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Post-mortem lung tissue: the fossil record of the pathophysiology and immunopathology of severe COVID-19

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The lungs are the main site that is affected in severe COVID-19, and post-mortem lung tissue provides crucial insights into the pathophysiology of severe disease. From basic histology to state-of-the-art multiparameter digital pathology technologies, post-mortem lung tissue provides snapshots of tissue architecture, and resident and inflammatory cell phenotypes and composition at the time of death. Contrary to early assumptions that COVID-19 in the lungs is a uniform disease, post-mortem findings have established a high degree of disease heterogeneity. Classic diffuse alveolar damage represents just one phenotype, with disease divisible by early and late progression as well as by pathophysiological process. A distinct lung tissue state occurs with secondary infection; extrapulmonary causes of death might also originate from a pathological process in the lungs linked to microthrombosis. This heterogeneity of COVID-19 lung disease must be recognised in the management of patients and in the development of novel treatment strategies.

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See Online for appendix

Introduction

SARS-CoV-2, the positive-sense, single-stranded RNA betacoronavirus, is responsible for the COVID-19 pandemic.[1] COVID-19 is a multisystem disease, but the respiratory system is the primary viral target and main site for disease progression. Increasingly described as a biphasic disease,[2] a progressive second phase seems to be driven by immunopathology. However, superimposed infection and thrombotic complications also precipitate clinical deterioration. The precise pathophysiological mechanisms that underlie all modes of deterioration remain unclear. Autopsies have been crucial in understanding pulmonary and extrapulmonary manifestations of COVID-19.[3] Although inferences can be made from clinical investigations such as peripheral blood sampling, post-mortem tissue analysis provides an unparalleled snapshot of tissue architecture, cellular constituencies, and cell gene-expression profiles, function, and interactions at the time of death. Several alternative approaches to tissue sampling in severe COVID-19 include mini-thoracotomy and transbronchial biopsy;[4,5] however, these methods are anatomically limited and not without risk. Analysis of explanted lung tissue from recipients of lung transplants after COVID-19 is hampered by low numbers and the picture is dominated by an advanced fibrotic disease stage.[6]

Post-mortem lung tissue can be analysed with conventional techniques, including light microscopy, immunohistochemistry, and immunofluorescence, as well as less conventional technologies such as imaging mass cytometry, electron microscopy, high-resolution tissue transcriptomics and proteomics, and digital spatial profiling.[7] SARS-CoV-2 proteins or RNA can be precisely localised within tissue by immunohistochemistry, imaging mass cytometry, electron microscopy, and in-situ hybridisation. A recurring theme across all these platforms is the finding of lung tissue heterogeneity both within and between patients who had COVID-19.[8] Establishing the overarching phenotype by conventional imaging approaches is essential to the subsequent interpretation of results from more advanced, deep-dive tissue imaging techniques.

In this Review, we aim to collate findings from the full range of approaches to post-mortem lung tissue analysis that have been used in COVID-19 autopsy studies—the fossil record of severe COVID-19 pathophysiology and immunopathology, from which trajectories of decline associated with the course of COVID-19 can be inferred. Details of key studies discussed in this Review are presented in the appendix; a glossary of terms is

Key messages

- Post-mortem lung tissue offers unrivalled insights into severe COVID-19 pathophysiology and immunopathology, providing a snapshot at the time of death of the cells present, their functional state(s), and their spatial relationship to damaged tissue and other cells
- Post-mortem lung tissue is highly heterogeneous, but three major tissue phenotypes have emerged, representing distinct pathological processes in the lungs: a classic phenotype characterised by progressive diffuse alveolar damage, bronchopneumonia resulting from secondary infection, and tissue thrombosis; these phenotypes are not mutually exclusive and often overlap
- Conventional tissue pathology techniques have been crucial in establishing tissue heterogeneity; however, a range of deep-dive technologies have generated novel insights through detailed architectural and immune phenotyping
- A combinatorial approach that integrates conventional techniques and deep-dive imaging approaches to dissect tissue is the optimal approach for contextualising and validating results from post-mortem tissue
- The pathologist's adage *mortui vivos docent*—Latin for "the dead teach the living"—should be remembered, because as the pandemic progresses, post-mortem tissue has the potential to provide essential information to guide treatment approaches for COVID-19

Panel 1: Glossary

Cell clustering
A means of analysing omics datasets by grouping cells on the basis of their expression profiles

Digital spatial profiling
An advanced pathology technique that uses oligonucleotide-linked tissue-bound probes to generate multiplexed readouts of RNA or protein expression patterns in tissue

Heatmap
A graphical representation to visualise the differential expression profiles of defined cell clusters

Imaging mass cytometry
A technique based on the simultaneous use of up to 40 metal-labelled antibodies, which are bound to tissue, laser-ablated, and spatially identified through the generation of multiplexed imaging data

In-situ hybridisation
A technique in which labelled nucleic acid-based probes bind to and highlight complementary strands in tissue

Multiplex pathology
Pathology techniques that involve labelling of structures of interest, enabling simultaneous analysis of two or more targets

Proteomics
Techniques that enable qualitative and quantitative analyses of proteins in tissue to investigate expression profiles

Region of interest
An area of tissue that has been selected for analysis, usually with conventional pathology techniques and often for analysis with advanced tissue pathology technologies

Single-cell segmentation
Bioinformatics separation of cells from multiplexed images on the basis of expression of markers and their location—eg, cell membrane, nucleus, extracellular location

Spatial analysis
Analysis of cell–cell interactions within tissue, including interactions between neighbouring and non-neighbouring cells and avoidances (ie, the absence of interactions between cells that usually interact)

Transcriptomics
Techniques that enable qualitative and quantitative analyses of RNA in tissue to investigate expression profiles

provided in panel 1. We consider the dominant disease phenotypes that have been identified through studies of post-mortem tissue, and the implications of these findings for future research directions and the development of targeted therapeutic options to improve the clinical management and health outcomes of patients with COVID-19.

