UNIVERSITÀ DI SIENA 1240

Department of Medical Biotechnologies

Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC)

XXXVI Cycle

Coordinator: Prof. Ilaria Meloni

Biomarkers in COVID-19 pandemic: implications for acute phase assessment and chronic management

Scientific disciplinary sector: MED/10

Candidate
Francesco Bianchi

Tutor
Prof. Elena Bargagli

Supervisor
Prof. Laura Bergantini

Academic year
2023/2024

INDEX

ABSTRACT

COVID-19 is the syndrome caused by SARS-CoV-2 infection. Severe phenotypes seem to be caused by a “cytokines storm” and pneumonia is one of the most important clinical features. COVID-19 could also be a trigger for fibrotic abnormalities.

Initially, we compared patients at hospitalization and healthy controls, focusing on acute phase biomarkers, especially IL-32, IL-8, IL-6 and IL-10. 64 patients underwent blood sample at hospitalization and 27 healthy controls were also included. The serum concentrations of IL-1β, IL-10, IFN-γ, TNF-α, IL-6, IL-8 and IL-32 were assessed. IL-8 was higher in COVID-19 patients than in controls, on the contrary IL-32 was lower. IL-6 was higher in patients with severe COVID-19, instead IL-10 was lower in this group.

Then, we evaluated a set of adipokines and cytokines in 108 hospitalized patients stratified according to clinical severity. 56 of them also underwent radiological and spirometry follow-up 3-6 months after discharge. Concerning the severity of disease, we found higher levels of TGF- β and IL-6 and lower levels of RBP-4 and IL-10 in the severe group. Subsequent analysis revealed that vaccinated patients showed higher levels of MCP-1 and IL-10. Considering the risk of fibrosis development, we observed higher levels of IL-1β, IL17A, TNF-α, TGF-β, IL-4 and IL-6 at hospitalization in the group with fibrotic alterations at follow-up. Regarding spirometry at follow-up, FVC% correlated inversely with TNF-α and directly with IL-32.

Finally, we chose a population of 89 follow-up patients previously hospitalized for COVID-19. Samples were collected during follow-up visits. The follow-up protocol included medical examination, HRCT of the chest, blood tests and lung function tests. The clinical data and medical history, also concerning the acute phase of COVID-19, were available for 80/89 patients. Severity of COVID-19 during hospitalization was recorded. Presence of fibrotic abnormalities did not affect spirometry values, but only DLCO. Direct comparison of cytokine levels in the two groups exhibited increased levels of IL-32 and decreased levels of IL-8 in patients with lung fibrotic alterations. IL-10 emerged as the only cytokine persistently decreased in previously severe patients.

INTRODUCTION

SARS-CoV-2 and COVID-19

Discovery and description

Coronaviruses (CoVs) are single-stranded positive-sense RNA (+ssRNA) viruses; seven species of this family cause acute respiratory illness in the human species. Family of Coronaviruses includes four genera: two of them, α-coronavirus and β-coronavirus, can infect humans (1,2). Their name is due to their aspect when observed in negative-stained electron microscopy: they appear crown-shaped (3).

Severe-Acute-Respiratory-Syndrome (SARS) CoV (a β-coronavirus lineage B) caused an epidemic spread in November 2002, originated from southern China, that showed a mortality rate about 9%(4). Middle-East-Respiratory-Syndrome (MERS) CoV (a β-coronavirus lineage C) has a relevant mortality rate. It has been described for the first time in Saudi Arabia (4) in 2012.

A new species of coronavirus, strictly related to SARS-CoV, caused a first severe epidemic spread in the Chinese province of Wuhan in the end of 2019 (2,4–6). International Committee on Taxonomy of Viruses (ICTV) named this CoV "SARS-CoV-2" on 11th February 2020 (7). The Wuhan Municipal Health Commission had reported this pathogen for the first time on 31st December 2019 and the whole genome of the new virus had been sequenced and so published on 10th January 2020 by the scientists of the Shanghai Public Health Clinical Center & School of Public Health coordinated by professor Yong-Zhen Zhang (8). The World Health Organization (WHO) named the disease caused by the infection of SARS-CoV-2 “COVID-19” on 11th February 2020, a contraction of “Coronavirus Disease 2019” (9). Since then, the disease quickly spread all over the world and so the WHO declared pandemic status on 11th March 2020 (10).

SARS-CoV-2, strictly genetically related to SARS-CoV, is probably a member of the wide family of zoonotic viruses, because it probably derived from bat coronavirus (11,12).

The viral envelope is glycoproteic and it shows the previously described “fringe” or “crown” morphology; the internal nucleocapsid shows a spiral configuration, but this changes into a spheric one when it is entering the host cell. Viral RNA replication is provided by the enzyme RNA polimerase RNA-dependent in the cytoplasm of the host cell (3,13). The enzyme ACE-2 (angiotensin-converting enzyme 2) is the receptor used by the virus to entry the cells (11,12,14). ACE-2 is a transmembrane protein almost ubiquitous, but it is principally expressed by type-2 pneumocytes, endothelial cells, enterocytes, kidney tissue, myocytes and myocardium (15). Also SARS-CoV uses ACE-2 as receptor (another proof of the strong relationship between the pathogens) (16), but this one binds its spike protein (specifically the S-ectodomain) with an affinity 10- to 20-fold lower than the one showed to S-ectodomain of SARS-CoV-2. This element can explain, at least partially, the significantly higher transmission rate of SARS-CoV-2 compared to SARS-CoV (17).

Moreover, SARS-CoV needs the activation of serine 2 transmembrane protease (TMPRSS2) to let S-protein bind ACE-2 (18,19), instead this protease is not essential for SARS-CoV-2 pathogenesis. Indeed, even if it is still important for the host cell entry process, the TMPRSS2 pathway can be partially replaced by the proteolytic pathway provided by

endosomal cysteine proteases cathepsin B and L (CatB/L). In fact, the association of camostat mesylate (inhibitor of TMPRSS2) plus E-64d compound (which inhibits CatB/L) firmly halts the entry process in the target cells (20).

SARS-CoV-2 transmission between humans is generally due to human-to-human close contact (21), mainly through respiratory droplets. Coughing, talking and sneezing can release respiratory secretions infected by the virus. Droplets generally spread all around two metres maximum from the patient (22). The presence of SARS-CoV-2 has been proved even in non-respiratory samples, as excrement, blood, ocular secretions and sperm, anyway the role of these possible transmission ways is not established (23–26). SARS-CoV-2 differs from SARS-CoV, which spread from a patient to another only during the symptomatic phase, because it can be transmitted also during the incubation period and by asymptomatic individuals (27,28). This element is obviously important concerning epidemiological studies and public health policies and it is confirmed by multiple studies, even if infectivity of asymptomatic patients could be weaker than symptomatic ones (29). Incubation period of COVID-19 can last up to 14 days since the exposure, most of the cases develop the disease 4 or 5 days after the infection (30,31).

Throughout the progression of the pandemic, the SARS-CoV-2 has evolved genetically with the development of new variants, including B.1.1.7 (Alpha), B.1.351 (Beta), P1 (Gamma), B.1.617.2 (Delta) and finally B.1.1.529 (Omicron), first detected in November of 2021. Since then, this variant has rapidly replaced Delta as the dominant variant of concern globally, further producing descendent lineages that include BA.1, BA.2, BA.3, BA.4, and BA.5 (32,33). The Delta variant is significantly more transmissible than the previous ones. It is also associated with higher rates of hospitalization and mortality, higher odds of oxygen requirement, need for intensive care unit (ICU) admission and death. The Omicron variant is even more transmissible, approximately 3.2 times that compared to the Delta variant, but it has been associated with reduced clinical severity (34).

Pathology and pathogenesis

COVID-19 is a clinically heterogeneous disease, and this phenomenon can be explained by its complex pathogenesis. Pathological alterations of the lungs in the early phase of the disease are consistent with alveolar damage (alveolar oedema and proteinaceous exudates), vascular congestion, patchy inflammatory infiltration with focal fibrin clusters mixed with mononuclear inflammatory cells and multinucleated giant cells in the airspaces, without significant neutrophil infiltration of the tissue. There is also patchy and severe pneumocyte hyperplasia and interstitial thickening, indicating reparative processes (35). In more advanced disease post-mortem autopsies show evident desquamation of pneumocytes and hyaline membrane formation, indicating acute respiratory distress syndrome (ARDS), with interstitial mononuclear inflammatory infiltrates, dominated by lymphocytes. Multinucleated syncytial cells with atypical enlarged pneumocytes characterised by large nuclei, amphophilic granular cytoplasm, and prominent nucleoli stand in the intra-alveolar spaces, showing viral cytopathic-like changes (36). Interestingly, a relevant amount of fibrin thrombi in the small arterial vessels seem to be a distinctive pathological feature of COVID-19 confirming that this illness is often complicated by coagulopathy. Histological examination of the main bronchi and bronchiolar branches have not specific alterations, just focal squamous metaplasia and mild transmural lymphocytic and monocytic infiltrates (37).

