Neutrophil Aggregates and Elastin Degradation Compromise Lung Architecture in Fatal COVID-19

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Abstract

Pulmonary fibrosis, profound alveolitis, and the failure to restore alveolar epithelial architecture are major causes of respiratory failure in fatal COVID-19. However, contributing factors to abnormal fibrosis in critically ill COVID-19 patients are yet to be understood. This study analyzed the histopathology of lung autopsy samples from eight COVID-19 and six non-COVID-19 post-mortems. The distribution and changes in extracellular matrix (ECM) proteins, including elastin and collagen in lung alveoli, were quantitatively assessed through morphometric analyses. These studies reveal massive degradation of elastin fibers along the thin-alveolar walls of the lung parenchyma that supersedes interstitial collagenous fibrosis and intra-alveolar fibrotic abnormalities. Injured lungs with collapsed alveoli and organized fibrotic regions exhibited widespread elastolysis. Further, immunoblotting of lung autopsy extracts validated extensive elastin degradation. Importantly, loss of elastin was correlated with induction of neutrophil elastase (NE), a potent protease that degrades ECM, and increased staining of peptidylarginine deiminase, a marker for neutrophil extracellular traps release, and extensive epithelial necrosis. Further, elevated plasma levels of NE-alpha1-antitrypsin complex in hospitalized COVID-19 patients indicate dysregulated neutrophil activity. These findings place elastin degradation at the center of alveolar structural disintegration and argue that elastolysis and alveolitis lead to abnormal ECM repair and fibrosis in fatal COVID-19.

Introduction.

The COVID-19 pandemic demonstrated the potentially devastating outcomes of respiratory viral infections. Although most infected individuals recover with relatively mild symptoms, many experience impaired lung function, require hospitalization, and as many as 2% of the infected patients succumb to the disease caused by the coronavirus CoV-2. To gain greater insight into the course of fatal COVID-19, lung autopsies from COVID-19 patients have been examined by histopathology. The disease reveals a complexity of pathologic features, including acute diffuse alveolar damage (DAD) and organized pneumonia and fibrosis.[1-6] Growing evidence indicates that during disease progression, fibrotic abnormalities become more predominant, especially in fatal COVID-19.[7-10] Similarly, post-acute phase sequelae of COVID-19 chest computed tomography (CT) scans find a variable degree of fibrotic abnormalities in a large number of recovered patients.[11] In fact,

pathologic lesions caused by fibrosis were also noted earlier in fatal avian influenza, 2003-SARS-COV, and Middle East respiratory syndrome coronavirus infections.[12-15] However, etiologic factors that drive virus-inflicted pulmonary fibrosis are yet largely unexplored.

Pulmonary fibrosis develops in a complex interplay between cell death, inflammation, and abnormal ECM remodeling that lead to alveolar architectural disorganization, irreversible lung dysfunction, and death.[16] Recent studies associate alveolar type II pneumocyte (AT-II) epithelial injury and endoplasmic reticulum (ER)-stress-induced impairment of AT-II cell regeneration with SARS-CoV-2-inflicted fibrotic abnormalities and reveal common features with idiopathic pulmonary fibrosis (IPF).[17] Persistent neutrophil accumulation and release of neutrophil extracellular traps (NETs) in the lower respiratory tracts and plasma samples of severely ill COVID-19 patients and associated with acute alveolar injury.[18-21] Therefore, factors that drive the disorganization of ECM proteins and abnormal ECM remodeling toward fibrotic changes in acute SARS-COV-2 infection deserve greater attention.

Elastin and collagen are major structural ECM proteins in the alveoli.[22] Elastin fibers consist of tropoelastin polymers, which are cross-linked by lysyl oxidase-mediated conversion of 4 lysine side chains into desmosine.[23-24] Elastin fibers align along the walls of the alveoli by association with fibrillin-1 and facilitate the expansion and recoiling of alveoli during normal breathing.[25] Unlike other ECM proteins, elastin has a half-life of 70-80 years, as synthesis of elastin fibers occurs during the postnatal lung alveolarization and continues during the first 10 years of life in humans.[26] The regeneration/replacement of damaged elastin is inefficient, thus damaged elastin is often replaced by collagen.[27-28] Elastin is a potent substrate for neutrophil elastase (NE), a serine proteinase produced by activated neutrophils.[29] NE-mediated elastolysis releases elastin degradative products (EDP), potent mediators of inflammation and interstitial fibrosis.[30]Cleavage of elastin is linked to the pathophysiology in bleomycin-induced fibrosis,[31] IPF,[32]and chronic obstructive pulmonary disease (COPD).[33]The activity of NE is regulated by an inhibitor called alpha-1 anti-antitrypsin (A1AT), which complexes with NE and blocks its activity.[34-35]

In this study, eight lung autopsy samples from COVID-19 patients and six non-COVID-19 lungs were compared microscopically. Micro-architecture with progressive elastin cleavage and collagen deposition in injured lungs correlated with lung pathophysiology, inflammation, and fibrosis. These observations reveal extensive degradation of elastin fibers in the alveolar interstitium that shows severe epithelial damage and massive neutrophil-specific inflammation. Further, loss of elastin and increased collagen deposits marked lesions with interstitial collagenous fibrosis and organized parenchymal fibrosis. Elastin degradation was also observed in areas of alveolar collapse indicating that elastolysis and alveolitis contribute to abnormal ECM repair and fibrosis in fatal COVID-19 patients. As proxy for elastase, NE-A1AT complexes in plasma of a second group of hospitalized COVID-19 patients were quantitated. Plasma samples from hospitalized patients had approximately 30-fold higher NE-A1AT complexes than plasmas from healthy donors, suggesting that such complexes may provide an early warning of host tissue damage due to inflammation in respiratory lung infections.

Materials and Methods

Materials.

Citrulline (C7629), 2,3-Butanedione monoxime (B0753), Antipyrine (A5882), Iron (lll) Chloride (157740), A1AT, Bovine serum albumin (A9418), and PNPP (S0942) were purchased from Sigma-Aldrich, MO. Antibodies including anti-NE (MAB9167, Novus Biologicals, CO), anti-A1AT (Thermo Fisher Scientific, MA), anti- peptidylarginine deiminase 4 (PAD4, ab128086, Abcam, MA), anti-MPO (14569P, Cell-signaling MA), anti-thyroid transcription factor-1 (TTF-1, SPT24, Biocare Medical, CA), anti-elastin (MA5-41583, Thermo Fisher Scientific, MA) and anti-histone H3 pan antibodies (07-690, Upstate, NY) were used in this study. Soluble elastin (Elastin Products Company, MO). Secondary antibodies for Western blot were purchased from Li-Cor Biosciences, NE, and anti-Rabbit IgG-AP (Southern Biotech, AL). Reagents including EnVision FLEX Target Retrieval Solution High pH (Dako/Agilent, CA) and low pH IHC Antigen Retrieval Solution (Invitrogen, MA), Biocare Decloaking Chamber (Biocare Medical, CA) and Tris-Buffered Saline

(TBS) (Dako/Agilent, CA) were used in immunohistochemistry.

Collection of the lung autopsy samples, plasma, and ethical committee approvals:

Lung autopsy samples from eight patients, who died of COVID-19, and six patients, who died of non-COVID-19-related deaths were examined. This study was conducted in compliance with approval by the ethical committee of Danylo Halytsky Lviv National Medical University, Ukraine (Protocol Numbers: 20180226/2; 20211122/9). Patients, who were hospitalized with positive COVID-19 test, with pneumonia, and with at least one complicating factor such as diabetes mellitus or hypertension and who died in the hospital after therapy including oxygen support, constituted the COVID-19 group. Non-COVID-19 samples were from hospitalized patients, who had no positive COVID-19 tests at the time of death and were characterized by oxygen saturation being >95%.