Conventional imaging approaches

Light microscopy

Light microscopy is used routinely in post-mortem examinations and is thus extensively reported. In an autopsy series of 38 patients who died with COVID-19 in hospitals in northern Italy early in the pandemic, Carsana and colleagues[9] identified the predominant histological pattern as diffuse alveolar disease (DAD). DAD refers to damage to pneumocytes and alveolar endothelial cells, the two main cell types populating lung alveoli.[10] The acute (exudative or early) stage is characterised by the microscopic hallmark of hyaline membranes, accompanied by interstitial and alveolar oedema, and by congested and haemorrhagic alveolar septa containing cellular debris and mononuclear inflammatory cell infiltrates (figure 1A). Mononuclear cells also infiltrate the perivascular space (figure 1B). The acute phase is followed by the organising phase, in which reparative pneumocyte hyperplasia and fibroblast proliferation result in septal and alveolar fibrin deposition, septal thickening, and grossly disrupted lung architecture (figure 1C).[11] Marked heterogeneity between patients has been reported, with both phases of DAD often coexisting and areas of transition to proliferative phases observed.[8,12] DAD was also the major finding in a study of three COVID-19 deaths in community settings.[13]

Light microscopy studies have provided the groundwork for the growing appreciation of other COVID-19 disease phenotypes that lead to decline. An early autopsy series of four patients from Wuhan, China, who died with COVID-19 noted that although DAD appeared to be the dominant histological lesion, a distinct pattern was seen in one patient, who had marked predominance of intra-alveolar neutrophils, which was thought by the authors to represent secondary bacterial infection.[11] Grosse and colleagues[8] reported superimposed acute broncho-pneumonia features in 11 of 14 patients, and this was thought to be the primary cause of death in two patients; bacterial cultures in these cases identified *Staphylococcus aureus* and *Enterococcus faecalis* species. This phenotype is characterised by areas of bronchiolo-alveolar neutrophilic suppuration[8] (figure 1D). In another series, 10 of 21 patients were found to have superimposed bronchopneumonia with diffuse and focal patterns.[14] A post-mortem study of eight patients reported systemic fungal infections in six cases, four having invasive lung mycoses (with *Aspergillus* spp identified in three patients and *Mucormycetes* spp in one patient).[15] Superimposed fungal infection might also represent a separate disease phenotype.

Further COVID-19 disease phenotypes are more difficult to differentiate by lung histology alone. An autopsy series from New York City, USA, noted that 11 of 40 patients had no acute lung injury, with just two having a definitive cause of death.[16] Indeed, there is a range of COVID-19-related sequelae, often involving thrombotic events, including pulmonary embolism, acute myocardial infarction, and deep vein thrombosis.[17] Grosse and

colleagues[8] observed that 3 of 14 autopsied patients had died with acute myocardial infarction. Acute myocardial infarction might be attributable to plaque destabilisation, cytokine or catecholamine dysregulation, or hypoxia during acute pneumonia. COVID-19 pneumonia itself might also contribute to a hypercoagulable state and an increased risk of thrombotic events. Acute cardiac failure has a distinct appearance in post-mortem lung tissue, featuring marked vascular congestion, prominent pulmonary veins, haemorrhage, interstitial and alveolar oedema (figure 1E), and features of increased back pressure from a failing heart.

The association between COVID-19 infection and thrombotic sequelae is under intense scrutiny.[17] Indicators of hypercoagulable states are more often elevated in non-survivors than in survivors of COVID-19.[18] Fibrin, platelet-rich microthrombi, or both are commonly reported in lung histology; for example, these structures were identified in 33 of the 38 patients studied by Carsana and colleagues,[9] and were evident in all nine autopsied patients in another study.[18] A platelet-rich thrombus in an intra-acinar vessel is shown in figure 1F. In a case series of 68 autopsies, 84% of patients had microthrombi and 42% had pulmonary arterial thrombi, despite 71% having been treated with anticoagulants.[19]

Pulmonary megakaryocytes are involved in platelet biogenesis[20] and might have a role in thrombosis generation. In one autopsy series, an increase in pulmonary megakaryocytes was seen in individuals who had DAD and died of COVID-19 compared with controls who had DAD but died of other causes,[21] although this increase in megakaryocytes was not statistically significant. Fox and co-workers[12] observed megakaryocytes undergoing thrombopoiesis in alveolar capillaries from COVID-19 autopsy sections, and megakaryocytes have also been noted in platelet aggregates and thrombi.[22] An increase in denuded megakaryocytes, indicating an exhaustive endpoint of thrombopoiesis, was reported by Roncati and colleagues.[23] They noted that interleukin-6 (IL-6), which is known to stimulate megakaryocytopoiesis, was elevated in the serum samples of all cases, suggesting an immunothrombotic effect. However, IL-6 elevation has been a variable feature in tissue studies.[24,25]

Pulmonary hypercoagulability might also be explained by endotheliitis and endothelial dysfunction, involving an imbalance between production or exposure of prothrombotic versus fibrinolytic factors, systemic cytokine overproduction, or the formation of prothrombotic neutrophil extracellular traps, all of which have been observed in COVID-19 post-mortem studies.[17,26]

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence allow more nuanced assessment of cell constituencies in COVID-19 post-mortem lung tissue than does light microscopy.[27] In particular, combining immunohistochemistry with haematoxylin and eosin stains highlights temporal differences in immune and structural cells in the context of evolving tissue architecture as disease progresses.[7] The key immune responders during early disease are macrophages ($CD68^+$) and T lymphocytes ($CD3^+$), which have alveolar and interstitial tissue distribution, respectively.[9] Later, fibroblast and myofibroblast proliferation leads to collagen deposition and fibrosis.[28] Immunohistochemistry highlights the varied distribution of immune cells in post-mortem lung tissue; in one autopsy series, $CD68^+$ macrophages were predominantly localised to areas with DAD.[25] Immunohistochemistry can also be used to detect soluble proteins such as cytokines and chemokines. An abundance of the proinflammatory cytokines IL-6, C-X-C motif chemokine ligand 10 (CXCL10), tumour necrosis factor (TNF), and IL-1β was detected in tissue sections, associated with