Ultrastructural examination can reveal particles suggestive of viral infection, especially in type 1 and type 2 pneumocytes; they are mainly localised along plasmalemmal membranes and within cytoplasmic vacuoles (37).

The direct viral toxicity is one of the pathways involved in this disease. Upon entry into the host cell, SARS-CoV-2 interacts with cellular molecules and modulates the metabolic activity of the cell, leading to various cytopathic effects. It has been shown that mitochondria could be one of the organelles most affected by SARS-CoV-2-derived cytopathy, as their alteration can lead to cellular stress (38). Known to play a role in the SARS-CoV-2 replicative cycle, the “hijacking” of endoplasmic reticulum (ER) by the viral replication process can trigger an ER stress response consequent to an altered accumulation of unfolded protein in the lumen of this organelle. It has also become clear that the virus could reconfigure the trafficking and structure of the ER through the interaction of its proteins with those of the host cell (38,39). Moreover, coronavirus infection can also cause severe ER membrane restructuring as a consequence of double-membrane vesicle formation during viral replication, as well as ER membrane exhaustion as a consequence of continuous viral particle synthesis (40,41). Some studies have shown the occurrence of an extensive Golgi apparatus fragmentation in infected lung epithelial cells, mainly triggered by SARS-CoV-2 S, M, E, nsp15, and ORF3a proteins; its function and structure may be altered by SARS-CoV-2- induced upregulation of trans-Golgi network integral membrane protein 2 (TGN46) and downregulation of Golgi re-assembly-stacking protein of 55 kDa (GRASP55) (41,42). SARS-CoV-2 seems especially prone to use the microtubule network and the Microtubule-Organizing Center (centrosome or MTOC) for host cell infection and its own proliferation. Thus, a profound remodelling of the cytoskeleton has been described in SARS-CoV-2-infected lung cells. Regarding the possibles effects on cell membrane, it has been described that the SARS-CoV-2 ORF3b protein interacts with Stomatin-like 2 (STOML2), whose dysfunction has been linked to altered formation of the T cell receptor (TCR) signalling complex. A recent study showed that the SARS-CoV-2 spike protein could directly suppress immune synapse formation in CD8+ T cells, which could be used by the virus to evade the cytotoxicity response against infected cells. Since activated T cells express the ACE2 receptor, this would, in turn, facilitate the entry of the SARS-CoV-2 virus, and when the cell is infected, the S protein is targeted to the immune synapse (38,43). The cellular nucleus can also undergo morphological and functional alterations when the cell is infected by SARS-CoV-2. In fact, some viral proteins alone cause some changes, some others block interferon-mediated responses in the host cell through different mechanisms, some of which are due to an alteration of nuclear-cytoplasmic transport (***Fig. 1***) (38).

Organelle	Cytopathic Manifestation(s)	Affected Host Protein(s)	Responsible SARS-CoV-2 Protein(s)
Mitochondria	Alteration of IFN-I responses	TOM70	ORF9b protein
	Dysregulating host antioxidant defense	SIRT1	Nsp14 protein
Endoplasmic reticulum	ER stress response, inhibition of IFN-β	IRE1	ORF8, S, E, M proteins
Golgi Apparatus	Golgi fragmentation	GRASP55, TGN46	S, M, E, nsp15, ORF3a proteins
Cytoskeleton	Cytoskeleton remodeling	Proteins of the MTOC	S protein
	Ciliary dysfunction	CUL2 complex	ORF10 protein
Cell membrane	Inhibition of immune synapse	T cell receptor	S protein
Nucleus	Inhibition of transcription factor entry into the nucleus	Transcription factors (such as STAT)	ORF6, ORF3b proteins
	Inhibition of the transcription of IFN-stimulated genes	STAT, IRF3	ORF6, ORF3b, N, nsp12 proteins
	Inhibition of host mRNA export from the nucleus	NXF1	nsp1 protein

Figure 1: Brief summary of the cytopathic effects of SARS-CoV-2. Modified from: Gonzalez-Garcia, P.; Fiorillo Moreno, O.; Zarate Peñata, E. et al. From Cell to Symptoms: The Role of SARS-CoV-2 Cytopathic Effects in the Pathogenesis of COVID-19 and Long COVID. *Int. J. Mol. Sci.* **2023**, *24*, 8290. https://doi.org/10.3390/ijms24098290

Since the first description of COVID-19, especially the severe cases, the so-called “cytokine storm” was depicted as the probably most important pathogenetic aspect, more than direct viral cytopathic effect. It’s important underline that not all the scientific community agree with this opinion (44), but the outstanding part does it. Cytokine storm is described as an exaggerated and dysregulated inflammatory response: a well-documented phenomenon considered to be the main feature of severe and critical COVID-19, even if it can be present also in other kinds of infection diseases. The term was first used in the early 1990s to describe the effects of graft-versus-host disease (GvHD) and later in the infectious disease setting (45). The cytokine storm has been suggested to be characterized by a positive feedback loop. Indeed, the initial wave of cytokines may induce a form of inflammatory cell death, that further induces the release of cytokines, eventually leading to the cytokine storm (46). Anyway, a specific molecular definition to delineate cytokine storm from normal inflammation and to describe the amounts and types of cytokines involved remains elusive. Increasing scientific evidences have described links between the pathogenesis of cytokine storm and programmed cell death processes. These processes include probably pathways other than

apoptosis in the recently described concept of PANoptosis: a composition of pyroptosis, apoptosis, and necroptosis (programmed necrosis) (**Fig. 2**) (45,47).

