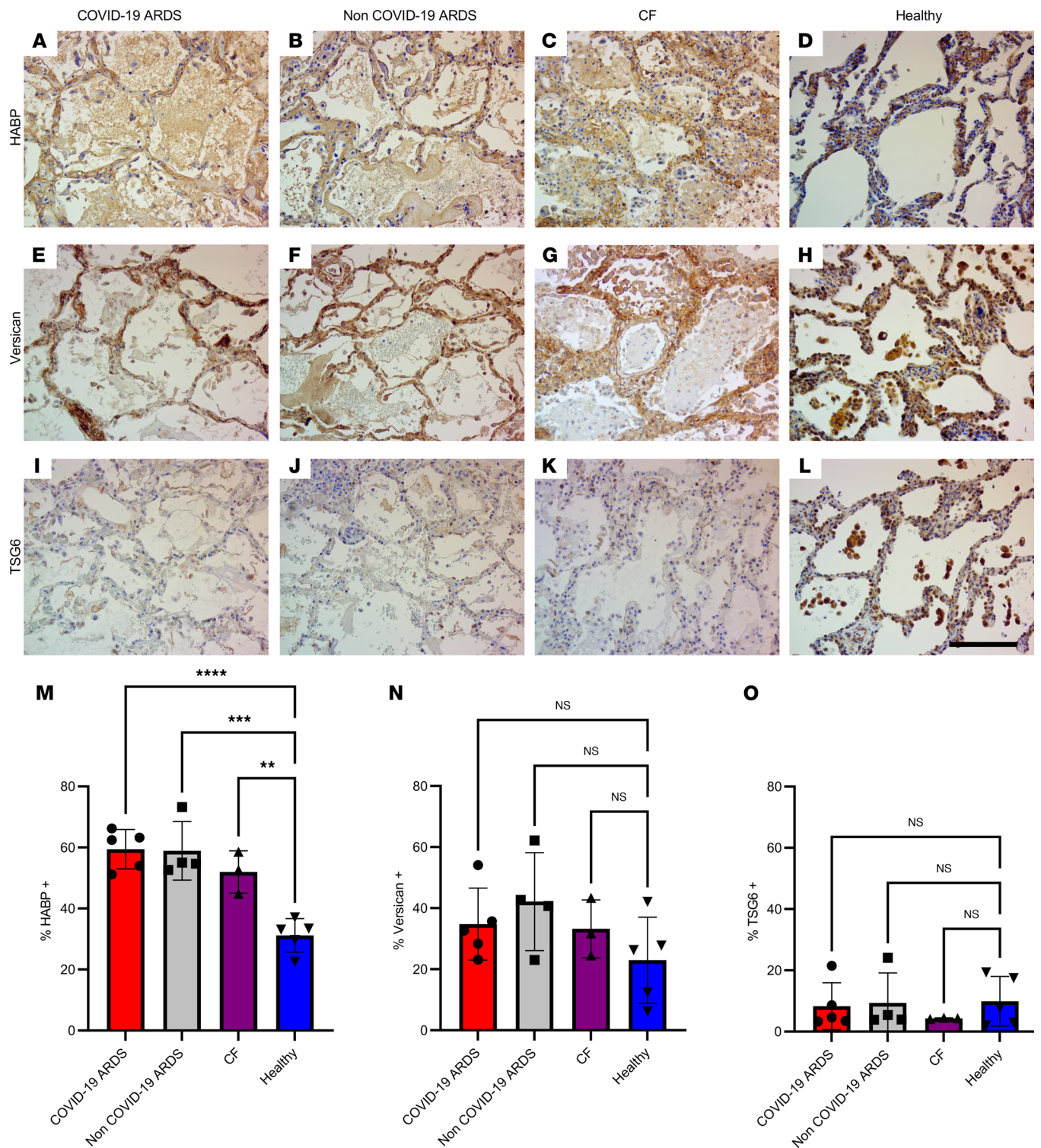


Figure 3. HA and hyaladherins are increased in blood vessels of COVID-19 lung sections. Representative histologic cadaveric lung sections from donors with COVID-19 ARDS, donors with non-COVID-19 ARDS, donors with CF, and healthy donors stained with (A–D) HABP, (E–H) versican, and (I–L) TSG-6 (original magnification, 40×). Nuclei are stained in blue, and HABP, versican, or TSG-6 are stained in brown. Scale bar: 400 μm (A–L). Tissues were examined using an Amscope T720Q microscope, and images (original magnification, 40×) were acquired using Amscope digital camera (MU1403) and imaging software. (M) Percentage HABP+, (N) versican+, and (O) TSG-6+ area in lung sections from COVID-19 ARDS (n = 5), non-COVID-19 ARDS (n = 4), CF (n = 3), and healthy donors (n = 5). One-way ANOVA with Dunnett’s multiple comparisons tests; **P < 0.01, ***P < 0.001, ****P < 0.0001.

in Methods and Supplemental Figures 6 and 7). Specifically, we examined the impact of enzymatic treatment with HAase (to degrade HA) or deoxyribonuclease (48) (DNase; to degrade dsDNA) on the flow properties of respiratory secretions. We hypothesized that enzymatic degradation of these biopolymers would lower the modulus (i.e., the resistance to flow) given the abundance of DNA and HA in these samples. As a nonenzymatic treatment control, samples were diluted with an equivalent volume of saline.