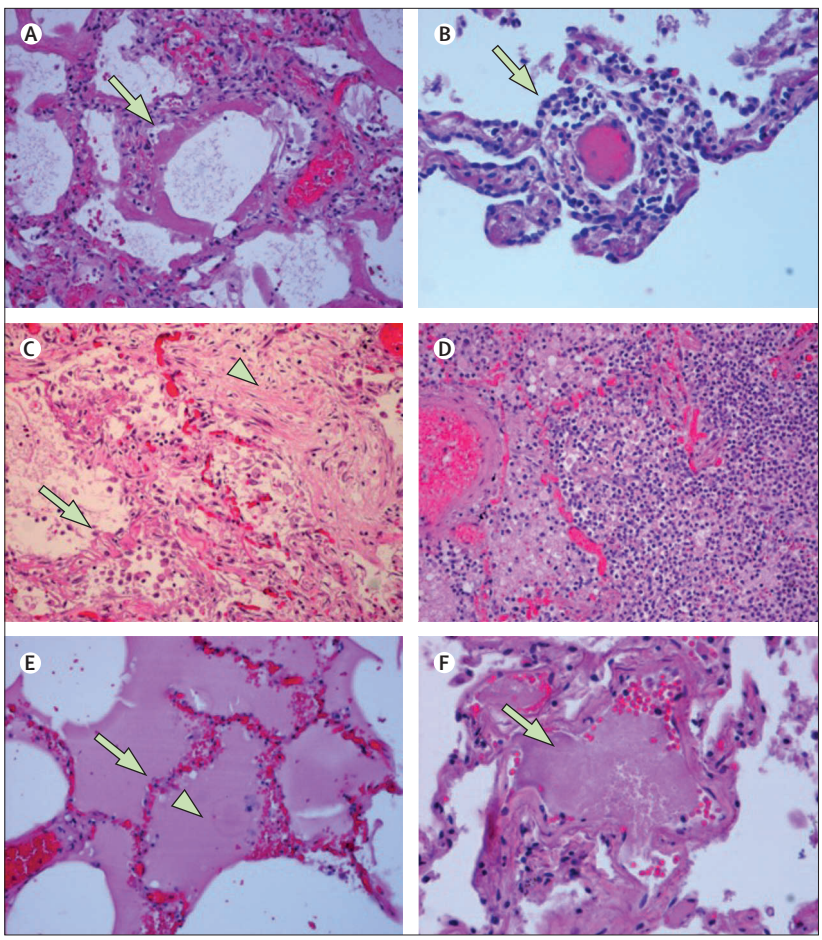

Figure 1: **Haematoxylin and eosin staining of post-mortem lung sections from patients who died with COVID-19**

(A) Exudative diffuse alveolar disease featuring prominent hyaline membranes (arrow) with mild interstitial infiltration by mononuclear cells, alveolar wall congestion, and cellular debris (original magnification ×400). (B) Perivascular lymphocytic infiltrate (arrow; ×400). (C) Organising diffuse alveolar disease with myofibroblast proliferation (arrowhead), fibrinous exudates, hyaline membrane remnants (arrow), and substantial alveolar architectural disruption (×200). (D) Acute bronchopneumonia featuring prominent intra-alveolar neutrophilic infiltration (×200). (E) Pulmonary cardiac oedema, characterised by eosinophilic fluid within the alveolar airspace (arrowhead), alveolar wall congestion (arrow), and absence of cellular damage (×200). (F) Platelet-rich thrombus (arrow) causing expansion of an intra-acinar vessel (×200).

diffuse immune cell infiltration.[25] Immunohistochemistry has also been used to further explore tissue coagulopathy in COVID-19, with findings of increased platelet deposition (CD61⁺) in COVID-19 lung tissue compared with influenza lung tissue. Staining was less prominent compared with that seen in bacterial and fungal pneumonia, perhaps indicating a role for secondary infection in promulgating platelet deposition.[29]

Immunohistochemical staining of viral components including spike and nucleocapsid proteins has allowed localisation and association of SARS-CoV-2 with components of post-mortem lung tissue. One autopsy series identified virus within hyaline membranes of tissue affected by acute DAD but not in late-stage DAD tissue, suggesting that disease progression might not be a direct response to SARS-CoV-2, but instead a result of the immunological repair response that it generates.[30]

Immunofluorescence has also had a key role in highlighting important pathophysiological steps in the course of COVID-19 lung disease. Leng and colleagues[31] demonstrated co-localisation of the SARS-CoV-2 spike protein and angiotensin-converting enzyme 2 (ACE2) using immunofluorescence staining. In an immunofluorescence study that stained for ACE2, the spike S1 domain, various cell-specific markers, and viral replication-associated non-structural protein 13 (nsp13) and nsp8, broad viral tropism—including tropism for some cells without ACE2 expression—was seen.[32] In a study in which perivascular mononuclear cell vasculitis of small and medium-sized pulmonary arteries was observed with light microscopy, immunofluorescence staining was used to characterise the infiltrating cells further, showing that the infiltrate was dominated by mononuclear cells positive for myeloid-related protein 8 (MRP8), which probably represent proinflammatory immunocytes and CD4⁺ and CD8⁺ T cells and macrophages.[33] Using immunofluorescence RNA and DNA staining to demonstrate architectural disruption, Fox and co-workers[12] observed fused pneumocytes containing abundant intracellular RNA within alveolar spaces that were thought by the authors to represent viral-infected cells. Another study used immunofluorescence stains for myeloperoxidase and citrullinated histone H3, showing the interplay between neutrophils and formation of neutrophil extracellular traps.[26]

Marked apoptosis has been demonstrated in the alveolar and vascular compartments of human COVID-19 lung tissue by use of the TUNEL assay.[34] Application of the TUNEL assay to lung tissue from a non-human primate model of COVID-19 disease showed co-localisation of cytochrome C and Fas ligand, markers of the intrinsic and extrinsic apoptotic pathways, potentially implicating both pathways in the disease process.[34] Formation of the inflammasome is understood to activate cell death pathways, including pyroptosis and apoptosis,[35] and immunofluorescence has been used to demonstrate the expression of inflammasome-associated proteins in COVID-19 lung tissue, which was greater than in control lung tissue and predominantly localised within leucocytes.[36] Bharat and colleagues[37] applied immunofluorescence to visualise the extracellular matrix of post-mortem or explanted lung tissue, noting COVID-19-associated fibrotic injury resembling end-stage pulmonary fibrosis.

Beyond highlighting aspects of the COVID-19 disease process, immunofluorescence has been a useful practical tool for digital segmentation of tissue compartments for differential analysis by advanced tissue pathology methods such as digital spatial profiling.[38] However, immunohistochemistry and immunofluorescence have some shortcomings. Owing to crossover in illuminated signals, these techniques are limited in terms of the number of targets that can be simultaneously analysed.[39] This limitation restricts the extent of spatial analysis of multiple cell types, identification of cell subpopulations, and assessment of functional status. Furthermore, a lack of standardisation in antibody choice and staining techniques exists, hampering interpretation of findings across studies.[27] Finally, there is some uncertainty about what the cell markers represent (eg, whether they define the cell type or whether they have a functional role), and markers are often shared between differing cell populations.