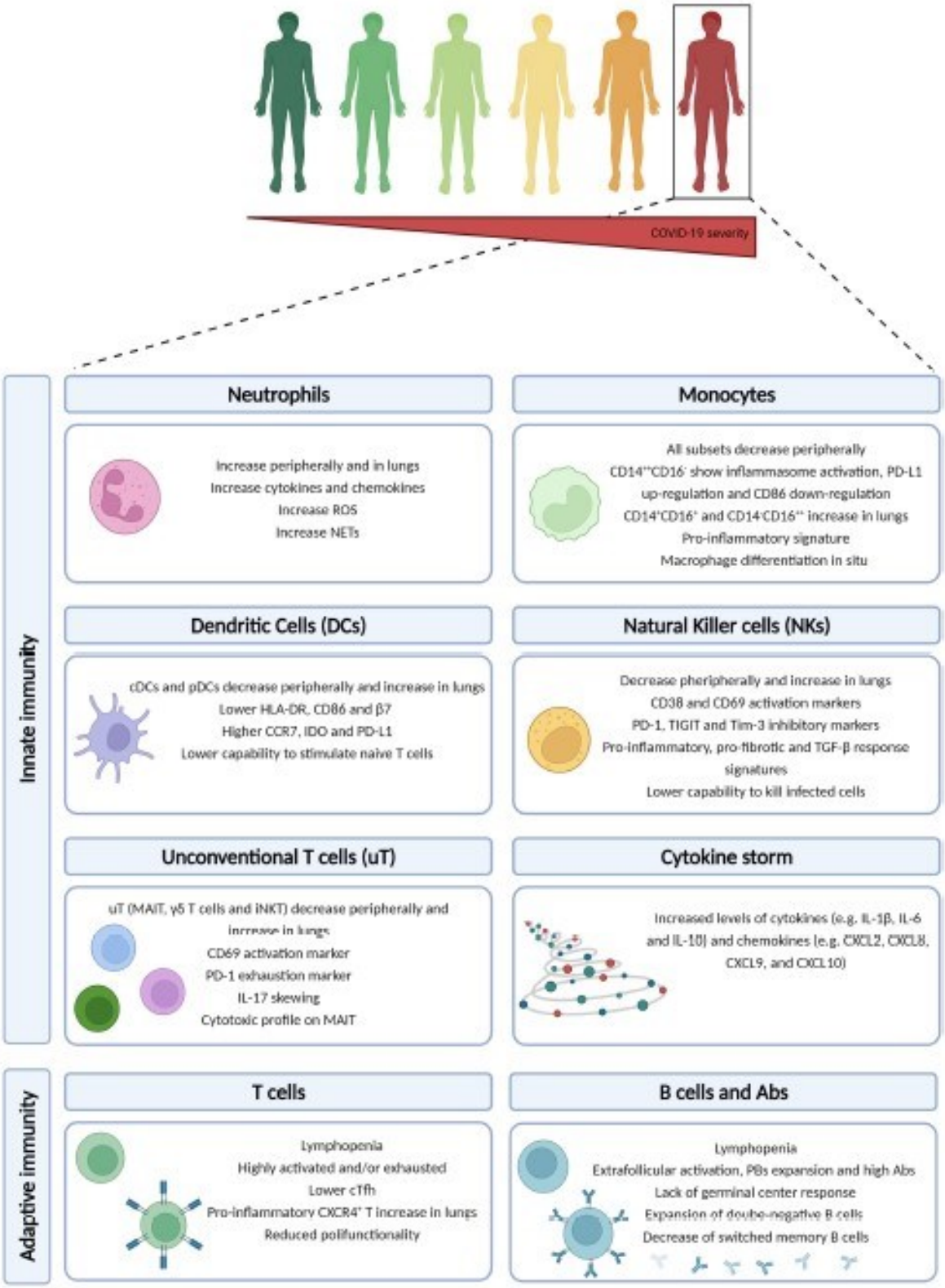

Figure 2: Summary of immune dysregulation involving both innate and adaptive immune responses in severe form of COVID-19 disease. From: Rovito, R.; Augello, M.; Ben-Haim, A. et al. Hallmarks of Severe COVID-19 Pathogenesis: *A Pas de Deux* Between Viral and Host Factors. *Front. Immunol.* **2022,** 13:912336. doi: 10.3389/fimmu.2022.912336

Immunopathology of severe COVID-19 implies both innate and adaptive immunity. Regarding the first one, some scientists tried to briefly describe all the process starting from the high activation of CD14+/CD16+ monocytes, which is responsible for the excessive production of proinflammatory cytokines tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6). The high neutrophil number promotes excessive formation of neutrophil extracellular traps (NETs) resulting in autoantibodies and more cytokine production, causing also possible activation of prothrombotic pathways. Furthermore, neutrophils promote CD4+ T-cell polarization toward IL-17-producing T-helper 17, responsible for a significant monocyte/macrophage recruitment to the site of infection and stimulation of interleukin 1β (IL-1β) and IL-6 cytokine cascades. In addition to the excessive proinflammatory responses, it is important to underline the presence of lymphopenia and exhaustion of natural-killer (NK) cells: distinctive traits of severe COVID-19 (48). Moreover SARS-CoV-2 infection reduces ACE2 expression. ACE2 is part of the ACE2/Ang(1-7)/MasR axis, and it is responsible for the hydrolysis of angiotensin II (Ang II) to Ang1-7, which directly influences the activation of the MasR receptor. The activation of this axis has anti-inflammatory and antifibrotic effects, because it reduces the expression of p38 Mitogen Activated Protein Kinases (MAPK) and nuclear factor kappa B (NF-κB) and inflammatory factors such as IL-6, TNFα and IL-8 (49). SARS-CoV-2 infection reduces ACE2 expression and ACE2/Ang(1-7)/MasR axis activity, so causing increased levels of Ang II, which promotes inflammation and fibrosis processes (50). Indeed, Ang II can activate the NF-κB pathway via stimulation of the phosphorylation of the p65 subunit of NF-κB. This will lead to increased production of IL-6, TNF-α, IL-1β and IL-10. NF-κB is also directly activated by SARS-CoV-2 itself through pattern recognition receptors. Besides, Ang II regulates MAPK (ERK1/2, JNK, p38MAPK), which have important functions on cellular processes including the release of cytokines such as IL-1, IL-10, IL-12 and TNF-α. Reduced expression of ACE2 also interferes with DABK/ bradykinin B1 receptor axis. The main ligand of bradykinin B1 receptor (BKB1R) is DABK and the ligand of bradykinin B2 receptor (BKB2R) is BK. Expression of BKB1R enhances the neutrophil attraction to tissue by release of chemokine C-X-C motif chemokine 5 (CXCL5) and the activity of this receptor leads to expression of FGF-2, and to increased levels of IL-1β and Monocyte Chemoattractant protein-1 (MCP1). DABK is a known pulmonary inflammatory factor. Notably, ACE2 cleaves terminal residue of DABK and this reaction causes deactivation of DABK. Therefore, it could be hypothesized that COVID-19-induced reduction of ACE2 activity would be accompanied with increased activity of DABK. Finally, we have to consider the activation of complement system. Firstly, the angiotensin type 1 receptor (AT1R) stimulation by Ang II seems to activate the complement cascade including Complement factor 5a (C5a) and C5b-9. Then viral-induced complement cascade activation promotes inflammatory processes. C5a induces release of pro-inflammatory cytokines and it can also induce secretion of TNF-α. Terminal products of the complement cascade can induce the production of cytokines such as TNF-α and IL-1. In particular, C5b-9 induces release of IL-6 via activation of NF-κB and MCP1 from vascular smooth muscle cells. Also, the increased production of C3a leads to production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (49).

The effects of COVID-19 on adaptive immune system are complex as much as the ones on innate immune system previously illustrated. During severe COVID-19 we can observe profound lymphopenia (characterized by marked reduction in CD4+ and CD8+ T cells and B cells), T cell hyperactivation and exhaustion, and scarce B cell maturation. The inflammatory cytokine storm probably plays a role in the observed lymphopenia. Indeed, high concentrations of IL-6 are associated with massive lymphocyte death. Furthermore, the impaired production of type I interferon (IFN), secondary to autoantibody production and decline of plasmacytoid dendritic cells, blocks the expression of B cell lymphoma 6 (Bcl-6)

in CD4+ T cells, preventing T follicular helper (Tfh) differentiation and the development of a mature humoral response. This scenario can explain the lack of B cell maturation and the