Plasma samples from hospitalized COVID-19 patients ($n = 62$) were obtained following a protocol approved by the institutional review board (IRB# 808542), University of Pennsylvania, between April and June 2020[36]; anonymous discarded plasma samples (n=15; gift of Donald Siegel, University of Pennsylvania)[37] and additional samples (n=11) were obtained following an IRB-approved protocol from the University of Tennessee Health Science Center.

Histopathology.

Lung tissues were fixed in neutral-buffered formalin and embedded in paraffin. Lung sections were cut at 4 μm, dewaxed, rehydrated, and stained with hematoxylin and eosin. Various lung pathologic features including acute or organizing diffuse alveolar damage (DAD), alveolar hemorrhage, thrombosis, and fibrotic remodeling described earlier in COVID-19 patients[38-40] were evaluated by an experienced pathologist (AK) in a blinded manner. Pathological lesions of acute DAD were characterized by disrupted alveolar epithelium, epithelial sloughing, formation of hyaline membranes, necrotizing bronchiolitis, interstitial inflammation, oedema, fibrin deposition, vasculitis, pulmonary hemorrhage, and microvascular thrombosis. The organizing form of DAD was defined as loosely organized fibrosis, interstitial fibrotic changes, and abnormal remodeling of epithelium with hyperplasic type II pneumocytes and proliferative airway epithelium. Fibrotic changes were defined as extensive and dense interstitial fibrosis replacing lung parenchyma and disintegrating alveolar architecture. The histopathological grading was 0-absent, 1-mild, 2-moderate, and 3-severe. Lung sections were stained with Verhoff van Gieson (VVG) and orcein for detecting elastin fibers and Masson's trichrome for identification of collagen depositions.

Immunohistochemistry.

Lung tissue sections were subjected to immunohistochemistry analysis using Bond^{TM} Polymer Refine Red Detection Kit (Leica Biosystems, IL) with primary antibodies for the detection of neutrophil granule proteins including NE (1:400 dilution), myeloperoxidase (MPO, 1:10000 dilution); alveolar type II epithelium marker, TTF-1, (1:1 dilution); and peptidylarginine deiminase 4 (PAD4, 1:300 dilution), a marker for neutrophil extracellular traps (NETs) induction. Antigen epitope retrieval was conducted with EnVision FLEX Target Retrieval Solution High pH (K800421-2, Dako/Agilent, USA) and low pH IHC Antigen Retrieval Solution (00-4955-58, Invitrogen, USA) using a Biocare Decloaking Chamber (Biocare Medical, USA) for 5 minutes at 120 °C. Following antigen retrieval, tissue sections were incubated with primary antibodies as described above, followed by Horse radish peroxidase-conjugated secondary antibodies. Color was developed using universal diaminobenzidine chromogen substrate. Whole slide images were captured using CaseViewer 3DHITECH Ltd. software.

Elastin fiber morphometry

Elastin fibers in the alveolar walls were detected by VVG and orcein staining and images were captured as described above. Lengths of the elastin fibers were measured using ImageJ software analysis (National Institute of Health, April, 2019; ImageJ with 64-bit Java 1.8.0_172). Images from 10 randomly selected fields at 400x were captured. The images were first normalized for scale, the actual lengths of the elastin fibers were measured in micrometers (μm) in a blinded manner, and the curvatures of the elastin fibers along the

walls of the alveoli were evaluated using freehand length measurement.[41] The bundle of elastin fibers was measured as a single fiber unless the fibers were distinctly separated from each other. Elastin fibers often appear as single detached, fragmented or degraded fibers in COVID-19 patients. These fibers were counted separately to measure their lengths. All data were exported into an Excel file and the average fiber length in μm was determined. Each fiber was counted only once and no extra weighting was used for larger/wider fibers. Elastin fibers staining in the airways or surrounding bronchioles or pulmonary blood vessels were excluded.

Quantification of collagen and measurement of collagen:elastin ratio in the lungs of COVID-19 patients.

Masson's Trichrome stained lung sections were imaged as described above, and images were analyzed by ImageJ software. For the measurement of collagen density, 10 random fields were selected from each lung section. The collagen staining was processed by selecting Masson's Trichrome specific staining using color deconvolution plugin software in ImageJ that separates red, blue, and green components from the image. Thresholds for collagen staining were established for each slide by enhancing the contrast to a point at which the collagen staining was easily identified by blue staining and the density of the collagen was measured over total lung area from the image.[42] The collagen was measured as percent collagen density in total lung area of the image. No weighting was performed for the intensity of blue staining. Further, adjacent serial lung sections were stained with Masson's Trichrome and orcein for quantitative measurement of collagen:elastin ratio as a fold change after both stains were normalized to the total lung area in the image captured in the same areas of the sections.

Protein extraction from formalin-fixed paraffin-embedded (FFPE) lung tissues and western blot analysis:

FFPE lung tissues (10 μm sections) from non-COVID-19 and COVID-19 patients were transferred into 1.5 ml centrifuge tubes, dewaxed in xylene for 10 min and centrifuged. T issue pellets were washed 3x with xylene and rehydrated with graded concentrations of ethanol (100 %, 85 % and 75 % for 2 min in each). After the ethanol wash, proteins in the tissue pellets were solubilized in Tris-SDS buffer, pH 8.0, by homogenization and sonication. Tissue homogenates were centrifuged at 10,000 g for 20 min and the supernatants were measured for total protein concentrations. Equal amounts of proteins were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer onto nitrocellulose membrane. Membranes were then blocked with 5% bovine serum albumin and incubated with anti-elastin (1:1000 dilution) and anti-histone H3 Pan antibodies (loading control, 1:4000 dilution) overnight at 4 ^{0}C. Next day, membranes were washed in TBST and incubated with IRDye 800CW and IRDye 600CW conjugated secondary antibodies. The membranes were washed 3 times and signal was measured using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Colorimetric determination of citrulline residues in proteins:

To measure the total citrullinated proteins in the lung homogenates, an equal amount of proteins from non-COVID-19 and COVID-19 patients were added to the chromogenic reagent (Diacetyl-monoxime Solution + Antipyrine Solution + Acid-Ferric Solution, at 1:2:3), mixed well, and the tubes covered with aluminum foil. For standard, 0.5 to 10 nmoles of citrulline were prepared as described above. The samples and standards were heated for 25 min in a boiling water bath and cooled to RT on ice. The color developed was read at 464 nm.[43]Quantification of the citrullinated proteins in the lung homogenates was determined using the citrulline-protein standard curve.

ELISA for evaluation of the NE-A1AT complexes in the plasma samples:

Standard NE-A1AT complexes were prepared by combining 2.5 μM NE with 10 μM A1AT in 100ul of PBS, and incubated at 37^{0}C for 30 and 60 min. After the incubation, aliquots were snap frozen and kept at -80^{0}C until further use. For ELISA, 96-well plates were coated with the anti-NE antibody at 1 μg/ml and incubated overnight at 4^{0}C. On the following day, the plates were washed once with PBS Tween-20, and blocked with 1 % BSA in PBS for 1 hour at RT. The plates were then incubated with plasma samples collected from non-COVID-19 or COVID-19 patients for 2 hours at RT, followed by 3 washes, and further incubation with

rabbit polyclonal anti-A1AT antibody at 1:10000 dilutions for 1 hour. Next, the plates were washed 3 times, and incubated with anti-rabbit-IgG-AP for 1 hour at 1:2000, followed by 3 washes, and further incubation with para-Nitrophenylphosphate substrate for 30min at RT in the dark. The color developed was read at 405 nm.[44,45] The NE-A1AT known standard complexes were used to quantitatecomplexes present in the COVID-19 and non-COVID-19 plasma samples.

Statistical analyses:

Data are expressed as the mean group value ± standard error mean (SEM). Analysis of the lung autopsies from COVID-19 positive and COVID-19 negative patients for measuring elastin fibers and collagen were performed using Image J software analysis ((National Institute of Health, April, 2019; ImageJ with 64-bit Java 1.8.0_172) Website: https://imagej.nih.gov/ij/ for performing densitometry measurements. NIH). Statistical differences between the two groups were determined by Student's t -test with a two-tailed comparison. A p-value of <0.05 was considered statistically significant.