Deep-dive imaging technologies

Imaging mass cytometry

Imaging mass cytometry has several advantages over immunohistochemistry and immunofluorescence. In this technique, rare earth metal-labelled antibodies are bound to paraffin-embedded tissue, where the metals are subject to laser ablation and then spatially identified by mass cytometry on the basis of particle mass.[39] As imaging mass cytometry is not affected by fluorescence crossover, up to 40 different epitopes can be targeted—limited only by the availability of rare earth metals and compatible antibodies—enabling the generation of highly multiplexed images that are amenable to deep bioinformatics analysis (figure 2).

Several studies have applied imaging mass cytometry to post-mortem lung tissue from patients with COVID-19. In an analysis of sections from two patients, one with DAD and another with superimposed bacterial pneumonia,[40] the first patient had substantial diffuse infiltration of CD4⁺ T cells and macrophages with focal infiltration of natural killer cells, whereas the second patient showed infiltrating macrophages, diffuse CD4⁺ T cells, and scattered natural killer cells and dendritic cells, as well as a cluster of neutrophils. A post-mortem study of tissue from three patients with COVID-19 showed substantial lung infiltration of monocytes or macrophages, as well as dendritic cells and natural killer cells, in all three cases.[39] The authors noted that a subset of CD11b⁺ macrophages that did not express the HLA DR isotype was spatially associated with IL-10, which they

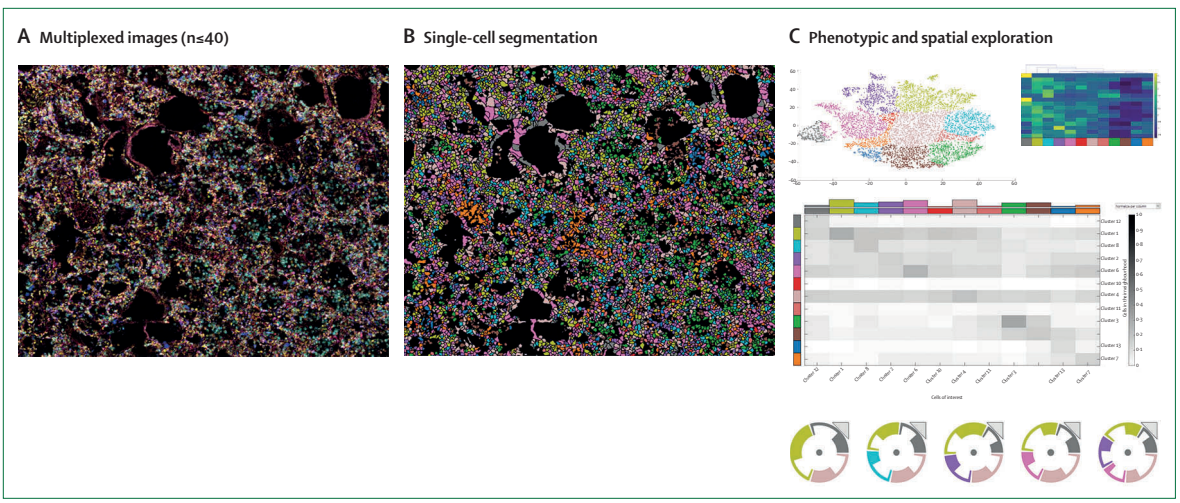

Figure 2: **Application of imaging mass cytometry to lung tissue from patients who died with COVID-19**
Lung sections are stained with a panel of rare earth metal-labelled antibodies, laser-ablated into an ionised plume, and spatially identified on the basis of particle mass. (A) Multiplexed images resembling immunofluorescence images are generated, with each colour representing a unique antibody target. (B) Analysis software is used for single-cell segmentation based on antibody binding, resulting in each cell being covered by a distinct mask. (C) Once segmented, analyses include cell clustering analysis according to expression profiles (top left panel), heatmap analysis based on differential expression (top right panel), and spatial analysis of neighbouring cell–cell interactions (middle and bottom panels) and avoidances (ie, the apparent absence of interactions between cells that usually interact).

suggested might be indicative of local suppression of a sufficient adaptive immune response.

Rendeiro and colleagues[41] compared lung tissue from ten patients who died with COVID-19 with tissue from control individuals with no lung disease at the time of death and from individuals who died with influenza or bacterial pneumonia. COVID-19-related deaths were divided into early deaths and late deaths depending on whether they occurred before or after 15 days following symptom onset. Significant reduction of lacunar space and infiltration with interstitial macrophages thought by the authors to be recruited from peripheral blood was seen in patients who had COVID-19 in both early and late stages compared with control individuals. Outlying data points indicated that some areas of tissue in COVID-19 cases had similar cellular composition and, in a few cases, had similar neutrophil levels as bacterial pneumonia cases, possibly representing the super-imposed bacterial infection phenotype. Unsurprisingly, substantial mesenchymal and fibroblastic proliferation was present in late-stage disease. Imaging mass cytometry was also used to detect virus and to functionally characterise the immune system and assess for interactions between cell types. The authors showed that spike protein was predominantly but not exclusively localised within alveolar epithelial cells; furthermore, these cells had a proinflammatory and apoptotic phenotype, with expression of phosphorylated signal transducer and activator of transcription 3 (STAT3), IL-6, and caspase 3, and were shown to be directly interfacing with macrophages.[41] Increased interactions were seen between macrophages and fibroblasts in late-stage disease, suggesting a link between immune cells and fibrosis.

Imaging mass cytometry is a powerful tool but is limited by the small area of tissue that can be analysed. Therefore, it is important to contextualise the regions of interest selected using other, higher-throughput and lower-resolution pathology imaging modalities.

Electron microscopy

Visualisation of post-mortem lung tissue at ultrastructural magnification has provided key insights into the localisation of viral particles and tissue damage associated with COVID-19. SARS-CoV-2 viral particles have been identified intracellularly and on the cell surface of both type I and type II pneumocytes;[9,19,22] intracellular virus has also been seen in macrophages[6] and in endothelial cells with surrounding features of endothelial injury.[42] In a post-mortem study combining scanning electron microscopy with microvascular corrosion casting, in which casting solutions were injected into cannulated specimens, substantial vascular endothelial distortion and altered angiogenesis function were observed in the lung tissue of patients with COVID-19.[42] A vascular bed with distorted architecture and altered function might provide an ideal setting for thrombotic events. Another electron microscopy study of vascular damage in COVID-19 lung tissue showed loosened junctional complexes in capillary endothelium, and a role was suggested for albumin leak in pulmonary oedema formation and possibly in hypoalbuminaemia, which is often seen in peripheral blood sampling of critical cases.[43] Notably, Roncati and colleagues[23] observed no viral particles within megakaryocytes to account for their altered behaviour.