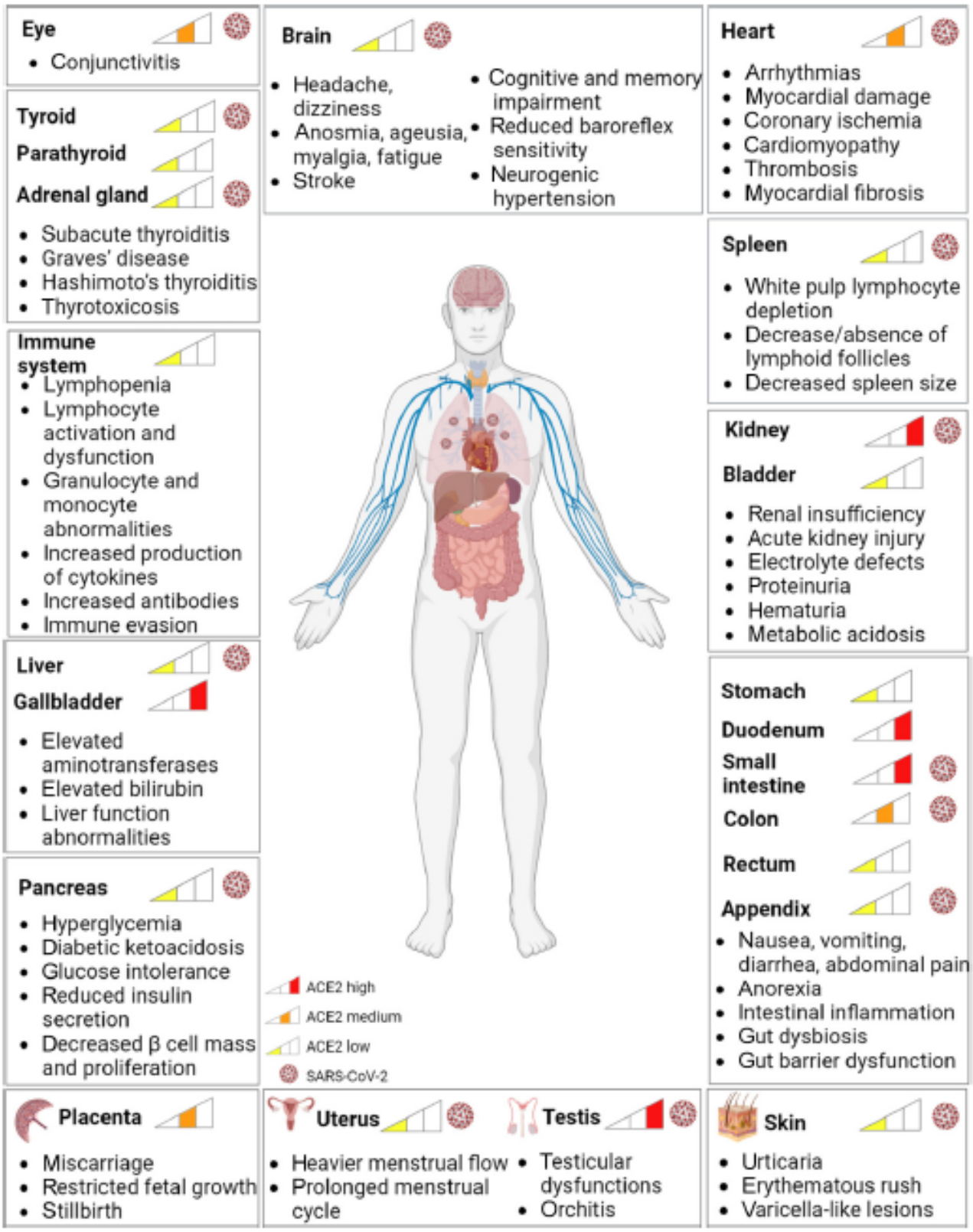

Figure 3: Quick overview of the extrapulmonary manifestations of COVID-19. The level of expression of ACE2 for each organ is indicated. Documented SARS-CoV-2 persistence is also indicated. From: Baldari, CT.; Onnis, A.; Andreano, E. et al. Emerging roles of SARS-CoV-2 Spike-ACE2 in immune evasion and pathogenesis. *Trends Immunol*. **2023** Jun;44(6):424-434. doi: 10.1016/j.it.2023.04.001.

production of a germline-like antibody response noticed in COVID-19 (**Fig. 3**) (48).

It should be noted that some immune cells, such as alveolar macrophages, are also target cells of SARS-CoV-2 infection. In these immune cells, centrosome is usually behind the nucleus playing a role of directional guidance and cell movement, but when interacting with antigen-containing cells, forming an immune synapse, centrosome moves to the front and is directly involved in the release of cytokines, interleukins, and tumour necrosis factor (TNF), among others. If SARS-CoV-2 takes control of the centrosome (as previously described), this release is uncontrolled, leading to an excessive inflammatory response that causes extensive cellular damage (51). SARS-CoV-2 is able to perform different immune evasion strategy and CD8+ cytotoxic T lymphocytes (CTLs) are one of the targets. For example, viral open reading frame (ORF) proteins ORF3a, ORF7a, and ORF8 have been reported to inhibit antigen presentation by the major histocompatibility complex I (MHC I) at different steps in human and primate kidney epithelial cell lines, leading to defective CTL activation. Baldari et al. noticed also that the viral spike protein suppresses immune synapse assembly in CTLs by interfering with T cell receptor (TCR) recruitment and activation of the tyrosine phosphorylation cascade required for lytic granule positioning and delivery beneath the immune synapse (48).

The interaction between innate and adaptive immunity is important to understand the molecular signature linked to the COVID-19. These branches of the immune response are not isolated one from the other, because the activation of cells implicated in adaptive responses depends on stimuli released by innate immunity (52). Also trained immunity could be important in COVID-19 pathogenesis because it involves epigenetic modifications and, above all, cytokines releasing (53–55).

A so complex pathogenesis is obviously susceptible to important influence of genetic factors. Indeed, established host risk factors for disease severity (eg: old age, male gender, obesity) do not explain all the variability in disease severity observed across individuals. The first genetic factors described to contribute to COVID-19 severity were rare loss-of-function variants in genes involved in type I interferon (IFN) responses (56), while the Italian GEN-COVID Multicenter Study contributed to the identification of rare variants and common polymorphisms associated with COVID-19 severity through the collection of more than two thousand biospecimens and clinical data from SARS-CoV-2-positive individuals and whole exome sequencing (WES) analysis (57–59).

Multiple studies have been conducted since now: several Genome-Wide Association Studies (GWAS) projects investigating the contribution of common genetic variation to COVID-19 have provided robust support for the involvement of various genomic loci associated with COVID-19 severity and susceptibility (60,61). Anyway, if GWAS studies can provide solid evidence of the host genetic factors individually associated with COVID-19 severity, they most often fail to provide an organic picture about their interplay. In this case, machine learning techniques have been applied to identify risk score predicting severity of COVID-19, as the Integrated PolyGenic Score (IPGS) elaborated from GEN-COVID Multicenter Study data (62,63).

Clinical features and impact

SARS-CoV-2 infection is a heterogeneous condition. A part of the infected people does not develop the clinical syndrome (COVID-19). The burden of asymptomatic patients is difficult to assess: mathematical models developed at the start of the pandemic, based on seroprevalence, hypothesized a significant proportion of asymptomatic or mild symptomatic

patients (64). Interestingly, studies conducted on the famous cruise ship “Diamond Princess”, where it was possible to accurately study an epidemic spread of SARS-CoV-2, concluded that the real percentage of asymptomatic was 17,9% (95%CrI: 15,5–20,2%). Anyway, study population cannot be considered representative of general population in any part of the world, because there was a prevalence of high age people (65,66). One meta-analysis concluded that asymptomatic infections are approximately 33% of those testing positive for SARS-CoV-2, but this number varies between different studies. More recently a third category of pre-symptomatic proposes that as many as half of these persons who do not declare symptoms at the time of positive testing develop symptoms later (34).

Clinical manifestations of COVID-19 are usually unspecific and heterogeneous. One of the first report, including 138 patients hospitalized for COVID-19 pneumonia in Wuhan city, described the following symptoms: fever (in almost entire population), asthenia, dry cough (both present in about one half of the population) and then anorexia, myalgias and dyspnoea in one third of the cases. Less common symptoms were vertigo, abdominal pain, diarrhoea, nausea and vomiting. Approximately 10% of the patients reported diarrhoea and nausea as initial manifestation, followed by fever and dyspnoea after 1 or 2 days (67,68). Subsequent case series and analysis generally confirmed this description. Classical initial symptoms, besides fever and asthenia, affect respiratory system. Reviewing medical literature some months after pandemic spread, Mehta and colleagues reported a median interval between the onset of initial symptoms to development of dyspnoea, hospital admission, and ARDS of 5, 7, and 8 days respectively. It’s important to underline that some patients with COVID-19 may have reduced oxygen saturation in blood but remained stable without significant distress: a condition termed as salient hypoxia or happy hypoxia (69). Interestingly, anosmia and dysgeusia can be present in COVID-19 and in some cases they are the only symptoms present. The pathophysiology of these conditions is still unclear, probably involving sensorineural mechanism and peripheral neurotropism of SARS-CoV-2. Anyway, the replacement of the previous viral variants by Omicron, which carries upward of 50 mutations on the spike protein alone, caused a significant reduction in appearance of anosmia and dysgeusia (70). Besides the typical presentation, we should remember that COVID-19, as evidenced since the first reports, is a systemic disease and many organs can be involved. This fact implies a wide range of possible manifestations at onset: arrhythmias, gastrointestinal symptoms, renal injury, delirium, conjunctivitis, maculopapular exanthem, papulovesicular rash, and many others (69).

Pneumonia, hypoxemic respiratory failure and ARDS are the typical severe manifestations of COVID-19, with hypoxemic respiratory failure the most common reason for ICU admission. Bacterial or fungal co-infections can affect a significant portion of patients, consisting in a major source of morbidity and mortality (34); in one study, half of those who died experienced a secondary infection (71). Literature suggests severe disease (defined as hypoxia or >50% lung involvement) can occur in over 15% of patients and critical disease (consisting of respiratory failure, multiorgan injury, or shock) in up to 5%, depending on patient population features (34).

There are different classifications of disease severity of COVID-19, but probably the most widely adopted is the one provided by WHO. Apart from asymptomatic patients, the listed subgroups are mild, moderate, severe and critical. Mild patients don’t have hypoxia or pneumonia, moderate ones show pneumonia without respiratory impairment, maintaining a SpO2≥90% on room air. Conversely, severe cases are characterized by a pneumonia with desaturation on room air or tachypnoea. COVID-19 can be considered critical when there are evidences of ARDS, sepsis, septic shock, acute cardiovascular events or in cases of specific hyperinflammation conditions (eg: the MIS-C described later) (72).