We evaluated the absolute impact of enzymatic treatment as a function of the measured pretreatment modulus of the respiratory secretions (Figure 5). Samples that had a higher pretreatment modulus (i.e., thicker samples that were more resistant to flow) had a larger response to enzymatic treatment by either DNase or HAase compared with a control saline dilution. We found a statistically significant linear relationship between the pretreatment modulus of the secretions and the difference between the change of modulus with dilution and change of modulus with enzymatic treatment ($\Delta G_{\text{Saline}} - \Delta G_{\text{Enzyme}}$). If the enzyme had no effect compared to the dilution control, then $\Delta G_{\text{Saline}} - \Delta G_{\text{Enzyme}} = 0$; if the enzyme treatment decreased the modulus of the sample, then $\Delta G_{\text{Saline}} - \Delta G_{\text{Enzyme}} < 0$. The BIC for comparing this linear model against a fit for random noise was much greater than 10 (BIC = 35.75 for DNase, and BIC = 36.92 for HAase), indicating strong statistical significance for this linear relationship. These data are consistent with the hypothesis that thicker COVID respiratory secretions are more sensitive to enzymatic treatments that degrade DNA and HA, resulting in a lower modulus (i.e., less resistance to flow). By comparison, healthy control samples had low pretreatment moduli and were not dramatically affected by enzymatic treatments.

We then evaluated the relationship between the pretreatment concentration of DNA and HA on the modulus change due to enzymatic treatment. Interestingly, we did not observe a correlation between biopolymer concentration and modulus change, as indicated by the BIC values being less than 10 (3.5 and 8.5 for DNA and HA, respectively) (Supplemental Figure 8). This suggests that the contribution of HA and DNA to the modulus of these samples is complex and may be influenced by the presence of HABPs in these samples, for example.

In addition to considering the absolute reduction in the modulus as a function of the pretreatment modulus, we also examined the percentage change from the initial pretreatment modulus. When compared with dilution, there was a trend toward a lower average modulus following enzymatic treatment with either HAase or DNase, but this did not reach statistical significance (Supplemental Figure 7). However, this approach does not account for the large variance in the modulus of pretreatment samples, a factor that is considered in the analysis shown in Figure 5.

Mucin expression is heterogeneous in COVID-19 respiratory secretions. We also assessed mucin glycoprotein content in the COVID-19 respiratory secretion samples. Mucins are high-molecular weight (HMW) and heavily glycosylated proteins lining mucosal surfaces that play an important role in innate defense, protecting the epithelium against invading pathogens (49). The major secreted airway mucins or gel-forming mucins are Mucin 5AC (MUC5AC) and Mucin 5B (MUC5B), which are produced by goblet cells and mucous cells within submucosal glands, respectively (50). We show representative results of MUC5AC and MUC5B expression following agarose gel electrophoresis in respiratory secretion samples from healthy, COVID-19, and CF participants (Supplemental Figure 9). We observed highly variable MUC5AC and MUC5B expression in the COVID-19 group, with samples showing undetectable expression, low expression, or high expression, compared with the healthy controls. Of note, in CF, mucin is reported to decrease during stable disease but increase during a pulmonary exacerbation (51, 52), which is consistent with our results.

One potential explanation for our COVID-19 results is that after acute infection, there may be less mucus production in some patients due to damage of mucus-producing glandular epithelial cells (49). In contrast to our study, in one published report, higher levels of MUC5AC (as measured by ELISA) were observed in airway mucus aspirated by bronchoscopy from COVID-19 intensive care unit (ICU) patients compared with induced sputum from healthy controls (53). Additional studies reported accumulation of mucins in patients with COVID-19, without comparison to mucins from healthy individuals (54, 55).

Overall, in our studies, we observed heterogeneous mucin expression in respiratory secretions from COVID-19 ARDS patients, compared with healthy controls.