In electron microscopy studies, targets are not highlighted with highly specific antibodies or stains, and

the definition of the structures depends to a large extent on the observer. Indeed, viral particles have been mistaken for organelles.[44] Thus, ultrastructural images must be interpreted with caution and in the context of the surrounding tissue.

In-situ hybridisation

RNA in-situ hybridisation is an alternative tool for the localisation of SARS-CoV-2 viral RNA in post-mortem lung tissue.[19] Using this technique, Delorey and co-workers[45] found that SARS-CoV-2 RNA was significantly enriched in myeloid cells and also (non-significantly) enriched in endothelial cells and other cell types, including some without co-expression of the viral target genes *ACE2* and transmembrane protease serine 2 (*TMPRSS2*). Some investigators have also used RNA in-situ hybridisation to cluster patients or samples into groups with high versus low or no viral RNA, enabling subsequent comparison of differential expression profiles using transcriptomic and proteomic platforms.[45,46] Bharat and colleagues[37] found that both positive-sense RNA and negative-sense RNA (representing active replication) were absent in all five post-mortem or explanted lung tissue samples studied (which was reassuring, given that three of the five cases had undergone lung transplantation), indicating further that late-stage disease cannot be directly attributable to active infection. RNA in-situ hybridisation might also be used to identify host cell phenotypes in COVID-19 lung tissue.

Omics platforms: whole lung tissue or single-cell analysis

Tissue transcriptomics and proteomics have added great depth to our understanding of lung tissue changes through the course of COVID-19. Traditionally, this technique involves the quantification of total amounts of RNA or protein from tissue, and measurement of their differential expression, which means that expression profiles are an amalgam derived from multiple cell types and are not spatially specific. A study using RNAseq transcriptome analysis in post-mortem lung tissue from patients with COVID-19 demonstrated the value of this technology in analysing multiple gene-expression pathways simultaneously, implicating genes related to viral infection, activation of reactive oxygen species, hypoxia-inducible factor-1α signalling, nucleotide-binding oligomerisation domain containing 1 (NOD)-like receptor signalling components, and monocyte-recruiting chemokines.[47] In another study using RNAseq, two transcriptomic phenotypes were identified on the basis of high or low expression of interferon-stimulated genes (ISGs).[48] Tissue with high ISG expression had high viral loads, and patients in this group tended to die earlier with less tissue damage, whereas tissue with low ISG expression had low viral loads, and patients in this group tended to die later with extensive DAD.

Explanted lung tissue from a recipient of a lung transplant after severe COVID-19 that was subjected to RNAseq analysis had differential expression of genes related to fibrosis, inflammation, and extracellular matrix disassembly, indicative of an active but possibly overwhelmed repair response.[6] Wu and co-workers[24] used RNAseq in an autopsy series of nine patients, and found differential expression of 4065 genes, with fibrosis again heavily implicated in COVID-19. *ACE2* expression was four-times greater than in control post-mortem lung tissue from individuals who did not have COVID-19, which might indicate enhanced viral attachment capacity in individuals who died of COVID-19. Contrary to expectations, *IL6* was not differentially expressed in lung tissue. On the basis of RNA expression profiles using RNAseq, Desai and colleagues[46] clustered 24 autopsy cases into three groups—a high viral-load group, a low viral-load group, and a third, mixed group. The high viral-load group had higher expression of genes related to proinflammatory signalling, MHC class I, and antiviral and wound-healing pathways, whereas the low viral-load group had higher expression of genes related to architectural cells such as the keratin (*KRT*) genes. Both groups expressed elevated platelet endothelial cell adhesion molecule 1 (*PECAM1*) and von Willebrand factor (*VWF*), markers of endothelial activation or injury, offering possible explanations for thrombosis.[46] Both groups also shared high expression of the macrophage marker gene *CD163*, although functional analysis indicated a relatively higher proportion of resting or uncommitted M0-like macrophages and polarisation towards M2-like macrophages associated with tissue repair in the low viral-load group, and a greater proportion of proinflammatory M1-like macrophages in the high viral-load group.

Using single-cell transcriptomics, Bharat and colleagues[37] identified several notable subsets of cells in post-mortem or explanted lung tissue, including a *KRT17*$^{+}$ epithelial cell group that is thought to represent a transitional stage between type II and type I pneumocytes, indicative of active lung repair and a profibrotic macrophage phenotype. *KRT17*$^{+}$ epithelial cells were suggested as a potential biomarker for irreversible fibrosis that could be obtained with bronchoscopy. Similarly, Delorey and co-workers[45] identified a group of *KRT8*$^{+}$ transitional state cells, a subset of which was *KRT17*$^{+}$ and thought to represent intrapulmonary basal-like progenitor cells, reflecting an emergency reserve activated in response to severe alveolar damage.

Melms and colleagues[49] also noted differential expression profiles in alveolar type I and type II cells and their transitional states, identifying a cluster of damage-associated transient progenitors (*KRT8*$^{+}$, claudin 4 [*CLDN4*]$^{+}$, cyclin dependent kinase inhibitor 1A [*CDKN1A*]$^{+}$) that were more common in COVID-19 tissue than in control tissue from patients who did not have COVID-19. Using conventional imaging methods for evidence of translation, this subset of cells was identified in non-COVID-19 lung tissue, in which immuno-fluorescence staining of KRT8^{+} and CLDN4^{+} epithelial cells

was also more prominent. Furthermore, these investigators functionally characterised the myeloid and lymphoid compartments using transcriptomic analysis. They found that myeloid cells tended to express genes associated with aberrant or dysregulated activation, including those associated with impaired T-cell immunity (nuclear enriched abundant transcript 1 [*NEAT1*], metastasis associated lung adenocarcinoma transcript 1 [*MALAT1*]) and impaired tissue regeneration (*AXL* receptor tyrosine kinase). Although plasma cells prominently expressed genes that encode variable heavy and light chain isotypes that are known to give rise to SARS-CoV-2-neutralising antibodies, there were only modest increases in genes associated with activation and tissue residency in the T-cell compartment, indicating a possible role for dysfunctional T-cell and myeloid immunity in disease progression. Furthermore, the investigators noted an overall increase in fibroblasts, predominantly a subset of pathological fibroblasts expressing collagen triple helix repeat containing 1 (*CTHRC1*), as well as genes associated with pathological extracellular matrix formation, including collagen type I alpha 1 chain (*COL1A1*) and *COL3A1*. Such cells have previously been implicated in idiopathic pulmonary fibrosis and scleroderma.[50] Bharat and colleagues[37] also noted a cluster of mesenchymal cells that were thought to be myofibroblasts, with upregulation of fibrosis-related genes such as *COL1A1*.