Laboratory tests can show several abnormalities in COVID-19. Firstly, we should notice an increase of inflammatory markers, like C-reactive protein, lactate dehydrogenase, ferritin, IL-6 and alterations of coagulation parameters (eg: D-dimer). Blood count often reveals lymphopenia and thrombocytopenia. Comprehensive alterations of coagulation parameters, including platelets count, are associated with poor prognosis (73). In some cases this can be related to the occurrence of disseminated intravascular coagulation (DIC) (74,75). Anyway, although rarely, also bleeding events can complicate COVID-19 and they can occur without a DIC context. Especially abdominal bleeding complications may be associated with COVID-19, apparently without predisposing causes (76). Lymphopenia too is associated to severity. Generally, we can find a global lymphopenia, affecting both T-CD4+ and T-CD8+ and also B-cells and NK-cells somehow (77–80). Anyway T-CD8+ count seems to be more accurate for stratifying patients in risk scores (81,82). Moreover, T-CD8+ count increase in the course of the disease associates with clinical recovery (83). Summing up, a community-acquired pneumonia (CAP) presenting with lymphopenia should be considered as a possible COVID-19 with an high risk for complications (84).

Initial radiological evaluation of COVID-19 patients with respiratory symptoms generally starts with chest radiography. A normal chest X-ray may be found in a significant proportion of patients, although different studies reveal significant variation in the frequency of their findings, and some cases can lately progress to abnormal findings on subsequent plain films. The most common abnormal X-ray findings include peripheral consolidations or ground-glass opacities. Generally, the lung involvement is bilateral. Computed tomography (CT) of the chest without contrast is much more sensitive for detecting lung abnormalities and should be considered in patients with an unremarkable or confusing X-ray findings (34). CT imaging features can be classified by a score designed by Pan and colleagues from stage 1 (early, 0-4 days after onset of symptoms, mainly characterized by subpleural ground-glass opacities in the lower lobes) to 4 (absorption stage, two weeks after the onset, without crazy-paving pattern, but with extensive ground-glass due to consolidation absorption). Stage 2 (progressive) and 3 (peak) are respectively characterized by extended bilateral diffuse ground-glass, crazy-paving pattern and consolidation and, in the third stage, dense consolidations and residual parenchymal bands, even if ground-glass and crazy-paving are still present. It is important to notice that this classification is based on not critical patients (85). Generally, the typical aspect of COVID-19 pneumonia is a bilateral “ground-glass” inflammation with a possible evolution toward consolidation variously extended (68). Ground-glass opacities are present in all symptomatic patients, instead consolidations, “crazy-paving” and pleural effusion are associated with severity. Interestingly, ground-glass opacities can be found also in CT-scan of asymptomatic or mild-symptomatic patients (28). Some authors described a new radiological sign in COVID-19, named “spider-web” sign, described as following: “*It showed a triangular or angular GGO under the pleura with the internal interlobular septa thickened like a net. The adjacent pleura were pulled and formed a spider web–like shape in the corner*” (86).

Lung ultrasound, an intriguing radiation-free technique of chest imaging, can be useful in the context of COVID-19 pandemic, especially with portable pocket-sized ultrasound scanners (87). Ultrasonographic features of COVID-19 are the following: thickening of the pleural line with pleural line irregularity, B-lines in a variety of patterns including focal, multifocal, and confluent, consolidations in a variety of patterns including multifocal small, non-translobar, and translobar with occasional mobile air bronchograms. Reappearance of A-lines occurs during recovery phase instead pleural effusions are uncommon (88).

Evaluating the real impact of COVID-19 over mortality rate is almost unfeasible. Confirmed reported case of infections cannot be considered a solid data, so many scientists

concentrated over mortality rate variations between different periods: before and after COVID-19 pandemic, assessing the so-called excess mortality. Excess mortality is defined as the difference in the total number of deaths in a crisis compared to those expected under normal conditions; it accounts for both the total number of deaths directly attributed to the infection and those resulting from the indirect impact (89). For example, an Omani study based on a retrospective cross-sectional analysis of daily mortality data, extracted from the Al-Shifa system (a comprehensive electronic healthcare information management system developed by the Ministry of Health) collected mortality data acquired from 1st January 2015 to 16th August 2020. They evidenced 15% increase in all-cause mortality in the pandemic period (16 March–16 August 2020) compared with baseline. This increase was particularly relevant for adult and old (over 60-year-old) people and most evident for hospital deaths than home deaths. The authors estimated that 10.8% of excess mortality can be attributed directly to COVID-19, leaving 5% of the total excess mortality (estimated as 15%) due to other causes, mainly unclassified (90). WHO agrees with this approach, and it released an estimate of 14.83 million excess deaths, with an uncertainty interval (UI) of 13.23 million to 16.58 million, for the period January 2020 to December 2021. This burden of deaths is 2.74 (UI 2.44 to 3.06) times higher than the 5.42 million COVID-19 deaths reported to the WHO for the same period. Concerning the P-scores (normalization of the excess estimates by the expected number of deaths for the analysed period, expressed as a percentage), there were 7.97% (UI 6.96% to 9.03%) and 18.30% (UI 15.99% to 21.15%) increases in deaths globally in 2020 and 2021, respectively, compared to what we would have expected if the pandemic had not occurred (89).

However, reported rates of hospitalization, mechanical ventilation, and mortality vary significantly due to several variables including patient age, healthcare and testing availability, and containment measures, among others. Early in the pandemic, overall mortality rates for admitted patients reached 20%, but in those admitted to the Intensive Care Unit (ICU) mortality approximated 40%. As the pandemic has progressed, ICU survival rates have improved up to 80%. More recent literature suggests the case fatality rate is under 2% in all patients with COVID-19, but it can rise to 6.4% in those over age 60 years, in those over age 80 years it is over 13% and in those over age 90 years mortality is over 25% (34).

According to the decreased pathogenicity of Omicron variant, in a retrospective Chinese study conducted over 445 confirmed cases of infected people with that variant (tested for being SARS-CoV-2 close contacts) no patients were admitted with severe or critical symptoms and all patients were discharged from the hospital after complete recovery without any serious complications or death. To be honest, we must underline that this study population comprised only 31 patients 60 years of age or older and more than 90% of the patients had received at least one dose of the COVID-19 vaccines (91). However, a decreased severity of Omicron infection compared to Delta one was observed also by a French retrospective research in various Paris emergency departments: the Omicron variant was associated with a reduced risk for ICU admission, mechanical ventilation, and in-hospital mortality, even if we should consider that in this study Omicron affected a younger population with a higher rate of vaccination (92).

The presence of comorbidities and older age are important risk factors for bad prognosis, as evidenced since the first pandemic wave. Many authors concentrated in trying to elaborate risk scores to predict risk-factors for death, generally integrating anamnestic data with clinical signs or biomarkers. For example, a European study described age, hypertension, obesity, renal insufficiency, any immunosuppressive disease, O2 saturation at presentation <92% and an elevated C-reactive protein as the most relevant risk-factors

for death. Moreover, the weight of every variable changed depending on the age stratum considered: for the younger cohort (<70 years), obesity and immunosuppression seemed to be more important, while hypertension was more relevant in the senior cohort; renal insufficiency and desaturation remained important in both (93). Role of obesity should be particularly underlined with one study where mortality was found to be more than doubled in patients with body mass index (BMI) over 40 and 4-fold higher in patients with a BMI >45 (94). Diabetes, cancer and prior cardiac or pulmonary disease have also been described as risk factors for severe disease (34).

Generally, COVID-19 is considered less important for children. Looking at January 2023, 4.817.426 cases in the population of individuals 0–19 years of age had been diagnosed and reported by the COVID-19 surveillance system of the Italian National Institute of Health (Istituto Superiore di Sanità, ISS) since the beginning of the pandemic. Around 25 thousands of them were hospitalized, but only 573 were hospitalized in intensive care, and 91 children died (95). Although COVID-19 in children is generally a mild respiratory infection, it may present respiratory distress like another viral bronchiolitis, even if child-age patients with SARS-CoV-2 infection have milder respiratory symptoms and a shorter duration of hospitalization compared to patients with respiratory syncytial virus (RSV) infection or co-infection between SARS-CoV-2 plus another virus (96).

The most relevant danger related to COVID-19 for children is the so-called Multisystem Inflammatory Syndrome in Children (MIS-C): diagnostic criteria are shown in ***Figure 4***. This disease was firstly described during the first pandemic wave in United Kingdom (UK): physicians noticed a cluster of children requiring admission to ICUs due to an unexplained multisystem inflammatory syndrome with features of Kawasaki disease and toxic shock syndrome. The majority of children affected weren't infected with SARS-CoV-2 at presentation, but were antibody positive, indicating past infection (97). The cause of the clinical syndrome was postulated to be a post-infectious inflammatory response following SARS-CoV-2 infection. Although a causal link between COVID-19 and MIS-C is yet to be confirmed the temporal, geographic and epidemiological features of MIS-C are strongly suggestive for this bond. Fortunately, MIS-C appears to be a rare phenomenon. The first systematic review of cases meeting the diagnostic criteria for MIS-C noticed that this condition has distinct epidemiological and clinical features comparing to acute severe COVID-19 infection in children. Indeed, the cases of MIS-C presented are older, did not frequently have comorbidities, and exordium often presents with gastrointestinal symptoms and significant cardiovascular dysfunction. In contrast, acute severe COVID-19 infection in children is associated with young age, a history of comorbidities, and presence of respiratory

symptoms and respiratory dysfunction. Same authors evidenced a mortality rate of 1,5% for this pathology (98).