Enhanced inflammatory burden in COVID-19 respiratory secretion samples. While substantial literature exists concerning systemic immune responses and inflammatory profile during SARS-CoV-2 infection derived from serum or plasma samples, there is very little information about the inflammatory profile of respiratory secretions in COVID-19. To better understand the lung tissue-specific immune responses, we measured 80

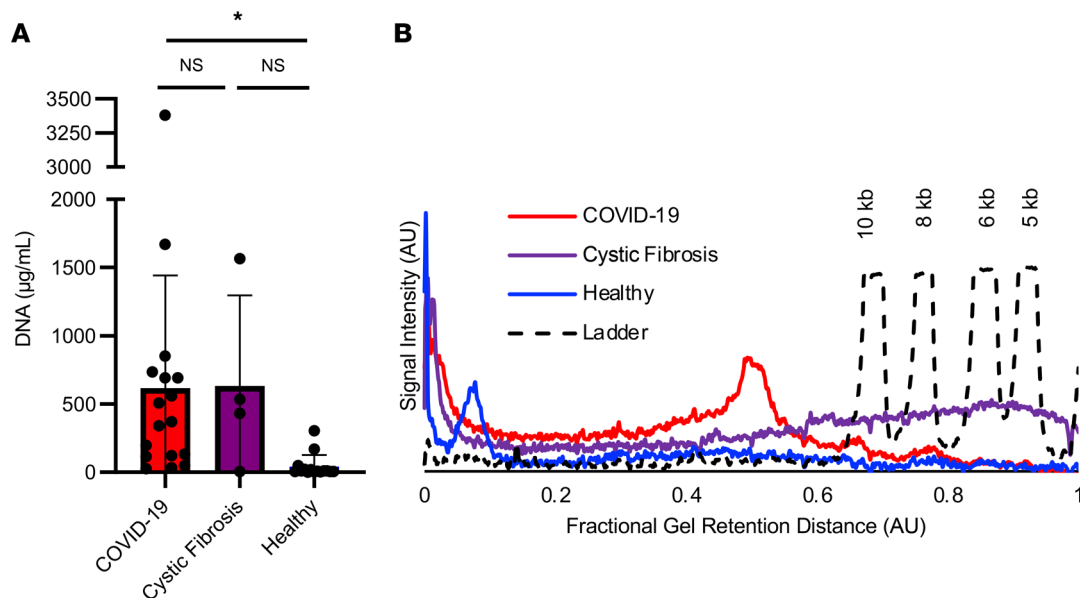


Figure 4. Increased levels of dsDNA in COVID-19 respiratory secretions. (A) Quantification of dsDNA in respiratory secretion samples. COVID-19 ($n = 17$), CF ($n = 4$), and healthy ($n = 15$) respiratory secretion samples. One-way ANOVA with Tukey's multiple comparisons tests. $*P < 0.05$. (B) Representative chromatogram of dsDNA molecular weight. Solid traces are the averages of COVID-19, CF, and healthy respiratory aspirate samples. The dashed line trace is the chromatogram of a DNA standard ladder with the dsDNA bp lengths labeled above the respective peaks.

cytokines, chemokines, adhesion molecules, and growth factor levels in COVID-19 respiratory secretion samples, compared with healthy controls.

We observed significant decreases in antiviral type I interferon IFN- $\alpha 2$ and PDGFAA in COVID-19 respiratory secretion samples, compared with the healthy controls (Figure 6 and Supplemental Figure 10). In contrast to a previous report, IL-13 was not significantly different from healthy controls in our samples (56). We observed significant upregulation of several other cytokines and chemokines in the COVID-19 cohort compared with healthy controls, including IL-6, tumor necrosis factor (TNF), IFN- γ , IL-10, IL-1 β , IL-18, M-CSF, RANTES/CCL5, MIP-1 β /CCL4, MIP-1 α /CCL3, and others. Although there was heterogeneity in the response among samples within a group, groups of samples (healthy or COVID-19) followed similar trends. These findings extend previous reports describing diminished type I interferon and hyper-inflammatory responses in the context of severe COVID-19 but show that lung cytokine responses are regulated in a distinct fashion. In contrast, we found that patients with CF had more generalized elevations in their sputum cytokine and chemokine profiles (Supplemental Figure 11).

Overall, our data support that increases in HA and DNA in COVID-19 ARDS respiratory secretion samples correlate with enhanced inflammatory burden in COVID-19.

Discussion

We report that respiratory secretions from patients with COVID-19 ARDS are thick and tenacious, comparable to the notoriously thick and tenacious sputum produced by patients with CF. COVID-19 respiratory secretions have significantly elevated levels of solids, with HA and DNA contributing to the elevated viscosity. Low molecular HA in particular is greatly increased in the respiratory secretion samples from intubated patients with COVID-19. Consistent with these findings in respiratory secretions, HA is abundant in histologic sections from cadaveric lung tissues from individuals with COVID-19-associated ARDS. Together, these data indicate that low molecular HA is elevated in the respiratory secretions of patients with COVID-19-associated ARDS.

We found that samples that had a higher pretreatment modulus (i.e., thicker samples that were more resistant to flow) had a larger response to enzymatic treatment by either DNase or HAase compared with a control saline dilution. However, this change did not scale with the pretreatment concentrations of these respective biopolymers. This may reflect the complex compositional nature of respiratory aspirates from patients with COVID-19 ARDS and interactions among the various components present.