Results:

Characteristics of Tissue Donors

The characteristics of the subjects with fatal COVID-19 are described in Table 1. Postmortem lung autopsy samples were collected at Lviv Regional Pathological Anatomy Office, CU ENT ≪Pulmonology Lviv Regional Diagnostic Center≫ from patients who were COVID-19 positive by RT-PCR and presented with bilateral pneumonia during admission into the hospital **(Table 1)** . The mean age of the subjects was 60.4 years with a range of 30 to 80 years (4 female and 4 male patients). Patients were admitted into the hospital on average 7.1 days from the onset of symptoms and 7 of 8 subjects were in the hospital for more than 10 days (range 10 to 21 days). One patient died on 5th day after hospitalization. The biochemical analyses revealed a high percentage of blood neutrophils (non-COVID-19 patients 57.5 % vs COVID-19 patients 79.8 %; P value <0.03) and decreased lymphocytes (non-COVID-19 patients 36 % vs COVID-19 patients 14.9 %, Pvalue <0.09). Erythrocyte sedimentation rate (ESR), a plasma marker for inflammatory response, was elevated in COVID-19 patients compared to non-COVID-19 patients (51.7±10.7 vs 17±9.8; P value <0.0004). Six lung autopsy samples collected from patients who died of non-COVID-19 related diseases were also analyzed.

Histopathological manifestations of acute DAD and variable fibrotic abnormalities in COVID-19.

In comparison of lung autopsy samples of 8 COVID-19 patients and 6 non-COVID-19 patients, progressive pathologic changes, including diffuse alveolar damage (DAD), fibrosing organizing pneumonia, were evaluated**(Figure 1A, B and Table 2)** . DAD was found in all 8/8 COVID-19 patients with major characteristic features of widespread alveolitis (**Figure S1A),** sloughing of the bronchiolar epithelium**(Figure S1B),** formation of a hyaline membrane, (4/8) of cases**(Figure S1C),** and Intra-alveolar edema (5/8) cases**(Figure S1D)** . Interestingly, lung fibrosis exhibited a variable degree of abnormal features in all COVID-19 patients. The onset of fibrotic changes appeared focally in the alveolar interstitium with increased deposition of collagenous fibers **(Figure 1C)** . Mild to moderate interstitial fibrosis was present in 3/8 cases, while intra-alveolar fibrosis was noted in all except 2 cases **(Figure 1D)** . In 5/8 cases, organizing fibrosis was evident, with fibrosis extending into the surrounding parenchyma, thus causing disorganization of alveolar architecture and loss of air spaces **(Figure 1E)** . The loose myxoid fibroblastic proliferation extended between the alveoli in an intercommunicating manner, at places forming Masson bodies in alveolar and bronchiolar regions **(Figure 1F)** . Formation of bronchiolitis obliterans organizing pneumonia (BOOP) was noted in one case **(Figure 1G).** The squamous cells formed distinct nodules resembling squamous morules **(Figure 1H)** . Fibrotic changes also appear in the perivascular regions with pulmonary blood vessels surrounded by loosely organized proliferating fibroblasts**(Figure 1I)** . One specimen showed evidence of high inflammation in the pleura **(Figure S1E)** .In two cases, a superimposed bacterial infection was present, which was characterized by the presence of bacterial colonies admixed with neutrophils within the alveoli**(Figure S1F)** . Vascular thrombi in the small vessels were observed in 6/8 cases **(Figure S1G)** .

Evidence for elastolytic activity in COVID-19 patients.

Recent studies linked alveolar epithelial senescence and impaired regeneration as inducing factors of fibrosis in COVID-19.[17] However, the changes in the ECM that trigges in fibrosis in unknown. These studies reveal widespread alveolar-elastolysis in COVID-1 patients. Morphometrically, the elastin fibers in the normal alveoli appear as slender fibers aligned with the alveolar walls. The fibers often appear as bundles of similar length and branch at intersections between alveoli in non-COVID-19 lungs**(Figure 2A)** . Abundant elastin fibers also were observed at the tips of the alveolar septa. The elastin fibers that align with alveolar walls are essential in providing mechanical support and elastic recoil to the air spaces during respiration. The elastin fibers in COVID-19 lungs exhibited extensive disintegration **(Figure 2B)** . The degradation of elastin was most notable along the walls of alveoli that displayed extensive necrosis of alveolar epithelium. Further, fragmented elastin formed shorter, and single fibers that separated from the elastin bundles in the alveolar interstitial lining. The individual elastin fibers often lost their alignment within the alveolar walls. Quantitative measurement of the lengths of the elastin fibers displayed significant reduction in the lengths in the COVID-19 patients**(Figure 2C)** . In parallel, elastin fibers in the perivascular regions also displayed extensive cleavage in COVID-19 patients**(Figure 2D).** The loss of elastin along the blood vessels is associated with severe endothelial necrosis and distortion of the blood vessel structures. Thus, a loss of breathing capacity was compounded by the lack of elasticity in blood vessels carrying blood needed for gas exchange. In support of these findings, Western blot analysis of elastin from protein extracted from FFPE lung tissues displayed degradation of tropoelastin band at 70 kDa. Lung extracts from COVID-19 negative patients showed a prominent tropoelastin **(Figure 2E)** . Densitometry analysis of Western blots using ImageJ software**(Figure 2F)** showed a significant reduction in tropoelastin reactivity in COVID-19 lungs, in agreement with the morphometric analysis of elastin degradation in the COVID-19 lungs.

Collagen accumulates and replaces elastin in the lung parenchyma of COVID-19 patients.

Unlike the collagen staining that appeared mainly in the alveolar walls of non-COVID-19 patients, dense accumulations of collagen were observed in the alveolar interstitium in COVID-19 patients, accompanied by thickening of the alveolar septa **(Figure 3A, B)** . A quantitative assessment of collagen was determined using ImageJ analysis by selecting Trichrome-specific signals that show significant increase in collagen accumulation within the alveolar regions in the COVID-19 patient**(Figure 3C)** . In normal ECM of alveolar intersitium, elastin fibers are generally interspersed with collagen fibers that provides elasticity and mechanical strength to the alveoli. To evaluate the relation between collagen and elastin in COVID-19 patients, especially during elastolysis, this study examined expression of collagen and elastin fibers in the thin-alveolar walls of COVID-19 positive and COVID-19 negative patients. As shown in **Figure 3 D-E** , epithelial injury and elastin degradation in the alveolar walls were found replaced with abundant collagen deposition. The thickened walls of the alveoli show dense interstitial collagenous fibrosis associated with degradation or complete loss of elastin fibers **(Figure 3 F-H)** . Interestingly, the interstitial regions showing degraded elastin also display epithelial necrosis, indicating that both elastolysis and epithelial injury are preceding events in pathologic development of interstitial fibrosis (**Figure 3 D-E)** . A recent study identified collapse induration associated with alveolar epithelial necrosis and denudation of basal lamina in a COVID-19 patient.[46]Accordingly, this study found widespread alveolar collapse with hallmarks of necrotic epithelium and narrowing of the alveolar lumen. Interestingly, the collapsed alveoli also displayed an extensive degradation of elastin fibers and, conversely, dense collagen deposition in the collapsed alveolar regions **(Figure 3I-J)** suggesting that epithelial damage and elastolysis may precede the fibrotic changes in the injured alveoli. Similarly, perivascular regions showing loosely proliferating fibroblasts exhibit thick collagen deposition and degradation of elastin fibers **(Figure S1H-J)** . In support of these observations, the collagen:elastin ratio was increased by four-fold in the lung sections of COVID-19 patients compared to COVID-19 negative patients **(Figure 4 A-E)** . The lung parenchymal regions with advanced fibrosis displayed dense collagen diffusion with complete absence of elastin fibers, thus indicating an extensive elastolysis occurring during progression of the fibrosis in COVID-19 patients. Areas of fibrosing organizing pneumonia displayed extensive loss of alveolar epithelium that correlates with the loss of elastin fibers in the lung parenchyma **(Figure 4 F-H)** .