Organization	Case definition
World Health Organization	**1. Age 0 to 19 years; AND** **2. Fever for ≥ 3 days; AND** **3. Clinical signs of multisystem involvement (at least two of the following):** • rash, bilateral nonpurulent conjunctivitis, or mucocutaneous inflammation signs (oral, hands, or feet); • hypotension or shock; • cardiac dysfunction, pericarditis, valvulitis, or coronary abnormalities (including echocardiographic findings or elevated troponin/BNP); • evidence of coagulopathy (prolonged PT or PTT; elevated D-dimer); • acute gastrointestinal symptoms (diarrhoea, vomiting, or abdominal pain); AND **4. Elevated markers of inflammation (e.g. ESR, CRP, or procalcitonin); AND** **5. No other obvious microbial cause of inflammation, including bacterial sepsis and staphylococcal/streptococcal toxic shock syndromes; AND** **6. Evidence of SARS-CoV-2 infection with ANY of the following: positive SARS-CoV-2 RT-PCR;** **positive serology; positive antigen test; contact with an individual with COVID-19.**
US CDC	1. Individual < 21 years presenting with fever, laboratory evidence of inflammation, and evidence of clinically severe illness requiring hospitalization, with multisystem (≥ 2) organ involvement (cardiac, renal, respiratory, haematologic, gastrointestinal, dermatologic or neurological); AND 2. No alternative plausible diagnoses; AND 3. Positive for current or recent SARS-CoV-2 infection by RT-PCR, serology, or antigen test; or exposure to a suspected or confirmed COVID-19 case within the 4 weeks prior to the onset of symptoms.
Royal College of Paediatrics and Child Health (RCPCR)	1. A child presenting with persistent fever, inflammation (neutrophilia, elevated CRP and lymphopaenia) and evidence of single- or multi-organ dysfunction (shock, cardiac, respiratory, renal, gastrointestinal or neurological disorder) with additional features. This may include children fulfilling full or partial criteria for Kawasaki disease. 2. Exclusion of any other microbial cause, including bacterial sepsis, staphylococcal or streptococcal shock syndromes, infections associated with myocarditis such as enterovirus. 3. SARS-CoV-2 PCR testing may be positive or negative.

Figure 4: Case definition of MIS-C. From: WHO/2019-nCoV/clinical/2021.2 annex 5

Clinical picture of MIS-C has been influenced by appearance of variants as well as COVID-19. A multicentre observational retrospective study conducted in France, Spain, UK and USA found that patients admitted during the Delta and Omicron eras were younger and less sick than those admitted in the Alpha era. Specifically, patients admitted during the Alpha era versus subsequent variant eras had more respiratory involvement, shock, and systemic inflammatory response syndrome (SIRS), higher CRP, absolute lymphocyte count, and troponin levels, lower albumin and longer hospitalization. However, variant classification of this study was based on historical criteria, not lab identification (99).

Shortly after the description of MIS-C a resembling disease was reported in adults, consequently named Multisystem Inflammatory Syndrome in Adults (MIS-A). This condition differs from classical severe COVID-19 for showing just minimal respiratory symptoms, hypoxemia, or radiographic abnormalities, even if also classical COVID-19 is characterized by a strong inflammation (100): the previously described “cytokine storm”.

Prevention and therapy

COVID-19 pandemic was probably the first time in the history of transmissible diseases that the process of the development of new vaccines was conducted on such a large scale and quickly. Timeline is impressive: within the first quarter of 2020, two candidate vaccines were in phase I clinical trials and 60 in the pre-clinical phase, at the end of the year, the first vaccines were approved for marketing. This event had been possible especially because drug authorization agencies, like the Food and Drug Administration (FDA) and the European Medicines Agency (EMA), had already elaborated procedures for fast-track approval in emergency situations. Generally, vaccine research and development usually take more than 10 years because the whole process is conducted in multiple sequential steps. In the fast-track emergency mode of vaccine development, the phases are simultaneously overlapped. Actual available COVID-19 vaccines are based on different technological platforms, like inactivated vaccine, protein subunit vaccines, mRNA vaccines (an innovative approach by delivering a nucleotide sequence encoding one or more antigens), vector-based vaccines (containing a modified viral vector into which a gene encoding an antigen is introduced, eg: the S spike protein), DNA vaccines (direct introduction into specific tissues of a plasmid containing the DNA sequence encoding the chosen antigens), and virus-like particle vaccines. Efficacy of different types of vaccines can vary among different variants, subvariants, and subpopulations of patients. Some platforms have been modified to ameliorate efficacy against the new variants (101).

Therapy of COVID-19 varies according to the severity of the disease. Optimization of therapeutic options for COVID-19 is still a “work-in-progress”, our purpose is just to briefly summarize the principal elements, underlining the most important correlation to pathogenetic processes.

Probably, the most important references for clinicians all around the world are WHO guidelines and National Institute of Health (NIH) guidelines, even if there are many other possible sources of information for the physicians. Symptomatic treatment is the correct choice for mild patients; for patients with risk factors for progression to severe disease who are not hospitalized, WHO suggest the use of pulse oximetry monitoring at home (72). Immediate administration of supplemental oxygen therapy to any patient with emergency signs during resuscitation to target SpO2 ≥94% is recommended, as to any patient without emergency signs and hypoxaemia (eg: stable hypoxaemic patient) to target SpO2 >90% (or ≥92–95% in pregnant women). Emergency signs are: obstructed or absent breathing, severe respiratory distress, central cyanosis, shock, coma and convulsions (72). There are increasing evidence for the use of continuous-positive airway pressure (CPAP), non-invasive mechanical ventilation (NIV or NIMV) or high-flow nasal cannula (HFNC) in hospitalized patients when conventional oxygen supply isn’t sufficient. WHO do not make a recommendation regarding HFNC versus CPAP versus NIV due to the uncertainty of the data. So, clinicians should therefore choose between these devices on the basis of considerations such as availability of devices and the supply of oxygen, their personal comfort and experience, and patient-specific considerations (72). Anyway, CPAP seem to be more effective in preventing mechanical ventilation compared to HFNC, which can be considered as a feasible alternative for patients who don’t tolerate CPAP (102). There are not specific recommendations for the initial flow rate, FiO2, or titration scheme when HFNC are applied. WHO suggests initial flow rates of between 50 and 60 L/min and initial FiO2 of 100%, titrated to patient SpO2 and work of breathing. In children, a fixed rate of 2 L/min/kg of body weight is suggested. It is important to remember that HFNC devices may require a higher oxygen flow compared with other non-invasive respiratory support devices (72).

Role of awake prone positioning has been extensively debated. Nowadays, WHO suggests it for severely ill patients hospitalized with COVID-19 requiring supplemental oxygen (includes HFNC) or non-invasive mechanical ventilation. Awake prone positioning can reduce rate of intubation and it could slightly also reduce mortality (72).

NIH guidelines recommend oral ritonavir-boosted nirmatrelvir as treatment for non-hospitalized patients with mild to moderate COVID-19 who are at high risk of progressing to severe disease. If this is not available or cannot be used a 3-day course of remdesivir intravenous infusions should be considered as alternative. When the preferred therapies indicated above are not available, feasible to use, or clinically appropriate clinicians could consider the use of molnupiravir (103), even if in some countries this drug is not provided by the health system due to uncertainty of benefits (104,105). All these drugs must be administered as early as possible, because their efficacy decrease over time since symptoms start (106–109). Nirmatrelvir is an orally bioavailable protease inhibitor which is active against a viral protease that plays a key role in viral replication, called chymotrypsin-like cysteine protease (3C-like protease or $3CL^{pro}$ or even Main protease: M^{pro}) (110). Remdesivir is a prodrug, converted in adenosine nucleoside analog. It can inhibit viral replication by stopping RNA transcription prematurely, indeed it binds to the viral RNA-dependent RNA polymerase nsp12. Unfortunately, some viral strains can acquire resistance to this drug (111,112). Molnupiravir is an oral prodrug, the active molecule is β-D-N4-hydroxycytidine, a ribonucleoside with antiviral activity against SARS-CoV-2 (113).