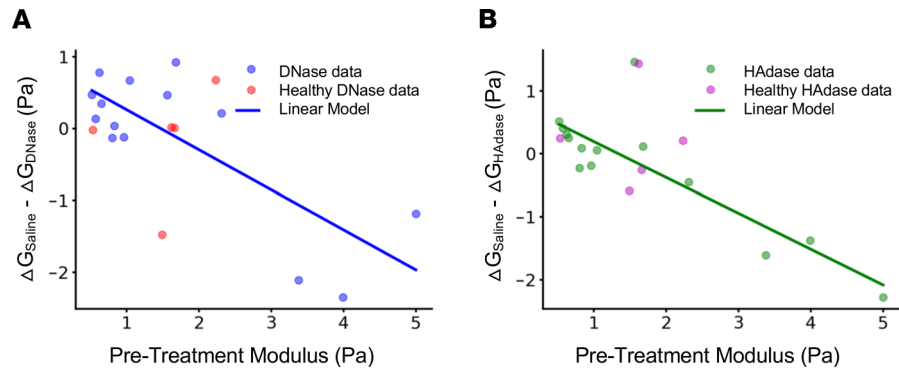


Figure 5. Enzymatic treatments affect the rheology of COVID-19 respiratory secretions in proportion to their pre-treatment moduli. (A) The difference in modulus of COVID-19 lung secretions ($n = 15$) upon control saline dilution and enzymatic DNase treatment ($\Delta G_{\text{Saline}} - G_{\text{DNase}}$) versus the initial, pretreatment modulus (blue). Healthy controls shown for comparison ($n = 6$). (B) The difference in modulus of COVID-19 lung secretions ($n = 15$) upon control saline dilution and enzymatic HAdase treatment ($\Delta G_{\text{Saline}} - G_{\text{HAdase}}$) versus the initial, pretreatment modulus (green). Healthy controls shown for comparison ($n = 6$) (magenta). The Bayesian Information Criterion (BIC) was used as the statistical metric to compare the impact of enzymatic treatment. (BIC = 35.75 for DNase, and BIC = 36.92 for HAdase.)

Nonetheless, upon targeting HA and DNA with enzymes, we did see a change in modulus, which points to an important functional contribution of these polymers to the modulus of these secretions.

Thinning of the fluid to improve lung clearance is a common goal across a range of diseases with respiratory inflammation (57–60). As we observed in our study, treatment of respiratory secretions with an enzyme to digest the biopolymers (and hence decrease the polymer entanglements) will decrease the flow resistance of thick samples with an initial high modulus. The impact of the DNase is established clinically, as it has been used in treating CF lung disease (61, 62) and is under investigation as a treatment for COVID (ClinicalTrials.gov NCT04359654, NCT04541979); however, the use of HAdase for improving the flow properties of respiratory secretions is a relatively new approach and requires further investigation. More research is needed to identify ideal treatment conditions, such as dosages and dosage regimens. Further, targeting the production of HA during infection may be more successful than relying on a postproduction degradation approach. Treatment with a pharmaceutical HA inhibitor, such as hymecromone (4-methylumbelliferone) (63), may be a viable approach to limit the deposition of HA during infection.

We also observed upregulation of many proinflammatory cytokines and chemokines in the COVID-19 cohort compared with healthy controls, including IL-6, TNF, IL-1b, IL-18, M-CSF, CCL5, CCL4, MIP-1 α /CCL3, and others. These data are consistent with an excessive proinflammatory macrophage activation phenotype and the contribution of myeloid cells to pathogenic inflammation as described by other reports (64–68). This is also consistent with published data correlating abundance of low molecular HA to a hyperinflammatory state (42, 43, 69).

Conversely, we observed a significant decrease in IFN- α 2 in COVID-19 respiratory secretion samples, compared with the healthy controls. Smith et al. likewise reported significantly lower expression of IFN- α 2 in the nasopharynx of patients with COVID-19, compared with healthy controls (70). Hadjadj et al. reported that plasma levels of IFN- α 2 decreased with disease severity but were not significantly different compared to healthy controls (71). Our results are different from a plasma study that reported higher IL-33 in a cohort with severe COVID-19, but our findings are consistent with a nasopharynx study (70, 72). IL-13 was also not significantly different from healthy controls in our samples, in contrast to a previous report (56). These differences may reflect distinct systemic versus local pulmonary immune responses in COVID-19 infection. To our knowledge, this is the first study to measure cytokines, chemokines, and other factors in respiratory secretions from intubated COVID-19 patients.