Using proteomics, Wu and co-workers[24] reported 637 differentially expressed proteins in their autopsy series of nine patients, further implicating translation of proteins related to neutrophil activation and pulmonary fibrosis. Leng and colleagues[31] detected multiple immune signalling proteins, including antiviral innate immune response receptor RIG-I, components of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway, IL-6 and TNF, intracellular adhesion molecule 1 (ICAM-1), and extracellular matrix and coagulation-related proteins, in COVID-19 post-mortem lung tissue. In another proteomics study, Nie and co-workers[51] demonstrated increased expression of cathepsin L1, a serine protease thought to function as a processor and potential activator of the SARS-CoV-2 spike protein, enabling the S1 domain to fuse with the endosomal membrane and allowing viral entry. An in-vitro experiment modelling SARS-CoV-2 infection showed that blocking cathepsin L1 led to a 76% reduction in viral entry into potential host cells.[52] Although Melms and colleagues[49] did not find differential expression of the cathepsin L1-encoding gene *CTSL* in their cohort, the deep exploration of processed tissue by Nie and co-workers[51] demonstrates the ability of these technologies to identify possible novel targets and potentially to inform new directions in therapeutics research and development.

Omics platforms: intact lung tissue

Digital spatial profiling is a form of molecular pathology that builds on traditional methods of transcriptomics and proteomics, enabling quantification of protein or gene expression in the context of intact tissue morphology.[46] Probes are used to quantify differential gene and protein expression in chosen regions of interest, allowing comparisons of expression patterns within and between patients (figure 3). Using this technology to study gene and protein expression patterns in six lobes of the lung from five patients, Desai and colleagues[46] found higher expression of interferon-responsive genes in SARS-CoV-2 virus-positive regions than in virus-negative regions. In a similar comparison of lung samples, Delorey and co-workers[45] reported enrichment of the viral genes open reading frame 1ab (*ORF1ab*) and spike protein gene (*S*), and viral response and innate immune genes, including *CXCL2* and *CXCL3*, in regions with high levels of viral

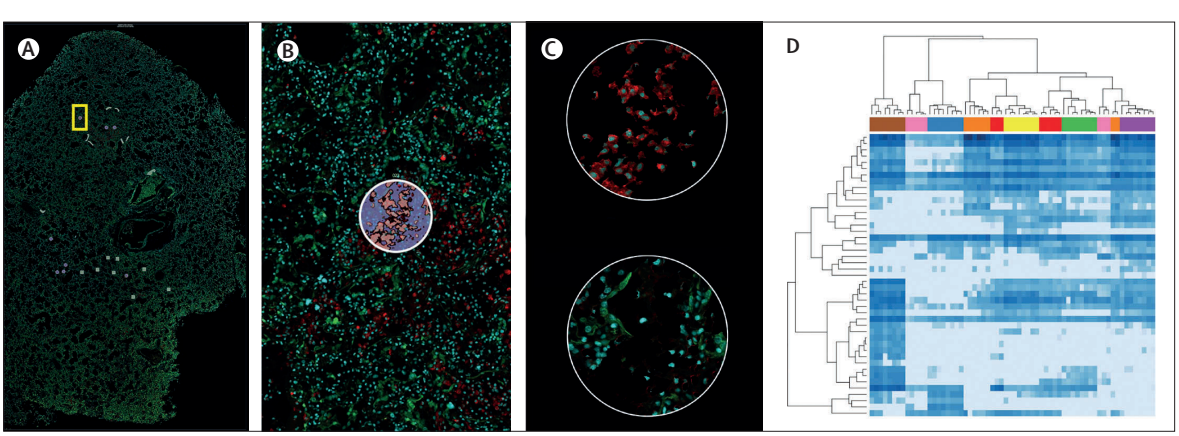

Figure 3: **Application of GeoMx digital spatial profiling to lung tissue from patients who died with COVID-19**
Lung sections were stained with the morphology markers pan-cytokeratin (green) and CD68 (red), and with a nuclear stain (blue). (A) Representative section showing pathologist-guided ROIs (small purple circles or green squares). Cells within ROIs can be analysed in their entirety for protein expression or mRNA abundance. (B) A single ROI (yellow box in part A) enlarged to show segmentation analysis. Isolating cell populations are defined by morphology markers (CD68 segmentation mask, pink; excluded cells, purple). (C) CD68 and pan-cytokeratin expression in $CD68^{+}$ segment (top) and $CD68^{-}$ segment (bottom). (D) Heatmap showing inter-patient heterogeneity in expression of 45 immune-associated protein targets. Each column represents a single ROI and eight ROIs were examined for each for the nine patients studied (colour coded as above). ROI=region of interest.