For all hospitalized patients anticoagulant therapy with heparin is suggested (unless contraindicated for clinical reason); generally the preferred dose is the prophylactic one, but in nonpregnant patients who requires conventional oxygen, with D-dimer levels above the upper limit of normal (ULN) and who do not have an increased bleeding risk the preferred regimen should be the therapeutic one (103). Antibiotic therapy or prophylaxis is not routinely suggested for mild and moderate patients (72).

In patients without needing oxygen therapy, but at high risk of progressing to severe COVID-19 or affected by moderate disease (requiring conventional oxygen therapy) treatment with remdesivir should be provided (103). Anyway, impact of remdesivir in patients with COVID-19 is not dramatic: it can be considered useful, but not crucial, especially over mortality outcome (114).

In patients needing oxygen supply corticosteroid treatment is considered pivotal (102) and the suggested molecule is dexamethasone. For patients with a respiratory failure treatable with conventional oxygen therapy remdesivir can be associated to steroids. When the severity of disease increase, clinicians should consider association of immunomodulators, especially baricitinib and tocilizumab, instead benefits of remdesivir are controversial for these categories, even if a full course of remdesivir, if already started, should be completed (103). Nigro and coll. conducted a systematic review of the literature to test if there are better approaches than standard regimen with 6 mg dexamethasone, but they didn’t find fully proven better alternative (115).

For critical patients, who need endotracheal intubation, shock management and vasopressor therapy specific suggestions by the different guidelines are provided (72). A summary of therapeutic management of COVID-19 in hospitalized patients is depicted in ***Figure 5***.

Disease Severity	Recommendations for Antiviral or Immunomodulator Therapy		Recommendations for Anticoagulant Therapy
	Clinical Scenario	Recommendation	
Hospitalized for Reasons Other Than COVID-19	Patients with mild to moderate COVID-19 who are at high risk of progressing to severe COVID-19	See Therapeutic Management of Nonhospitalized Adults With COVID-19.	For patients without an indication for therapeutic anticoagulation: • **Prophylactic dose of heparin**, unless contraindicated **(AI)**; **(BIII)** for pregnant patients
Hospitalized but Does Not Require Supplemental Oxygen	All patients	The Panel **recommends against** the use of **dexamethasone (AIIa)** or other systemic corticosteroids **(AIII)** for the treatment of COVID-19.	
	Patients who are at high risk of progressing to severe COVID-19	**Remdesivir (BIIb)** for patients who are immunocompromised; **(BIII)** for other high-risk patients	
Hospitalized and Requires Conventional Oxygen	Patients who require minimal conventional oxygen	**Remdesivir (BIIa)**	For nonpregnant patients with D-dimer levels above the ULN who do not have an increased bleeding risk: • **Therapeutic dose of heparin (CIIa)** For other patients: • **Prophylactic dose of heparin**, unless contraindicated **(AI)**; **(BIII)** for pregnant patients
	Most patients	Use **dexamethasone plus remdesivir (BIIa)**. If remdesivir cannot be obtained, use **dexamethasone (BI)**.	
	Patients who are receiving dexamethasone and who have rapidly increasing oxygen needs and systemic inflammation	Add 1 of the following immunomodulators: *Preferred* • **PO baricitinib (BIIa)** • **IV tocilizumab (BIIa)** *Alternatives* • **IV abatacept (CIIa)** • **IV infliximab (CIIa)**	
Hospitalized and Requires HFNC Oxygen or NIV	All patients	**Dexamethasone** should be administered to all patients **(AI)**. If not already initiated, promptly add 1 of the following immunomodulators: *Preferred* • **PO baricitinib (AI)** *Preferred Alternative* • **IV tocilizumab (BIIa)** *Additional Alternatives (Listed in Alphabetical Order)* • **IV abatacept (CIIa)** • **IV infliximab (CIIa)** Add remdesivir to 1 of the options above in certain patients	For patients without an indication for therapeutic anticoagulation: • **Prophylactic dose of heparin**, unless contraindicated **(AI)**; **(BIII)** for pregnant patients For patients who are started on a therapeutic dose of heparin in a non-ICU setting and then transferred to the ICU, the Panel recommends switching to a **prophylactic dose of heparin**, unless there is another indication for therapeutic anticoagulation **(BIII)**.
Hospitalized and Requires MV or ECMO	All patients	**Dexamethasone** should be administered to all patients **(AI)**. If the patient has not already received a second immunomodulator, promptly add 1 of the following (listed in alphabetical order): • **PO baricitinib (BIIa)** • **IV tocilizumab (BIIa)**	

Figure 5: Therapeutic Management of Hospitalized Adults With COVID-19. Modified from: COVID-19 Treatment Guidelines Panel. Coronavirus Disease 2019 (COVID-19) Treatment Guidelines. National Institutes of Health. Available at https://www.covid19treatmentguidelines.nih.gov/. Accessed on 11/9/2023.

Convalescent plasma from recovered people is a classical approach to any new infectious disease, even if a meta-analysis conducted for viral epidemics other than COVID-19 did not find conclusive results (116). The role of convalescent plasma with proved neutralizing high-titre for immunocompromised patients is arguable. Many case reports are available (112,117), but there are not clear statement about it. NIH guidelines only allow this option, without suggesting, instead Infectious Diseases Society of America (IDSA) suggest using it among ambulatory patients with mild-to-moderate COVID-19 at high risk for progression to severe disease who have no other treatment options (118). Actually, this last condition (no other treatment options) is very unlikely in economically developed country.

Monoclonal antibodies against viral antigen, especially SARS-CoV-2 spike protein, were developed. They provided clinical benefits in treating SARS-CoV-2 infection, but laboratory studies have found that anti-viral activity of them against specific variants and subvariants can vary dramatically. This obviously interferes with their use in clinical practice. In USA the association bamlanivimab plus etesevimab, casirivimab plus imdevimab, sotrovimab, and bebtelovimab were registered as clinical progression preventive treatments in outpatients. Of them, the association casirivimab plus imdevimab was authorized also for hospitalized

patients. The combination tixagevimab plus cilgavimab was allowed to be used as COVID-19 pre-exposure prophylaxis. Anyway, due to their lack of efficacy against many Omicron subvariants, they are currently unavailable in USA (103). In other countries the picture can be slightly different. For example, in Italy casirivimab plus imdevimab association is still included among usable therapies for hospitalized patients, but an alert is declared about the possible inefficacy. The same alert is delivered for the products authorized for outpatient treatment, like sotrovimab, tixagevimab/cilgavimab, regdanvimab and the already reported casirivimab/imdevimab. Previous authorization of bamlanivimab alone and bamlanivimab/etesevimab has been completely withdrawn (119,120). There are also other products that could be authorized in USA or in Europe in the next future. Susceptibility of the different SARS-CoV-2 variants and subvariants to the monoclonal antibodies is continuously under study, Stanford University provides update in *https://covdb.stanford.edu/susceptibility-data/table-mab-susc/* (121).

It's interestingly to notice as actual suggested therapy of COVID-19 conceptually agrees with our pathogenetic interpretation of the disease: direct contrast of viral replication in the early phase followed by anti-inflammatory agents. Indeed, dysregulated inflammatory response is the pivot of severe and critical COVID-19, as previously described.

Pulmonary fibrosis

Description and classification

Pulmonary fibrosis, along with lung inflammation, is one of the features of a group of heterogeneous conditions commonly known as interstitial lung diseases (ILDs). This denomination derives from the common involvement of lung interstitium in the pathogenesis. However, one should realize that also diseases associated with alveolar filling or vascular abnormalities are classified under the umbrella term of “ILD” (122).