These studies have several limitations. Most notable is the small numbers of cases and samples of secretions involved. These findings need to be confirmed in larger, multicenter studies involving individuals with diverse backgrounds and case presentations. The underlying mechanisms that lead to increased HA would also benefit from further research to identify the causative cell types and signaling pathways. In addition, data in SARS-CoV-2 animal models would enable improved understanding of the contribution of HA to pathogenesis in this disease. Finally, to safely acquire the rheology data, the COVID-19 samples

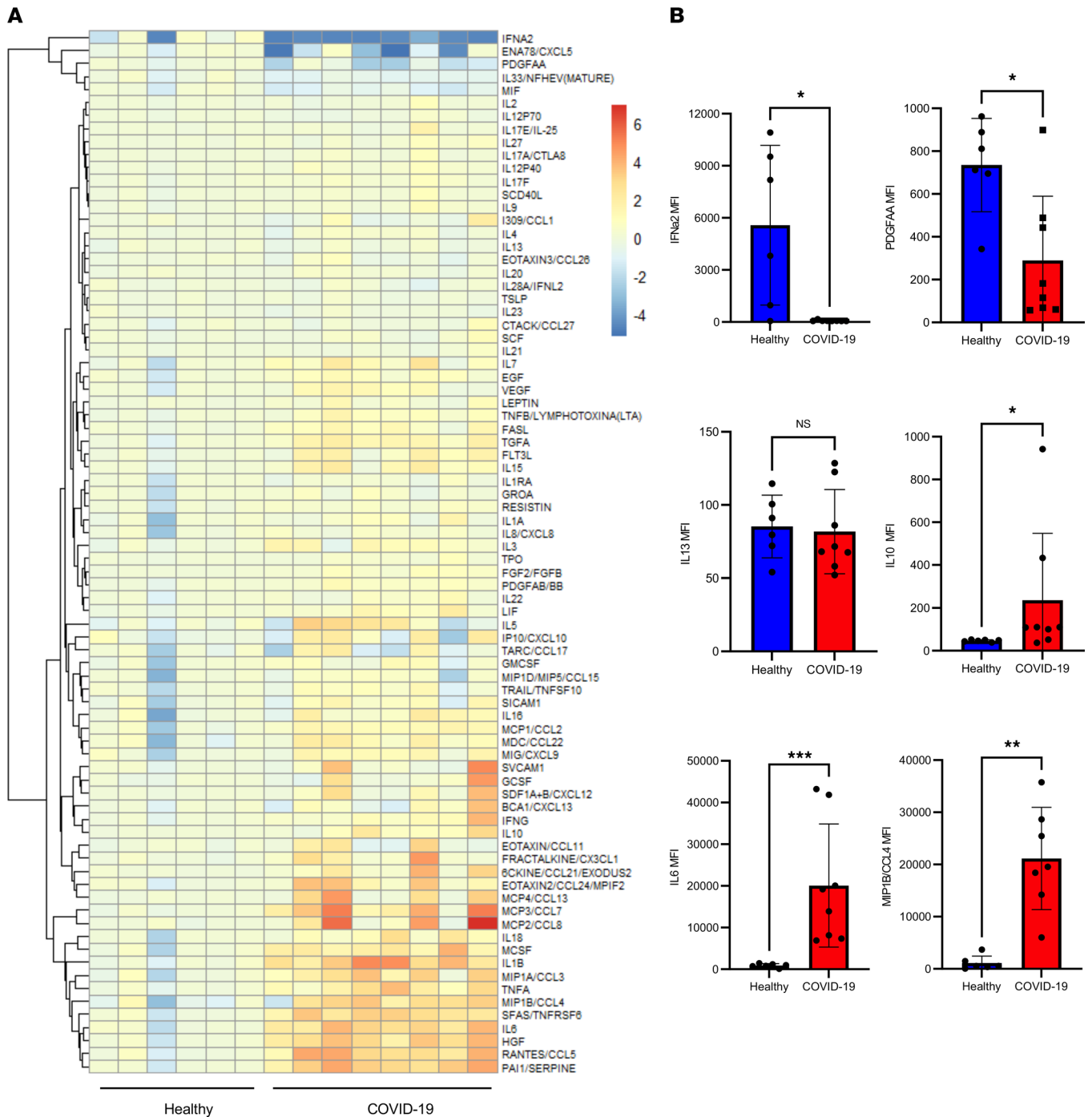


Figure 6. Immunological characterization of respiratory secretions from COVID-19 ARDS patients. (A) Heatmap of mean fluorescence intensity (MFI) data, log₂ transformed and normalized to average of the healthy controls per cytokine. Data are ordered by k-nearest neighbors (KNN) clustering of the cytokines (y axis). Cytokines, chemokines, adhesion molecules, and growth factors were measured in the respiratory secretion samples of healthy controls (n = 6) and in COVID-19 ARDS patients (n = 8) using a bead-based multiplexed immunoassay system, Luminex-EMD Millipore Human 80 Plex assays. Upregulated cytokines are shown in orange and downregulated in blue. (B) Bar graphs of raw MFI values for representative cytokines (IFN-α2, PDGFAA, IL-13, IL-10, IL-6, and MIP-1β/CCL4) in healthy control and COVID-19 ARDS groups (not normalized). Mann-Whitney test; *P < 0.05, **P < 0.005, ***P < 0.0005.

were heat-treated to render the samples noninfectious. In control CF samples, this same heat treatment was found to decrease the modulus (Supplemental Figure 4A), presumably due to the denaturation of biopolymers in the sample. Since we observed that higher modulus samples had larger responses to enzymatic treatment, the true effect of enzymatic treatment on COVID-19 lung secretion may be larger than that reported here using heat-treated samples. Future studies should further evaluate a range of enzymatic

treatment dosages and durations to assess the rheological effects on non-heat-treated COVID-19 lung secretions. Additionally, the necessity of using induction to collect healthy sputum is a limitation.