Neutrophil aggregates and active NETosis were prominent features in the lungs of COVID-19 patients.

Although neutrophil influx and NET release were reported in COVID-19 patients, their pathogenic role is yet to be understood. Massive neutrophil aggregates were found within the airways and alveolar spaces that displayed widespread alveolar architectural disintegration**(Figure 5A-D)** , The lung neutrophils displayed strong immunostaining for granule proteins including NE, MPO, and PAD4 co-localized within the regions of neutrophil aggregates in the alveoli**(Figure 5E)** . Accordingly, we measured an increase in total citrullinated proteins in COVID-19 lungs compared to non-COVID-19 lungs**(Figure 5F)** . Strong NE staining in the extracellular space within the disintegrated alveoli is consistent with the degranulation of neutrophils in the inflammatory microenvironment.

Increased NE-A1AT complexes were found in the plasma samples of COVID-19 patients:

Given the accumulation of neutrophils in COVID-19 patient lungs and the abundant evidence of NE expression and activity in the tissues, it was of interest to determine whether plasma from COVID-19 patients showed indications of increased NE. Because NE activity quickly dissipates, due to the high levels of the A1AT protease inhibitor that binds to NE and blocks its activity,[47] an appealing alternative is to measure concentrations of NE-A1AT complexes that have an extended half-life. A sandwich ELISA was developed to determine levels of NE-A1AT complexes in the plasma samples of COVID-19 patients as well as healthy individuals. As shown in **Figure 6,** a significant increase in NE-A1AT complexes was observed in COVID-19 patients.

Discussion

Although the COVID-19 pandemic subsided after two years of global devastation, sporadic outbreaks continue to recur in different parts of the world, especially during winter seasons.[48-50] The mortality rates remain high in hospitalized patients with severe infections. Findings of lung autopsy samples of patients, who died of COVID-19, revealed a wide spectrum of pathologic manifestations including acute DAD, impaired lung repair, and fibrosis. Notably, lung fibrosis was more prominent in the critically ill COVID-19 patients with prolonged hospitalization and extended progression of the disease. The underlying mechanisms of how SARS-CoV-2 infection progresses to fibrotic abnormalities in injured lungs is still largely unknown and has motivated the current study.

Pathophysiological development of pulmonary fibrosis involves a series of events that include massive inflammation, alveolar type II epithelial damage, subsequent surfactant deficiency, and abnormal ECM remodeling, which result in alveolar collapse, collapse induration, and fibrosis.[41-52] Alveolar epithelial loss and insufficient epithelial regeneration trigger expression of pro-fibrotic cytokines such as transforming growth factor beta, IL-6, and tumor necrosis factor-alpha, all known to induce fibrotic remodeling.[53] Recent observations highlight AT-II epithelial injury, and ER-stress-induced arrest of AT-II cell regeneration as promoters of fibrotic changes in COVID-19 patients.[17,54] The present study investigates structural integrity of ECM proteins including elastin and collagen and their correlation with pathologic manifestations of fibrotic abnormalities in the lung autopsy samples of COVID-19 patients.

The most notable result of this study was the overall degradation of elastin in the extracellular matrix of COVID-19 lungs. This degradation included the cleavage and disintegration of the elastin fibers of the alveolar wall, as well as within the critical vascular network in the lung parenchyma. Damaged lungs with pathologic manifestations of fibrosing organizing pneumonia displayed complete degradation or absence of the elastin network. Elastin is a polymer of tropoelastin units that scaffold onto fibrillin-1 along the walls of the alveoli.[23] Structurally, elastin provides elasticity and tensile strength essential for normal stretching and contraction of alveoli during respiration. Thus, a loss of elastin fibers potentially destabilizes alveoli promoting their collapse. Elastolysis has been associated with several chronic and acute clinical conditions.[34] The dysregulated neutrophil activation and overwhelming NE activity contribute to elastin degradation and disruption of alveolar basement causing emphysema in COPD patients. NE-mediated release of elastin degradative products such as desmosine and isodesmosine are potent inducers of fibrosis because they enhance

myofibroblast differentiation, proliferation and collagen expression.[28,32,55] In the present study, analysis of lung autopsies displayed a dense neutrophil congregates within the areas of DAD with overwhelming expression of NE and MPO in the airways and alveolar airspaces. Further, an increase in PAD4 expression, indicates active NETosis in the infected lung microenvironment of COVID-19 patients. Consistent with NETosis, high levels of total citrullinated proteins were observed in lung autopsy samples of COVID-19 patients compared to non-COVID-19 patients. In fact numerous clinical reports also found increased neutrophilia, high neutrophil-lymphocyte ratios,[56-58] and NETosis with progressive lung damage.[59] These results indicate an extensive elastin degradation in a short period of time attests to the shocking aggressiveness of invading neutrophils in the lungs and their attack on the extracellular matrix in the lung parenchyma.

Interestingly, morphometric analysis of alveolar interstitium showing elastin degradation found replaced with excessive collagen deposition thus forming interstitial fibrotic changes. In support of this, collagen:elastin ratios showed diffuse collagen deposition in the lung parenchyma in advanced fibrotic regions, which displayed an absence of elastin fibers and widespread alveolar epithelial disintegration in COVID-19 patients. Thus, a combination of elastolysis with epithelial and endothelial injury disrupts the alveolar-capillary barrier; increased alveolar rigidity may cause remodeling of the alveolar architecture, eventually inducing collagen expression, and, ultimately, engulfing fibrosis. These results support the view that elastolysis combined with epithelial injury precede collagen deposition and likely progress to interstitial fibrosis. The disintegration of alveolar architecture compromises gas exchange at the thin epithelial-capillary network in the alveoli. Thus, the results of this study indicate that elastolysis and alveolitis could contribute to collagenous fibrotic abnormalities, ultimately leading to respiratory dysfunction in severely ill COVID-19 patients.

These findings also draw a direct connection between pulmonary sequestration and degranulation of neutrophils, with the destruction of alveolar architecture. Many views of COVID-19 affected lungs show granulocytes and identify colocalized granule enzymes.[18-20] Among these, NE is the obvious candidate for the massive degradation of elastin fibers that precedes fibrotic abnormalities. Clearly, NE can be placed at the scene of matrix dissolution by immuno-fluorescence. However, it is difficult to measure changes in NE more distantly, such as in plasma from affected individuals. This is because NE that is locally released is rapidly inhibited by A1AT through the formation of NE-A1AT complexes. Interestingly, analysis of plasma samples of COVID-19 patients showed high levels of NE-A1AT complexes indicating elevated extracellular release of NE. The formation of NE-A1AT complexes thus may be used for diagnosis of neutrophil activation, NET release, and indicate tissue damage in COVID-19 patients. These observations need further investigation to validate and correlate their association with COVID-19.

In summary, the present study provides insights into the pathologic manifestations of COVID-19 and highlights that elastolytic activity plays a key role in exacerbating pulmonary pathogenesis in COVID-19 patients, especially in the pathologic development of pulmonary fibrosis. Dysregulated neutrophil activity and NETosis contribute to elastin degradation, ECM remodeling, and fibrotic changes in COVID-19. A better understanding of the mechanism of elastin degradation and its significance for the pathology of fibrosis will likely help to identify novel therapeutic targets and prevent pulmonary fibrosis in COVID-19 patients.

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Authors Contributions

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Table Legends

Table 1. Summary of the autopsy cases.

Clinical and biochemical signatures in COVID-19 positive and COVID-19 negative hospitalized patients during admission and progression of the COVID-19 disease in intensive care units.