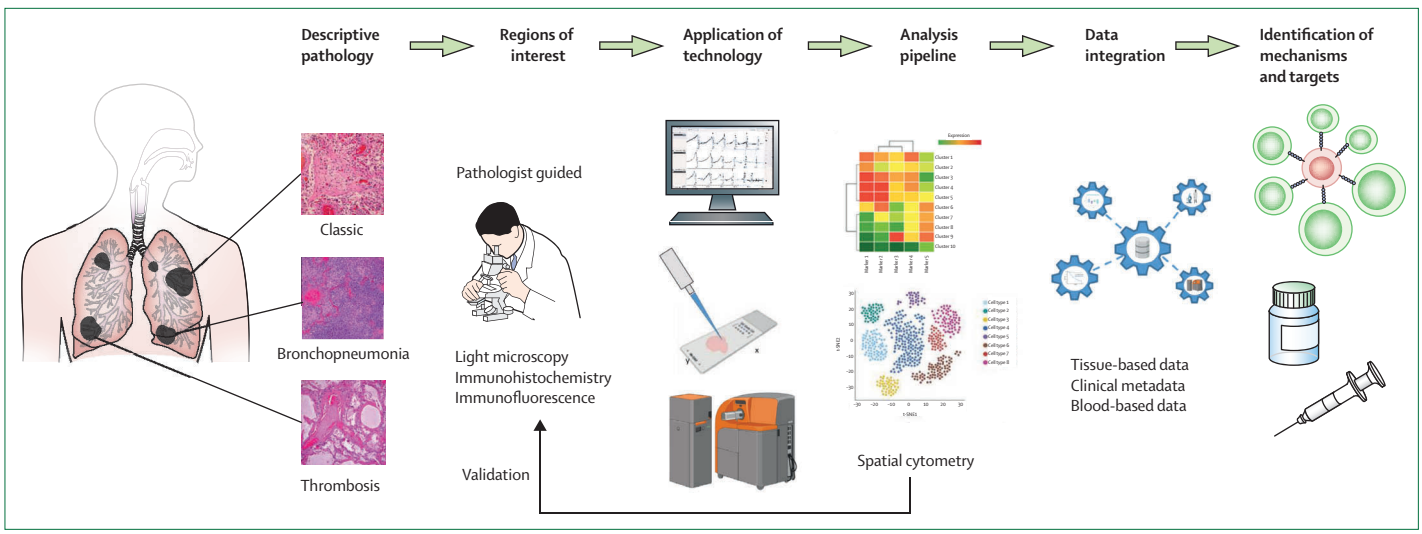

Figure 4: **Outline of a workflow combining conventional and advanced pathology approaches**
Post-mortem lung tissue sections from patients who died with COVID-19 have shown diffuse alveolar disease, bronchopneumonia, or tissue thrombosis (alone or in combination) using conventional pathology techniques. Advanced tissue pathology technologies are used to generate multiplexed images that are amenable to deep bioinformatics analyses, including cell clustering analysis, heatmap analysis showing expression patterns, and interactional analyses. As the investigation progresses, the cells present at the time of death are identified and characterised to determine the cell types, their functional states, and interactions and avoidances. Validation of findings might be sought using conventional pathology techniques. The ultimate goal is to identify clues (ie, mechanisms of action and targets) that could inform future treatment strategies. Some figure components were created with BioRender.com.

RNA versus those with low levels or no viral RNA. A further comparison of regions of interest with and without inflammation showed differential enrichment of innate and inflammatory genes such as *IFNG* and interleukin signalling genes. Tight junction genes were downregulated, which is consistent with alveolar architectural disruption. In another study, regional gene expression was evaluated in post-mortem lung tissue from patients who died with acute respiratory distress syndrome related to COVID-19 or influenza.[38] Compared with the influenza group, the COVID-19 group had increased epithelial compartment expression of genes associated with extracellular matrix and epithelial–mesenchymal transition, consistent with greater collagen deposition on light microscopy with Masson's trichrome staining, whereas in vascular tissue, this group had increased differential expression of genes related to endothelial injury and coagulation. Macrophages appeared to show alternative activation profiles with expression of M2 phenotype programmes. These findings suggest a possible role for antifibrotic therapeutics in ameliorating disease progression.

Importantly, most omics studies published so far have been descriptive, highlighting changes in the presence of specific cell types and protein and gene expression patterns. Therefore, further research on large datasets is needed to yield insights into pathway activation or deactivation, and causal relationships between immune cells and end-organ damage.

Implications for research and clinical practice

The ultimate goal of comprehensive and spatial mapping of post-mortem lung tissue from patients with COVID-19 is to determine which cells are present at the time of death, their functional state(s), and their location in relation to areas of tissue damage or other cell types. To achieve this goal, combinatorial approaches involving conventional methods and deep-dive techniques for detailed architectural and immune phenotyping are appropriate: conventional methods can provide the phenotypic context for interpreting the results of the more advanced technologies (figure 4 outlines a workflow that combines conventional and advanced pathology methods). Whereas traditional pathology is often qualitative, advanced approaches can provide quantitative outputs and enable a greater depth of analysis when evaluating potential biomarkers and investigating targeted treatment regimens. Studies that correlate findings from tissue pathology and peripheral blood sampling—enhancing understanding of the tissue targets of therapeutic candidates and the effects of their use on blood parameters that are surrogates of tissue status or function—will provide insights into the mechanisms by which the immunopathological lung landscape can be modified therapeutically and enable translation to the bedside in the care of critically ill patients. Imaging studies that correlate findings with pathological tissue states also have translational implications; for example, one study mapped early, proliferative, and late stages of DAD to distinctive CT findings (ground glass opacity or normal in the early group, crazy paving pattern in the proliferative group, and consolidation pattern in the late group), which in living patients might have implications for treatment strategies.[53]

Several challenges are associated with the use of post-mortem lung tissue for COVID-19 research. There is a risk of artifacts related to autolysis occurring during the

Panel 2: **Priorities for research with post-mortem lung tissue**

- Application of multiple tissue imaging technologies, including conventional and deep-dive methods for detailed architectural and immune phenotyping, to the same regions of interest in post-mortem lung tissue samples to comprehensively map tissue changes caused by SARS-CoV-2, and for combined analyses of complementary proteomic, genomic, and metabolomic data at the single-cell level, to validate findings, generate new hypotheses, and identify potential therapeutic targets
- Use of rapid autopsies to optimise tissue pathology investigations by mitigating artifacts due to autolysis and enabling molecular and genetic analyses
- Comparisons of findings from tissue pathology and clinical investigations (eg, peripheral blood and imaging studies) to identify clinical correlates of pathological states, which might have implications for treatment strategies in living patients
- Comparisons of pathological findings in post-mortem lung tissue from patients with COVID-19 with findings from a panel of controls (eg, healthy lung tissue, tissue from COVID-19-positive cases without severe lung injury, or tissue from COVID-19-negative cases with an alternative cause of severe lung injury) to determine the specificity of any findings to lung involvement in fatal COVID-19
- Investigation of lung tissue pathology in patients who have died with COVID-19 outside the hospital setting to enhance understanding of disease progression in individuals who have not received major pharmacological or medical interventions that might obscure data interpretation
- Assessment of lung tissue obtained from patients who had milder COVID-19 at the time of death but who died due to other causes to understand immune differences across the disease severity range
- Investigation of lung tissue from patients who survived severe COVID-19 lung disease and either underwent lung transplantation or died of other causes to determine the long-term consequences of severe lung injury, including repair and fibrotic processes
- Investigation of the lung microbiome to shed light on the interplay between commensal bacteria, fungi, viruses, and parasites in COVID-19 infection, and to provide insights into the differences in COVID-19 disease severity between individuals
- Comparisons of patterns of injury, inflammation, and immune cell phenotypes in lung tissue from patients who have died with COVID-19 due to different SARS-CoV-2 variants to determine whether they elicit different pathophysiological responses
- Assessment of the effects of second-wave and third-wave SARS-CoV-2 infections on patterns of lung injury to identify any differences in immune responses associated with vaccine escape and the use of therapies aimed at reducing mortality