These studies may inform the development of much-needed therapeutics for patients with COVID-19. Indeed, a study of oral hymecromone as a potential tool for HA inhibition was recently completed in healthy individuals (ClinicalTrials.gov NCT02780752) (for results, see ref. 73). Developing treatments that render the respiratory secretions of lungs less viscous, and thus easier to clear via natural mucociliary clearance, could be pivotal to improving clinical outcomes in severe COVID-19 and ARDS.

Methods

Histologic staining of lung tissues for HA, versican, and TSG-6. For Figure 2 describing HA histologic staining, human lung tissue was obtained from a deidentified autopsy specimen provided through the Stanford Department of Pathology in the form of a formalin-fixed, paraffin-embedded histologic specimen. Histologic staining for HA was performed as described previously (74). In brief, 5 μm thick sections were cut on a Leica RM 2255 Microtome (Leica Microsystems Inc.). For HA affinity histochemistry, the Bond Intense R Detection kit, a streptavidin-HRP system (Leica Microsystems, Inc.), was used with 4 $\mu\text{g}/\text{mL}$ biotinylated HABP in 0.1 % BSA in phosphate-buffered saline (PBS) as the primary. All images were collected using the BZ-X710 inverted fluorescence microscope (Keyence) at 20 \times original magnification. Montages were generated using the Keyence BZX Analyzer software's stitching function. For Figure 3, HA histologic staining was performed similarly, except for the use of 4 PLUS Streptavidin HRP label (Biocare Medical), instead of the Bond Intense R Detection kit.

For Figure 3 and supplemental figures, most of the lung tissue samples from healthy, COVID-19 ARDS, and non-COVID-19 ARDS groups were obtained in collaboration with clinical partners at University of Texas Health Science Center at Houston (HSC-MS-15-1049 and HSC-MS-08-0354) and Houston Methodist Hospital (Pro00003392). Discarded donor lungs for transplantation that served as healthy controls were obtained from LifeGift Organ Procurement (Houston, Texas, USA). Lung tissues were collected from the midportion of the upper and lower lobes as described previously (75). The details of the study population are summarized in Supplemental Table 1. The CF tissue samples were provided through the Stanford Department of Pathology. All histologic specimens were formalin-fixed, paraffin-embedded, 5 μm thick sections. HA staining was performed as detailed above. Histologic staining for versican and TSG-6 was performed as described previously (76). Rabbit anti-versican antibody, clone EPR12277 (ab177480; Abcam), and rabbit anti-TSG-6 antibody (ab204049; Abcam), were used at 1:50 dilution. Positive and negative controls were included in each staining experiment.

Whole-section imaging was performed using an Aperio (Leica) AT2 Digital Pathology whole-slide scanner at the Stanford University Department of Pathology, Human Pathology/Histology Service Center. Slides were scanned in bright-field at a 20 \times objective and the digital images imported for analysis using the Aperio Imagescope v12.4.3.5008 viewing software. All the images were taken under the same experimental settings. Tissues were also examined using an Amscope T720Q microscope, and higher magnification images (40 \times) were acquired using Amscope digital camera (MU1403) and imaging software (for Figure 3).

Histology quantification. A total of 150 regions of interest (ROIs), 500 \times 500 pixels, were randomly sampled from each tissue section imaged with the Aperio AT2 Digital Pathology whole-slide scanner. The RGB image was deconvolved into its hematoxylin and DAB components using an algorithm (77) implemented in the `rgb2hed` function in the `scikit-image` Python package. The frequency of pixels in the DAB channel above a predefined threshold was computed per ROI. The threshold was calibrated on tiles randomized across groups to optimally detect positive DAB staining. The mean DAB⁺ frequency across ROIs was calculated per sample and plotted as % DAB⁺ area.

Collection of human respiratory secretions. We collected respiratory secretions from patients enrolled in the Stanford University sputum biobank study from March 2020 to March 2021 (IRB approvals 28205 and 55650). COVID-19 samples were respiratory secretions obtained during the course of routine clinical care. All COVID-19 samples were collected from ventilated patients who were diagnosed with ARDS. Eligibility criteria included admission to Stanford Hospital with a positive SARS-CoV-2 nasopharyngeal swab by reverse transcription PCR. Patients admitted to the ICU were included. Patients were phenotyped for ARDS using the Berlin criteria (acute onset of hypoxemic respiratory failure with a PaO₂/FIO₂ ratio [i.e., the ratio of the partial pressure of arterial oxygen to the percentage of inspired oxygen] of less than 300 on at least 5 cm of positive end-expiratory pressure, with bilateral infiltrates on chest x-ray). For controls, sputum was collected from asymptomatic adult donors. Healthy controls were asymptomatic, aged 24–50 years.