Table 2 . Histopathologic signatures of the lung autopsies of COVID-19 patients. Semiquantitative histopathology scores of the lung autopsy samples from COVID-19 positive and COVID-19 negative patients.

Figure Legends

Figure 1. Lung histopathology analyses of COVID-19 patients.

Autopsy lung samples collected from COVID-19 patients show major patterns of (A) widespread diffuse alveolar damage, and (B) fibrosing organizing pneumonia. Fibrotic changes in COVID-19 patients show variable patterns including (C) interstitial fibrotic development (black arrows), (D) intra-alveolar fibrin deposition and organizing fibrosis showing spreading of fibrosis in alveoli (white arrows) and (E) diffuse alveolar fibrosis within small airways (arrowhead). (F) The loose myxoid fibroblastic proliferation extended between the alveoli forming Mason bodies in alveolar and bronchiolar regions (yellow frame). (G) Bronchiolitis obliterans organizing pneumonia (BOOP)-like areas (red frame). (H) The squamous cells formed distinct nodules resembling squamous morules were noted in COVID-19 patients (blue circles). (I) Disrupted small blood vessels were also displayed perivascular fibroblast proliferations (Hash). Scale Bars = 100 μm.

Figure 2. Elastin degradation in the lungs of the COVID-19 patients.

The quantitative measurement of elastin fibers length in μm. The elastin fibers present in the alveolar walls were measured using ImageJ analysis. (A) Among COVID-19 negative patients. Elastin fibers appear as bundles in the alveolar walls (red arrows). (B) In COVID-19 patients, fragmentation of elastin fibers was observed (open red arrow). (C) Lengths of elastin fibers in COVID-10 positive and COVID-19 negative patients. (D) Elastolysis (red arrowhead) in the perivascular regions of the COVID-19 patients compared to COVID-19 negative patients. To validate elastolysis observed in morphometric analyses, lung tissue lysates prepared from COVID-19 positive and COVID-19 negative patients were evaluated by western blot analysis. (E) The lung tissue lysates from COVID-19 negative (2-5 lanes) and COVID-positive (6-11 lanes), and soluble elastin (EL) (12) were probed with anti-elastin antibodies and the immunoblot shows a strong band at 70 kDa representing tropoelastin. Lung tissue lysates of COVID-19 patients show degradation of tropoelastin. (F) The densities of tropoelastin were normalized to the histone protein levels in the lung homogenates. The values of elastin fibers lengths were represented as means ± SD in lung sections from 8 COVID-19 patients and 6 non-COVID-19 patients (2C). Data was represented as mean ± SD from lung sections from 6 COVID-19 patients and 4 non-COVID-19 patients (2F). Scale bar=100 μm.

Figure 3. Loss of elastin found replaced with collagenous fibrosis in alveolar interstitium and perivascular regions.

The density of collagen distribution in the lung parenchyma was measured in (A) COVID-19 negative and (B) COVID-19 positive patients using Masson's trichrome collagen specific staining. (C) The density of collagen in the lung was quantified using ImageJ analysis as described in the methods. The values of collagen density were represented as means ± SD in lung sections from 8 COVID-19 patients and 6 non-COVID-19 patients. (D) Necrotic and denuded alveolar epithelial lining displayed elastolysis and (E) dense collagen deposition

(red arrows - loss of elastin in the alveolar walls; red asterisk - epithelial denudation; black arrows - collagen accumulation). (F, G, H) Collagen replacing elastin fibers in formation of interstitial fibrotic changes in COVID-19 patients evident by H&E, collagen deposition, and elastin degradation respectively (red frame). (I, J) Lung parenchyma shows alveolar collapse (red asterisks) and fibrotic development and reduction of elastin fibers. Scale bar=100 μm.

Figure 4. Measurement of collagen:elastin ratio.

The density of collagen and elastin fibers distribution in the lung parenchyma was measured in COVID-19 positive and COVID-19 negative patients. Adjacent lung sections stained with Masson's trichrome and orcein from (A, B) non-COVID-19 patient (A, collagen - black arrow) and (B, elastin – black arrowhead). COVID-19 patient lungs displayed widespread (C) collagen deposition, (D) complete loss of elastin. (E) The collagen:elastin ratio was quantified using ImageJ analysis as described in the methods. (F-H) Areas of fibrosing organizing pneumonia also displayed (G) extensive loss of alveolar epithelium stained with TTF-1 (yellow line), while undamaged areas show positive staining for TTF-1 and elastin (red arrow). Scale bar=100 μm.

Figure 5. Neutrophil influx, and NETosis in the lung parenchyma of COVID-19 lung autopsy cases.

(A) H&E staining of lung tissues with massive neutrophil influx. (B) Neutrophils show high expression of neutrophil elastase. (C) Insert from panel B depicts extracellular release of NE (white asterisk) in the alveoli. (D) Lung sections with immunohistochemistry for MPO also indicate neutrophil activation. (E) PAD4 staining. (F) Increased levels of total citrullinated proteins in the lungs of COVID-19 patients compared to COVID-19-negative patients. Scale Bar=100 μm.

Figure 6. Evaluation of NE-A1AT complex in the plasma samples by ELISA.

The levels of NE-A1AT complexes were found significantly higher in COVID-19 patients compared to healthy individuals.

Figure S1 Pathological lesions of alveolitis, bronchiolitis, inflammation thrombosis and pleuritic in COVID-19. (A) Alveolar epithelial denudation is evident by H&E staining (open arrow). (B) Extensive bronchiolitis with sloughing of bronchiolar epithelium was noted in all cases (black arrow). (C) Denudation of epithelial lining and formation of hyaline membranes in COVID-19 patients (black arrowhead), (D) edema with filling of alveolar air spaces with protein exudates (asterisks), (E) One specimen showed evidence of pleuritic (yellow frame). (F) In two cases, bacterial superinfections were noted with presence of bacterial colonies admixed with neutrophils within the alveoli (hash). (G) Blood vessels also show prominent fibrin thrombus. (H- I) Perivascular regions showing loosely proliferating fibroblasts, accumulation of collagen, and (H) degradation of elastin fibers (red arrows). Scale bar=100 μm.

References

1. Ravaglia C, Doglioni C, Chilosi M, Piciucchi S, Dubini A, Rossi G, Pedica F, Puglisi S, Donati L, Tomassetti S, Poletti V. Clinical, radiological and pathological findings in patients with persistent lung disease following SARS-CoV-2 infection. Eur Respir J. 2022;60(4):2102411.
2. Mauad T, Duarte-Neto AN, da Silva LFF, de Oliveira EP, de Brito JM, do Nascimento ECT, de Almeida Monteiro RA, Ferreira JC, de Carvalho CRR, do Nascimento Saldiva PH, Dolhnikoff M. Tracking the time course of pathological patterns of lung injury in severe COVID-19. Respir Res. 2021;22(1):32.
3. Merdji H, Mayeur S, Schenck M, Oulehri W, Clere-Jehl R, Cunat S, Herbrecht JE, Janssen-Langenstein R, Nicolae A, Helms J, Meziani F, Chenard MP; CRICS TRIGGERSEP Group (Clinical Research in Intensive Care, Sepsis Trial Group for Global Evaluation, Research in Sepsis). Histopathological features in fatal COVID-19 acute respiratory distress syndrome. Medicina Intensiva. 2021;45(5):261-270.