time between death and post-mortem sampling, which can extend to several days. Major autolytic changes can be identified by an experienced histopathologist, and such cases can be excluded from analysis, but a more subtle effect on immune marker expression cannot be ruled out. The recent introduction of rapid autopsies will help to further mitigate artifacts due to autolysis, and support accurate molecular and genetic analysis. Another issue concerns the definition of appropriate control tissue, which could be healthy lung tissue, or could be tissue from COVID-19-positive cases without severe lung injury or from COVID-19-negative cases with an alternative cause of severe lung injury. Ideally, a panel of controls would be used to establish the specificity of any findings to lung involvement in fatal COVID-19.

COVID-19 has led to many deaths outside the hospital setting, which are not adequately represented in the literature.[13] Increased recruitment of this group for post-mortem research would offer a clearer view of how disease progresses in individuals who have not received major pharmacological or medical interventions that might obscure data interpretation. Post-mortem studies might also have value in the investigation of milder disease, using lung tissue obtained from patients who clearly died from other causes while also having COVID-19.

The important question of why COVID-19 manifests as severe lung disease for some people but not others requires innovative approaches. Attempts must be made to separate the direct effects of SARS-CoV-2 infection from the uncontrolled downstream immunological consequences that are independent of the presence of virus in susceptible individuals. The contribution of the lung microbiome might be an area of interest in this context. The healthy lung is not sterile but hosts multiple resident microbial populations, and their relative prevalence has been implicated in susceptibility to new infections, inflammatory tone, and chronic respiratory diseases including those involving fibrotic processes such as idiopathic pulmonary fibrosis.[54] Extension of this line of investigation to the potential interplay between commensal bacteria, fungi, viruses, and parasites in COVID-19 infection might be a worthwhile direction for future research. We propose several other priorities for future research in panel 2.

At the time of our literature search (July 10, 2021), the major advance in the treatment of severe COVID-19 lung disease had been use of the broad-spectrum anti-inflammatory and immunomodulatory agent dexamethasone;[55] targeted antiviral therapies or single-target anti-inflammatory agents had also shown modest effects.[56,57] Despite these advances, the mortality rate for patients with COVID-19 and severe lung disease remains high, and improved treatments that target key effector cells or pathways are urgently needed. A deeper understanding of the phenotypes of immune cells that

Search strategy and selection criteria

References were identified through a search of the Scopus database for journal articles published between Dec 1, 2019, and July 10, 2021, using the following search terms: "COVID-19" with "post-mortem", "autopsy", "lung", "pathology", or their derivatives. Articles were deemed to be relevant if they reported the application of any tissue imaging technique to COVID-19-affected lung tissue, were published in English, and were peer-reviewed. Some articles containing light microscopy findings alone that were replicated in other studies were excluded to maintain brevity. Post-mortem studies of animals were also excluded.

are present in severe lung disease, afforded by studies of post-mortem tissue, and correlations between identified cell types and clinical characteristics, including interventions used, will inform the design of trials of novel targeted therapies for patients who do not respond to current treatment options.

As we move through the next phases of the SARS-CoV-2 pandemic, with effective vaccination programmes in many countries, the predominant features of COVID-19-related lung injury appear to be changing as vaccination provides substantial protection against severe disease.[58] However, there are still many deaths among patients with severe disease, highlighting the continued relevance of post-mortem lung tissue in these cases. Post-mortem tissue could also provide valuable opportunities to study the effects of vaccination on the immune response at the tissue level in patients who had milder active COVID-19 at the time of death but who died due to other causes. Furthermore, collection of post-mortem tissue from patients with persistent respiratory symptoms months after the onset of COVID-19 who died from other causes—a group within the population of patients with long COVID—might provide immunological insights beyond those from cases of fatal severe COVID-19.

Conclusions

Research using post-mortem lung tissue is vital to our understanding of the pathogenesis of severe COVID-19. From the point of initial infection in the lungs, there appear to be several distinct phenotypic pathways that lead to death. In many patients, the histological pattern is characterised by classic DAD. However, in some people, an overlapping infection drives a distinct inflammatory milieu. Others have extrapulmonary manifestations of COVID-19 caused by thrombosis, which might originate in the lungs. Some people have faster, more aggressive disease, and die earlier than others with slower progression, each having distinct immune and repair signatures.

Much of our knowledge of COVID-19 pathophysiology to date comes from clinical investigations such as peripheral blood sampling. However, inferences about pathophysiology from peripheral blood findings can be made only at the tissue level. For example, a change in a circulating immune cell count does not provide information about the organ or tissue origins or direction of travel of these cells, or about tissue-restricted resident cell populations. Correlations between findings from peripheral blood sampling and autopsy studies can be particularly informative and, crucially, autopsies can be done safely given appropriate protective equipment and techniques.[3] Although findings from post-mortem studies might not always be applicable to living patients owing to potential pathophysiological differences between survivors and non-survivors, post-mortem lung tissue provides the best model available to understand the mechanisms of severe COVID-19. Continued collection and prospective study of post-mortem lung tissue from patients infected with SARS-CoV-2 who die due to COVID-19 or other causes promises to provide valuable information about the pathophysiological and immunopathological pathways that underlie the short-term and long-term health effects of COVID-19. Just as the fossil record provides clues to the location, evolution, and relationships of species, post-mortem lung tissue provides a snapshot of COVID-19 respiratory disease—the cells present, their functional state(s), and their spatial relationship to damaged tissue and other cells—at the time of death, allowing us to piece together trajectories of decline and providing essential insights that might inform improvements in treatment.

Contributors

LM, AF, and AJF conceptualised the article. LM did the literature search and wrote the original draft. LM and JM developed the figures and appendix table. JM, NC, PMK, OB, AF, and AJF contributed to the writing, review, and editing of the manuscript.

Declaration of interests

We declare no competing interests.

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