Sputum samples from CF patients were collected during routine care. All samples were frozen at -80°C immediately after collection. Samples were thawed slowly on ice, then heat-treated at 65°C for 30 minutes to render the virus inactive and the sample noninfectious prior to further analyses.

Compositional characterization of respiratory secretions. The solids content of the human respiratory secretions was determined by taking the ratio of the freeze-dried mass and wet mass of the samples following at least 2 days of lyophilization. Protein concentrations were determined using the Pierce Bicinchoninic Acid Protein Assay (Thermo Fisher Scientific) following manufacturer's instructions. HA concentration was determined using a modified HA ELISA as previously described (78). DNA concentrations were determined using Quant-iT dsDNA Broad-Range Assay Kit (Molecular Probes, Thermo Fisher Scientific) following manufacturer's instructions. The pH of the samples was measured using pH indicator strips (Supelco).

Gel electrophoresis to characterize HA molecular weight. Respiratory secretion samples were treated with 250 U benzonase for 30 minutes at 37°C for nucleic acid digestion, followed by an incubation with 1 mg/mL proteinase K for 4 hours at 65°C for further digestion. Proteinase K was heat inactivated by incubating the samples at 100°C for 5 minutes. Insoluble material was removed by centrifugation at $17,000g$ for 10 minutes before further processing. Samples were precipitated with ethanol overnight at -20°C by adding 4 volumes of prechilled 200-proof ethanol to each sample. The following day, the samples were centrifuged at $17,000g$ for 10 minutes. The supernatant was discarded, and the pellet was washed by adding 4 volumes of prechilled 75% ethanol. Samples were centrifuged at $17,000g$ for 10 minutes and the resulting pellet air-dried at room temperature for 20 minutes. Each sample was resuspended in 100 μL of 100 mM ammonium acetate in water, lyophilized, and resuspended in 10 μL of 10 M formamide. Samples were separated on a 1% Tris-acetate EDTA agarose gel run at 100 V, then stained with Stains-All (1.25 mg/200 mL in 30% ethanol) (MilliporeSigma). The gel was imaged on a BioRad GS-800 Calibrated Densitometer. Twice the volume of healthy control samples was loaded in each gel lane compared with CF and COVID-19 samples. We chose 250 kDa as the cutoff for low molecular HA.

Gel electrophoresis to characterize DNA molecular weight. Respiratory secretion samples were mixed with loading solution and separated on a 1% agarose gel with 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide at 120 V. Samples were separated on the same gel with a 2-Log DNA Ladder (New England Biolabs). Gels were imaged on a BioRad ChemiDoc MP imaging system.

Microrheology measurements. Dynamic light scattering (DLS) microrheology data was collected as previously described (46, 79) with minor modifications as described below. Due to the presence of naturally occurring particulates within all samples, no additional beads were required to induce light scattering. Light scattering was collected from a Malvern Panalytical Zetasizer Nano with a 633 nm laser operated in 173° backscatter mode. The raw intensity autocorrelation function of a respiratory secretion sample was measured at a specified measurement position for 30 minutes at 37°C . Following initial microrheology measurements, the same respiratory secretion sample was then treated with 1) benzonase nuclease (250 U/mL) for 1 hour at 37°C , 2) HAase (50 mg/mL, MilliporeSigma) for 2 hours at 37°C , or 3) $1\times$ PBS for 1 hour at 37°C as a dilution control. After the allotted reaction time, the DLS measured the raw intensity autocorrelation function of the sample at the same settings as before. To safely determine the effect of heat on the rheological behavior of respiratory secretions, we measured the intensity autocorrelation function of CF sputum, which is similar to COVID-19 respiratory secretions in both composition and rheological behavior, before and after the same heat treatment that all COVID-19 respiratory secretion samples were subjected to prior to handling. The heat treatment significantly decreased the resistance to flow (i.e., the elastic modulus) of the CF sputum (Supplemental Figure 4A).