4. Batah SS, Fabro AT. Pulmonary pathology of ARDS in COVID-19: A pathological review for clinicians. Respir Med. 2021;176:106239.
5. Valdebenito S, Bessis S, Annane D, Lorin de la Grandmaison G, Cramer-Bordé E, Prideaux B, Eugenin EA, Bomsel M. COVID-19 Lung Pathogenesis in SARS-CoV-2 Autopsy Cases. Front Immunol. 2021;12:735922.
6. Yao XH, Luo T, Shi Y, He ZC, Tang R, Zhang PP, Cai J, Zhou XD, Jiang DP, Fei XC, Huang XQ, Zhao L, Zhang H, Wu HB, Ren Y, Liu ZH, Zhang HR, Chen C, Fu WJ, Li H, Xia XY, Chen R, Wang Y, Liu XD, Yin CL, Yan ZX, Wang J, Jing R, Li TS, Li WQ, Wang CF, Ding YQ, Mao Q, Zhang DY, Zhang SY, Ping YF, Bian XW. A cohort autopsy study defines COVID-19 systemic pathogenesis. Cell Res. 2021;31(8):836-846.
7. Li Y, Wu J, Wang S, Li X, Zhou J, Huang B, Luo D, Cao Q, Chen Y, Chen S, Ma L, Peng L, Pan H, Travis WD, Nie X. Progression to fibrosing diffuse alveolar damage in a series of 30 minimally invasive autopsies with COVID-19 pneumonia in Wuhan, China. Histopathology. 2021;78(4):542-555.
8. Wells AU, Devaraj A, Desai SR. Interstitial lung disease after COVID-19 infection: a catalog of uncertainties. *Radiology.* 2021;299(1):E216–e8.
9. Berezowska S, Schmid A, Losmanová T, Trippel M, Blank A, Banz Y, Jakob SM, Langer R. Frequency and Significance of Pathologic Pulmonary Findings in Postmortem Examinations-A Single Center Experience before COVID-19. Diagnostics (Basel). 2021;11(5):894.
10. Spagnolo P, Balestro E, Aliberti S, et al. Pulmonary fibrosis secondary to COVID-19: a call to arms? *Lancet Respir Med.* 2020;8(8):750–752. [PMC free article] [PubMed] [Google Scholar]
11. Caruso D, Guido G, Zerunian M, Polidori T, Lucertini E, Pucciarelli F, Polici M, Rucci C, Bracci B, Nicolai M, Cremona A, De Dominicis C, Laghi A. Post-Acute Sequelae of COVID-19 Pneumonia: Six-month Chest CT Follow-up. Radiology. 2021;301:E396-405.
12. Huang, W.J., Tang, X.X. Virus infection induced pulmonary fibrosis. *J Transl Med* 19, 496 (2021).
13. Qiao J, Zhang M, Bi J, Wang X, Deng G, He G, Luan Z, Lv N, Xu T, Zhao L. Pulmonary fibrosis induced by H5N1 viral infection in mice. Respir Res. 2009;10(1):107.
14. Wu X, Dong D, Ma D. Thin-Section Computed Tomography Manifestations During Convalescence and Long-Term Follow-Up of Patients with Severe Acute Respiratory Syndrome (SARS). *Med Sci Monit* 2016;22:2793–2799.
15. Arabi YM, Balkhy HH, Hayden FG, Bouchama A, Luke T, Baillie JK, Al-Omari A, Hajeer AH, Senga M, Denison MR, Nguyen-Van-Tam JS, Shindo N, Bermingham A, Chappell JD, Van Kerkhove MD, Fowler RA. Middle East Respiratory Syndrome. N Engl J Med. 2017;376(6):584-594.
16. Chanda D, Otoupalova E, Smith SR, Volckaert T, De Langhe SP, Thannickal VJ. Developmental pathways in the pathogenesis of lung fibrosis. Mol Aspects Med. 2019;65:56-69.
17. Sinha S, Castillo V, Espinoza CR, Tindle C, Fonseca AG, Dan JM, Katkar GD, Das S, Sahoo D, Ghosh P. COVID-19 lung disease shares driver AT2 cytopathic features with Idiopathic pulmonary fibrosis. EBioMedicine. 2022 Aug;82:104185.
18. Ackermann M, Anders HJ, Bilyy R, Bowlin GL, Daniel C, De Lorenzo R, Egeblad M, Henneck T, Hidalgo A, Hoffmann M, Hohberger B, Kanthi Y, Kaplan MJ, Knight JS, Knopf J, Kolaczkowska E, Kubes P, Leppkes M, Mahajan A, Manfredi AA, Maueröder C, Maugeri N, Mitroulis I, Muñoz LE, Narasaraju T, Naschberger E, Neeli I, Ng LG, Radic MZ, Ritis K, Rovere-Querini P, Schapher M, Schauer C, Simon HU, Singh J, Skendros P, Stark K, Stürzl M, van der Vlag J, Vandenabeele P, Vitkov L, von Köckritz-Blickwede M, Yanginlar C, Yousefi S, Zarbock A, Schett G, Herrmann M. Patients with COVID-19: in the dark-NETs of neutrophils. Cell Death Differ. 2021;28(11):3125-3139.
19. Werner J D Ouwendijk, Matthijs P Raadsen, Jeroen J A van Kampen, Robert M Verdijk, Jan H von der Thusen, Lihui Guo, Rogier A S Hoek, Johannes P C van den Akker, Henrik Endeman, Thomas Langerak, Richard Molenkamp, Diederik Gommers, Marion P G Koopmans, Eric C M van Gorp, Georges M G M Verjans, Bart L Haagmans, High Levels of Neutrophil Extracellular Traps Persist in the Lower Respiratory Tract of Critically Ill Patients With Coronavirus Disease 2019, *The Journal of Infectious Diseases* . 2021;223: 1512–1521.
20. Seren S, Derian L, Keleş I, Guillon A, Lesner A, Gonzalez L, Baranek T, Si-Tahar M, Marchand-

Adam S, Jenne DE, Paget C, Jouan Y, Korkmaz B. Proteinase release from activated neutrophils in mechanically ventilated patients with non-COVID-19 and COVID-19 pneumonia. Eur Respir J. 2021;57(4):2003755.

21. Wang Y, Schughart K, Pelaia TM, Chew T, Kim K, Karvunidis T, Knippenberg B, Teoh S, Phu AL, Short KR, Iredell J, Thevarajan I, Audsley J, Macdonald S, Burcham J, McLean A; PREDICT-19 consortium; Tang B, Shojaei M. Blood transcriptome responses in patients correlate with severity of COVID-19 disease. Front Immunol. 2023;13:1043219.
22. Gagiannis D, Steinestel J, Hackenbroch C, et al. Clinical, serological, and histopathological similarities between severe COVID-19 and acute exacerbation of connective tissue disease-associated interstitial lung disease (CTD-ILD) *Front Immunol.* 2020;11
23. Kozel BA, mecham RP, Rosenbloom J. Elastin. In: Mecham RP, editorBiology of Extracellular Matrix. Springer-Verlag; Berlin-Heidelberg: 2011. 267–301.
24. Davidson JM. Biochemistry and turnover of lung interstitium. Eur Respir J. 1990;3: 1048–68.
25. Mecham RP. Elastin in lung development and disease pathogenesis. Matrix Biol. 2018;73:6-20.
26. Deslee G, Woods JC, Moore CM, Liu L, Conradi SH, Milne M, Gierada DS, Pierce J, Patterson A, Lewit RA, Battaile JT, Holtzman MJ, Hogge JC, Pierce RA. Elastin expression in very severe human COPD. Eur Respir J. 2009;34:324–31.
27. mShapiro SD, Endicott SK, Province MA, Pierce JA, Campbell EJ. Marked longevity of human lung parenchymal elastic fibers deduced from prevalence of D-aspartate and nuclear weapons-related radiocarbon. J Clin Invest. 1991;87(5):1828-34.
28. Liu X, Ma S, Turino G, Cantor J. The pattern of elastic Fiber breakdown in Bleomycin-induced pulmonary fibrosis may reflect microarchitectural changes. Lung. 2017;195:93–9.
29. Benjamin JT, Plosa EJ, Sucre JM, van der Meer R, Dave S, Gutor S, Nichols DS, Gulleman PM, Jetter CS, Han W, Xin M, Dinella PC, Catanzarite A, Kook S, Dolma K, Lal CV, Gaggar A, Blalock JE, Newcomb DC, Richmond BW, Kropski JA, Young LR, Guttentag SH, Blackwell TS. Neutrophilic inflammation during lung development disrupts elastin assembly and predisposes adult mice to COPD. J Clin Invest. 2021;131: e139481.
30. Starcher B, Peterson B. The kinetics of elastolysis: elastin catabolism during experimentally induced fibrosis. Exp Lung Res. 1999;25(5):407-24.
31. de Brouwer B, Drent M, van den Ouweland JMW, Wijnen PA, van Moorsel CHM, Bekers O, Grutters JC, White ES, Janssen R. Increased circulating desmosine and age-dependent elastinolysis in idiopathic pulmonary fibrosis. Respir Res. 2018;19:45.
32. Araos J, Ayala P, Meneses M, Contreras R, Cutiño A, Montalva R, Tazelaar H, Borzone G. Resolution of lung injury after a single event of aspiration: a model of bilateral instillation of whole gastric fluid. Am J Pathol. 2015;185: 2698–708.
33. He J, Turino GM, Lin YY. Characterization of peptide fragments from lung elastin degradation in chronic obstructive pulmonary disease. Exp Lung Res. 2010;36:548–57.
34. Wewers MD, Crystal RG. Alpha-1 antitrypsin augmentation therapy. COPD. 2013;10 Suppl 1:64-7.
35. D'Souza RF, Masson SWC, Woodhead JST, James SL, MacRae C, Hedges CP, Merry TL. α1-Antitrypsin A treatment attenuates neutrophil elastase accumulation and enhances insulin sensitivity in adipose tissue of mice fed a high-fat diet. Am J Physiol Endocrinol Metab. 2021;321:E560-E570.
36. Chang SE, Feng A, Meng W, Apostolidis SA, Mack E, Artandi M, Barman L, Bennett K, Chakraborty S, Chang I, Cheung P, Chinthrajah S, Dhingra S, Do E, Finck A, Gaano A, Geßner R, Giannini HM, Gonzalez J, Greib S, Gündisch M, Hsu AR, Kuo A, Manohar M, Mao R, Neeli I, Neubauer A, Oniyide O, Powell AE, Puri R, Renz H, Schapiro J, Weidenbacher PA, Wittman R, Ahuja N, Chung HR, Jagannathan P, James JA, Kim PS, Meyer NJ, Nadeau KC, Radic M, Robinson WH, Singh U, Wang TT, Wherry EJ, Skevaki C, Luning Prak ET, Utz PJ. New-onset IgG autoantibodies in hospitalized patients with COVID-19. Nat Commun. 2021;12:5417.
37. Bar KJ, Shaw PA, Choi GH, Aqui N, Fesnak A, Yang JB, Soto-Calderon H, Grajales L, Starr J, Andronov M, Mastellone M, Amonu C, Feret G, DeMarshall M, Buchanan M, Caturla M, Gordon J, Wanicur A, Monroy MA, Mampe F, Lindemuth E, Gouma S, Mullin AM, Barilla H, Pronina A,

Irwin L, Thomas R, Eichinger RA, Demuth F, Luning Prak ET, Pascual JL, Short WR, Elovitz MA, Baron J, Meyer NJ, Degnan KO, Frank I, Hensley SE, Siegel DL, Tebas P. A randomized controlled study of convalescent plasma for individuals hospitalized with COVID-19 pneumonia. J Clin Invest. 2021;131:e155114.

38. Mauad T, Duarte-Neto AN, da Silva LFF, de Oliveira EP, de Brito JM, do Nascimento ECT, de Almeida Monteiro RA, Ferreira JC, de Carvalho CRR, do Nascimento Saldiva PH, Dolhnikoff M. Tracking the time course of pathological patterns of lung injury in severe COVID-19. Respir Res. 2021;22:32.
39. Merdji H, Mayeur S, Schenck M, Oulehri W, Clere-Jehl R, Cunat S, Herbrecht JE, Janssen-Langenstein R, Nicolae A, Helms J, Meziani F, Chenard MP; CRICS TRIGGERSEP Group (Clinical Research in Intensive Care, Sepsis Trial Group for Global Evaluation, Research in Sepsis). Histopathological features in fatal COVID-19 acute respiratory distress syndrome. Med Intensiva (Engl Ed). 2021;45:261-270.
40. Valdebenito S, Bessis S, Annane D, Lorin de la Grandmaison G, Cramer-Bordé E, Prideaux B, Eugenin EA, Bomsel M. COVID-19 Lung Pathogenesis in SARS-CoV-2 Autopsy Cases. Front Immunol. 2021;12:735922.
41. Zweers MC, van Vlijmen-Willems IM, van Kuppevelt TH, Mecham RP, Steijlen PM, Bristow J, Schalkwijk J. Deficiency of tenascin-X causes abnormalities in dermal elastic fiber morphology. J Invest Dermatol. 2004;122:885-91.
42. Kottmann RM, Sharp J, Owens K, Salzman P, Xiao GQ, Phipps RP, Sime PJ, Brown EB, Perry SW. Second harmonic generation microscopy reveals altered collagen microstructure in usual interstitial pneumonia versus healthy lung. Respir Res. 2015;16:61.
43. Dwivedi N, Hedberg A, Zheng YY, Neeli I, Satoh M, Morel L, Rekvig OP, Radic M. B Cell Tolerance to Deiminated Histones in BALB/c, C57BL/6, and Autoimmune-Prone Mouse Strains. Front Immunol. 2017;8:362.
44. Neumann S, Hennrich N, Gunzer G, Lang H. Enzyme-linked immunoassay for human granulocyte elastase in complex with alpha 1-proteinase inhibitor. Adv Exp Med Biol. 1984;167:379-90
45. Engelmaier A, Weber A. Sensitive and specific measurement of alpha$_1$-antitrypsin activity with an elastase complex formation immunosorbent assay (ECFISA). J Pharm Biomed Anal. 2022;209:114476
46. Ochs M, Timm S, Elezkurtaj S, Horst D, Meinhardt J, Heppner FL, Weber-Carstens S, Hocke AC, Witzenrath M. Collapse induration of alveoli is an ultrastructural finding in a COVID-19 patient. Eur Respir J. 2021;57(5):2004165
47. McElvaney OJ, McEvoy NL, Boland F, McElvaney OF, Hogan G, Donnelly K, Friel O, Browne E, Fraughen DD, Murphy MP, Clarke J, Choileáin ON, O'Connor E, McGuinness R, Boylan M, Kelly A, Hayden JC, Collins AM, Cullen A, Hyland D, Carroll TP, Geoghegan P, Laffey JG, Hennessy M, Martin-Loeches I, McElvaney NG, Curley GF. A randomized, double-blind, placebo-controlled trial of intravenous alpha-1 antitrypsin for ARDS secondary to COVID-19. Med. 2022;3(4):233-248.e6.
48. Mahroum N, Seida I, Esirgün SN, Bragazzi NL. The COVID-19 pandemic - How many times were we warned before? Eur J Intern Med. 2022;105:8-14.
49. Voskarides K. SARS-CoV-2: tracing the origin, tracking the evolution. BMC Med Genomics. 2022;15:62.
50. Nugent MA. The Future of the COVID-19 Pandemic: How Good (or Bad) Can the SARS-CoV2 Spike Protein Get? Cells. 2022;11:855.
51. Burkhardt A. Pathogenesis of pulmonary fibrosis. Hum Pathol 1986;17: 971–973.
52. Spencer H. Pathogenesis of interstitial fibrosis of the lung. Progr Respir Res. 1975;8: 34-44.
53. Froese AR, Shimbori C, Bellaye PS, Inman M, Obex S, Fatima S, Jenkins G, Gauldie J, Ask K, Kolb M. Stretch-induced Activation of Transforming Growth Factor-β1 in Pulmonary Fibrosis. Am J Respir Crit Care Med. 2016;194(1):84-96.
54. D'Agnillo F, Walters KA, Xiao Y, Sheng ZM, Scherler K, Park J, Gygli S, Rosas LA, Sadtler K, Kalish H, Blatti CA 3rd, Zhu R, Gatzke L, Bushell C, Memoli MJ, O'Day SJ, Fischer TD, Hammond TC, Lee RC, Cash JC, Powers ME, O'Keefe GE, Butnor KJ, Rapkiewicz AV, Travis WD, Layne SP, Kash JC, Taubenberger JK. Lung epithelial and endothelial damage, loss of tissue repair, inhibition of