Microrheology data analysis. The intensity autocorrelation data acquired above were analyzed using the custom analysis package found at dlsur.readthedocs.io. The size of the particulates was assumed to be 500 nm in diameter for all samples. While this assumption affects the absolute value of the modulus derived from the scattering autocorrelation function, it has the same proportional effect across all samples. Thus, the trends observed in the microrheology data, along with the conclusions drawn from those trends, are unaffected by this assumption. All rheological measurements in this study obtained the complex modulus over a wide range of frequencies (from about 10^1 to 10^6 Hz), but only the modulus value at 1 frequency was used when comparing the modulus across samples and conditions. This is a common approach when comparing rheological results of lung secretions (25). To determine this frequency, the complex moduli of the pre- and posttreatment were compared. In the spectrum with the higher complex modulus (typically the pretreatment), a single frequency was determined by selecting either a) the middle of the "plateau" region

of the elastic modulus (Supplemental Figure 3A) or, in the case of no plateau region, b) the lowest frequency for which there were data (Supplemental Figure 3B). Some samples had limited-frequency ranges due to the fast decay of the measured autocorrelation function, which often corresponds to solutions with less resistance to flow. For a single sample, the same frequency was chosen for the pretreatment modulus and posttreatment modulus. The change in modulus with dilution, ΔG_{Saline} , was determined by subtracting the modulus of the sample after dilution from before dilution. The change in modulus with enzyme (DNase or HAdase) treatment, ΔG_{Enzyme} , was determined by subtracting the modulus of the sample after enzyme addition from the modulus before enzyme addition. The measured moduli pretreatment, G_{Pre} , and posttreatment, G_{Post} , for all individuals are shown in Supplemental Figure 4B.

Luminex-EMD Millipore human 80 Plex assays. Luminex assays were performed by the Human Immune Monitoring Center (HIMC) at Stanford University. The COVID-19 respiratory secretion samples and the respective healthy controls were inactivated with 1% Triton-X (vol/vol) (80, 81). Samples were spun down rigorously and diluted 1:6 for the Luminex assay. Kits were purchased from EMD Millipore and used according to the manufacturer's recommendations with modifications described as follows: H80 kits include 3 panels: panel 1 is Milliplex HCYTA-60K-PX48; panel 2 is Milliplex HCP-2MAG-62K-PX23; and panel 3 includes the Milliplex HSP1MAG-63K-06 and HADCY MAG-61K-03 (resistin, leptin and HGF) to generate a 9-plex. The assay setup was performed as recommended by the manufacturer. Briefly, samples were mixed with antibody-linked magnetic beads on a 96-well plate and incubated overnight at 4°C with shaking. Incubation steps were performed on an orbital shaker at 500–600 rpm. Plates were washed twice with wash buffer in an ELx405 washer (BioTek Instruments). Following a 1-hour incubation at room temperature with biotinylated detection antibody, streptavidin-PE was added for 30 minutes with shaking. Plates were washed as described above and PBS added to wells for reading in the Luminex FlexMap3D Instrument with a lower bound of 50 beads per sample per cytokine. Custom Assay Chex control beads were purchased and added to all wells (Radix BioSolutions). Wells with a bead count < 50 were flagged, and data with a bead count < 20 were excluded. MFI, an estimate of analyte concentration, was used to compare expression in each sample. The cytokines/chemokines/adhesion molecules measured by the Luminex assay are presented in Figure 6, and the raw data are available in Supplemental Figure 10.

Statistics. Data are expressed as mean \pm SD of n independent measurements. Significance of the difference between the means of 2 or 3 groups of data was evaluated using a 1-way ANOVA followed by Tukey's or Dunnett's post hoc test, as indicated. A t test (2-tailed) with Welch's correction or Mann-Whitney test was used to determine the significance between the means of 2 groups of data, as indicated. A P value less than 0.05 was considered statistically significant. The small number of samples and large amount of scatter in our rheological data necessitated a statistical measure that could capture the significance of any correlation. Therefore, we chose to use the BIC as the statistical metric because it can establish correlation while accounting for measurement error in the analysis, which is generally not the case for the more commonly seen R^2 metric in linear regressions and paired t tests. More simply put, the BIC tests the significance of any trend in the data relative to there being no trend, and a BIC value greater than 10 shows that the trend is statistically significant relative to the hypothesis that there is no trend in the data.

Study approval. All secretion samples were obtained under the auspices of research protocols approved by the Stanford IRB (approvals 28205, 53685, 55650, 37232, and 43805). Human respiratory secretion studies were approved under Administrative Panel on Biosafety protocol 2379. Samples were collected after receipt of written informed consent from patients or their surrogates prior to inclusion in the study.

Additional details may be found in Supplemental Methods.

Author contributions

GK, PLB, AJS, and SCH conceived the study. EBB, MRN, MGO, DPR, AEPN, SY, HKQ, AMP, BZ, MLB, Stanford COVID Biobank, CEM, and AJR identified, enrolled, and consented eligible patients/patient samples. MJK, GK, SD, and PLB processed patient samples. MJK, GK, SD, PCC, GLB, and PYJ performed experiments. VADJP provided the histopathology descriptions and analysis. MJK, GK, SD, PCC, EBB, MP, GLB, MRN, NN, RBV, TNW, CEM, AJR, AJS, SCH, and PLB performed data analysis and interpreted data. MJK, GK, SD, CEM, AJS, SCH, and PLB wrote the manuscript with input from all authors. Authorship order for co-first authors was determined via mutual agreement among MJK, GK, and SD.

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