fibrinolysis, and cellular senescence in fatal COVID-19. Sci Transl Med. 2021;13:eabj7790.
55. Nagel D, Clough R, Sime PJ , Kottamann RM. Elastin degradative products induce myofibroblast differentiation Am J Resp Crit Care Med 2019, 199:A5333.
56. Gong J, Dong H, Xia QS, Huang ZY, Wang DK, Zhao Y, Liu WH, Tu SH, Zhang MM, Wang Q, Lu FE. Correlation analysis between disease severity and inflammation-related parameters in patients with COVID-19: a retrospective study. BMC Infect Dis. 2020;20(1):963.
57. Wang X, Li X, Shang Y, Wang J, Zhang X, Su D, Zhao S, Wang Q, Liu L, Li Y, Chen H. Ratios of neutrophil-to-lymphocyte and platelet-to-lymphocyte predict all-cause mortality in inpatients with coronavirus disease 2019 (COVID-19): a retrospective cohort study in a single medical centre. Epidemiol Infect. 2020;148:e211.
58. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. JAMA. 2020 Mar 17;323(11):1061-1069. doi: 10.1001/jama.2020.1585. Erratum in: JAMA. 2021;325:1061–9.
59. Barnes BJ, Adrover JM, Baxter-Stoltzfus A, Borczuk A, Cools-Lartigue J, Crawford JM, Daßler-Plenker J, Guerci P, Huynh C, Knight JS, Loda M, Looney MR, McAllister F, Rayes R, Renaud S, Rousseau S, Salvatore S, Schwartz RE, Spicer JD, Yost CC, Weber A, Zuo Y, Egeblad M. Targeting potential drivers of COVID-19: Neutrophil extracellular traps. J Exp Med. 2020 ;217(6):e20200652.

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Table 1 **Summary of the autopsy cases**

No	sex	Age	Upon hospitalization	Days in the hospital	Disease duration	SARS-CoV-2 PCR positive, at day	ESR		Lymphocytes				Neutrophils			
all - cause of death is Acute hemorrhagic tracheobronchitis, respiratory distress syndrome (according to local classification).							upon hospitalization	last record	upon hospitalization	upon hospitalization	last record	last record	upon hospitalization	upon hospitalization	last record	last record
									x10^9/L	%	x10^9/L	%	x10^9/L	%	x10^9/L	%
COVID-19	M	61	bilateral pneumonia COVID19	10	16	6	55	46	18.3	72.5	26.6	61.2	4.6	18.3	12.5	24
COVID-19	F	64	bilateral pneumonia COVID19	21	27	6	72	49	0.2	14.8	0.4	8.7	1	80.8	4.2	87.4
COVID-19	M	59	bilateral pneumonia COVID19, DM	5	8	2	45	42	0.76	13	0.5	8.1	4.63	79	58	89
COVID-19	F	80	bilateral pneumonia COVID19	19	26	7	47	24	0.2	n/a	0.9	6.1	11.4	84.1	13	91
COVID-19	F	61	bilateral pneumonia COVID19	19	24	5	42	17	1.1	10.6	1	14.9	8.6	81.9	5.5	80.30
COVID-19	M	30	pneumonia (unilateral, R) COVID19	17	26	9	53	42	0.6	9	0.5	2.5	11.6	95	17.4	93.8
COVID-19	M	71	pneumonia (unilateral, R) COVID20	16	24	8	37	9	0.7	6.9	1.1	10.8	9.6	89.2	5.36	85.5
COVID-19	F	56	bilateral pneumonia COVID19	13	27	14	49	29	0.4	7.2	0.4	6.5	5	89.1	5.7	87.7
mean		60.3		15.0	22.3	7.1	50.0	32.3	2.8	19.1	3.9	14.9	7.1	77.2	15.2	79.8
non-COVID-19	F	81	Caecum cancer pT3N0M0G2	4		0	28		4.6	32				58		
non-COVID-19	M	85	Atherosclerotic aneurism of ifranenal aortha with rupture	1	1	0	9		6.2	37				62		
non-COVID-19	F	66	Hypertensive microangiopaty of brain, kidney and pancreas	21		0	14	12	5.4	39				52		
mean		77.3		8.7	1.0	-	17.0	12.0	5.4	36.0				57.3		

Note: Additional 3 non-COVID-19 lung autopsy samples were received from UTHSC Memphis Tennessee. The samples were received from non-COVID-19 patients and the clinical background of these patients is not available.

Table 2

Variables	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Alveolar changes								
Hemorrhage	1	1	1	3	0	1	1	2
Edema	1	2	0	0	1	3	0	3
Fibrin deposition	1	0	0	3	1	3	1	1
Hyaline membrane	1	1	0	0	0	1	0	1
Exfoliation	1	1	1	2	2	1	1	1
Necrosis of epithelium	1	1	1	0	0	1	1	1
Type 2 Pneumocyte hyperplasia	3	3	1	3	1	3	3	3
Organization (fibrosis)	0	2	3 Bacterial colonies, BOOP Masson bodies	3	3	1	2	0
Inflammation	0	2	3	2	3	3	0	1
Multinucleate giant cells	0	0	0	0	0	1	0	0
Others	Atypia of type II pneumocytes	Atypia of type II pneumocytes	Reed Sternberg like cells Sqamous morules	Atypia of type II pneumocytes	Acute eosinophilic pneumonia	Corpora amylacea	-	-
Interstitial changes								
Hemorrhage	2	2	3	3	0	1	1	0
Expansion	2	2	3	3	2	1	2	1
Inflammation	2	2	3	1	1	1	1	1
Fibrosis	1	2	2	3	0	0	3	1
Fibrin depostition	1	1	1	3	0	1	1	1
Vasculitis	1	1	0	1	0	1	1	1
Thrombi	1	1	0	2	0	1	1	0
Pleura	1	0	0	0	0	0	0	0
Bronchiolitis	1	1	1	1	1	1	1	1

Alveolar changes

- Hemorrhage – Absent-0, Mild -1, Moderate-2, Severe-3
- Edema - Absent-0, Mild -1, Moderate-2, Severe-3
- Fibrin deposition- Absent-0, Mild -1, Moderate-2, Severe-3
- Hyaline membrane formation- Absent-0, Mild -1, Moderate-2, Severe-3
- Type II pneumocyte hyperplasia- Absent-0, Mild -1, Moderate-2, Severe-3
- Organization (fibrosis)- Absent-0, Mild -1, Moderate-2, Severe-3
- Inflammation- Absent-0, Mild -1, Moderate-2, Severe-3
- Epithelial necrosis-Absent-0, Present-1
- Exfoliation- Absent-0, Present-1
- Atypia in alveolar - Absent-0, Present-1
- Multinucleate giant cells- Absent-0, Present-1
- Bronchiolitis obliterans organizing pneumonia-BOOP

Interstitial changes

- Expansion- Absent-0, Mild -1, Moderate-2, Severe-3
- Inflammation – Absent-0, Mild -1, Moderate-2, Severe-3
- Fibrosis – Absent-0, Mild -1, Moderate-2, Severe-3
- Fibrin deposition- Absent-0, Mild -1, Moderate-2, Severe-3
- Vasculitis- Absent-0, Present-1
- Thrombi- Few vessels, many vessels (Absent -0, Few vessels (0-3) -1 , Many vessels (>4) - 2)
- Pleura -Chronic inflammation - Absent-0, Present-1
- Bronchiolitis- Absent-0, Present-1