ILD classification has evolved substantially since the first descriptions of pulmonary fibrosis more than a century ago. ILDs were often classified based on pathologic features until the turn of the century, with increasing use and understanding of computed tomography (CT) that let us to confidently provide ILD diagnosis without histopathology. The contemporary approach to ILD classification has been established in a series of consensus statements and clinical practice guidelines produced over the 20 years. The first major change in ILD classification over the past 2 decades has been the adoption of an integrated multidisciplinary approach, specifically based on the conclusions arising from a multidisciplinary discussion (MDD) that includes an ILD clinician (pulmonologist), a chest radiologist, and a lung pathologist. A second major change has been the increasing emphasis on disease behaviour. Anyway, still now there are multiple overlapping approaches to ILD classification, and it is therefore common for patients to simultaneously be classified based on multiple approaches. So, we can identify an aetiology-based classification, a morphology-based one (which classifies patients on radiologic and/or histopathologic patterns) and a behaviour-based approach (123). Generally, clinicians use an integrated approach to classify patients into specific diagnosis. In ***Figure 6*** is reported one of the most common used classifications of diffuse parenchymal lung diseases

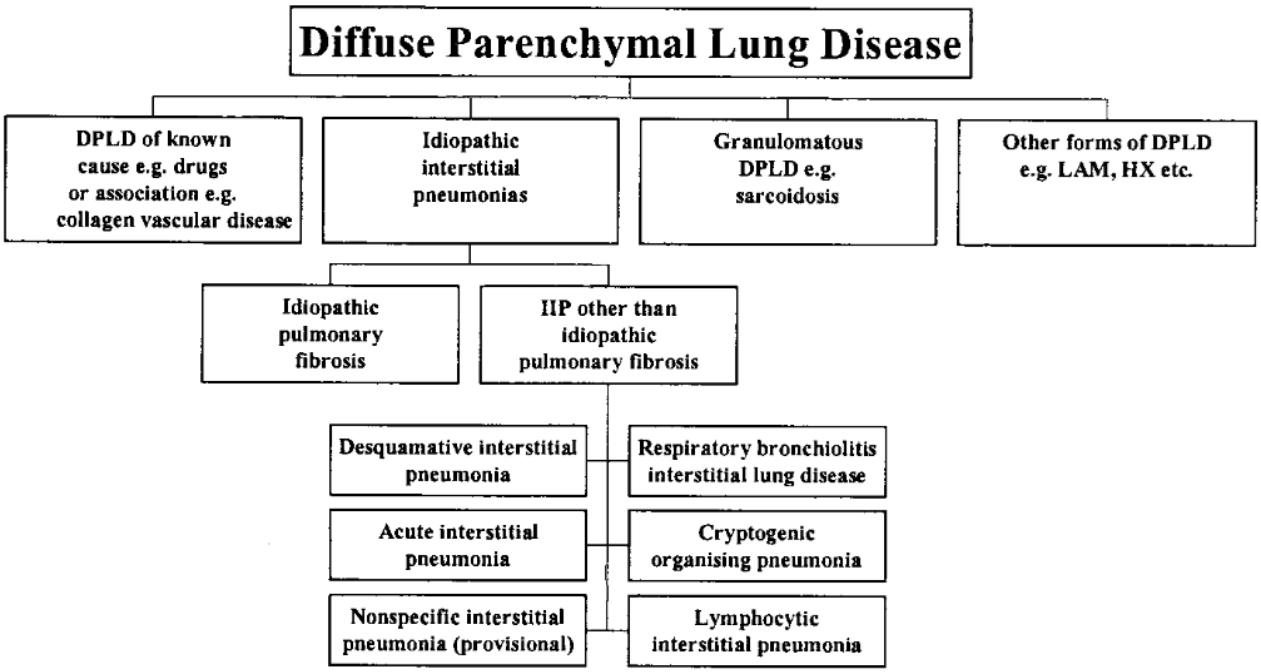

Figure 6: commonly accepted classification of diffuse parenchymal lung diseases (DPLDs or ILDs). From: ATS/ERS. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. Adopted by the ATS board of directors in June 2001 and by the ERS Executive Committee in June 2001. *Am J Respir Crit Care Med*. **2002** Jan 15;165(2):277-304. doi: 10.1164/ajrccm.165.2.ats01. Erratum in: *Am J Respir Crit Care Med* **2002** Aug 1;166(3):426.

(DPLDs), which include ILDs and other diseases (124). Generally, more attention has been adopted concerning the group of idiopathic interstitial pneumonias (IIPs), for which a revised and more detailed classification is available (***Fig. 7***) (125).

Probably, idiopathic pulmonary fibrosis (IPF) represents the most known example of ILD, being also one of the more severe and more frequent ones. According to European Respiratory Society (ERS) and the others major scientific societies around the world IPF is a chronic, fibrosing interstitial pneumonia of unknown cause that is associated with radiological and histologic features of usual interstitial pneumonia (UIP). It occurs primarily in older adults, is characterized by progressive worsening of dyspnoea and lung function and has a poor prognosis. Even if the radiological UIP pattern is a hallmark of IPF (IPF-UIP), it can also be seen in patients with other

REVISED AMERICAN THORACIC SOCIETY/EUROPEAN RESPIRATORY SOCIETY CLASSIFICATION OF IDIOPATHIC INTERSTITIAL PNEUMONIAS: MULTIDISCIPLINARY DIAGNOSES

Major idiopathic interstitial pneumonias
- Idiopathic pulmonary fibrosis
- Idiopathic nonspecific interstitial pneumonia
- Respiratory bronchiolitis–interstitial lung disease
- Desquamative interstitial pneumonia
- Cryptogenic organizing pneumonia
- Acute interstitial pneumonia

Rare idiopathic interstitial pneumonias
- Idiopathic lymphoid interstitial pneumonia
- Idiopathic pleuroparenchymal fibroelastosis

Unclassifiable idiopathic interstitial pneumonias

Figure 7: Modified from: Travis, WD.; Costabel, U.; Hansell, DM. et al. ATS/ERS Committee on Idiopathic Interstitial Pneumonias. An official ATS/ERS statement: Update of the international multidisciplinary classification of the idiopathic interstitial pneumonias. *Am J Respir Crit Care Med*. **2013** Sep 15;188(6):733-48. doi: 10.1164/rccm.201308-1483ST.

IPF suspected		Histopathology pattern			
		UIP	Probable UIP	Indeterminate for UIP or biopsy not performed	Alternative diagnosis
HRCT pattern	UIP	IPF	IPF	IPF	Non-IPF dx
	Probable UIP	IPF	IPF	IPF (Likely)	Non-IPF dx
	Indeterminate	IPF	IPF (Likely)	Indeterminate	Non-IPF dx
	Alternative diagnosis	IPF (Likely)	Indeterminate	Non-IPF dx	Non-IPF dx

Figure 8: IPF diagnosis on the basis of HRCT and biopsy patterns. Modified from: Raghu, G.; Remy-Jardin, M.; Richeldi, L. et al. Idiopathic Pulmonary Fibrosis (an Update) and Progressive Pulmonary Fibrosis in Adults: An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline. *Am J Respir Crit Care Med*. **2022** May 1;205(9):e18-e47. doi: 10.1164/rccm.202202-0399ST.

diseases, like fibrotic hypersensitivity pneumonitis (HP), connective tissue disease (CTD) (CTD-UIP), or exposure-related ILDs. The histopathological criteria that characterize UIP are the following: patchy dense fibrosis with architectural distortion (eg: honeycombing), a predilection for subpleural and paraseptal lung parenchyma, presence of fibroblast foci and the absence of features that suggest an alternative diagnosis. The integrated assessment of radiological and histopathological elements leads to the diagnosis of IPF (***Fig. 8***) (126).

Organising pneumonia (OP) is a morphological description of an interstitial lung disease pattern (123), which includes cryptogenic organising pneumonia (COP), a well-defined clinical and etiological entity (124), but also many other secondary forms. The term organising pneumonia has replaced the previously used term bronchiolitis obliterans with organising pneumonia (BOOP). The classification of OP into COP and secondary OP is clinically important, as the management of patients with secondary OP includes not only the treatment of OP but also the management of underlying disease and avoidance of any known offending agents. To date, it remains unclear if COP and secondary OP represent two distinctive clinical entities or if they are a common entity of unspecific lung injury and repair (127). Pathology of organising pneumonia is characterized by accumulation of inflammatory debris in alveolar ducts and spaces with the presence of endoluminal fibro-inflammatory buds that are typical of organising pneumonia: Masson's bodies. Interstitial inflammatory infiltrates are present. Generally, OP resolve with *restitutio ad integrum* after steroid treatment, but recurrences can happen and a proportion of the patients (up to 25%) develop lung fibrosis. Pulmonary infections are possible causes of secondary organising pneumonia (128,129).

As previously evidenced, the evaluation of disease evolution over time is now considered a pivotal element for a correct assessment of ILDs. Therefore, it has been introduced the concept of progressive pulmonary fibrosis (PPF). PPF is defined as at least two of the following three criteria occurring within the past year with no alternative explanation: worsening respiratory symptoms, physiological evidence of disease progression and radiological evidence of disease progression. The physiological aspects consist in forced vital capacity (FVC) or diffusing capacity for carbon monoxide (DLCO) decrease; radiological

extension is evaluated as percentage of lung volume containing fibrotic features in the upper, mid, and lower lung zones (***Fig. 9***) (126).

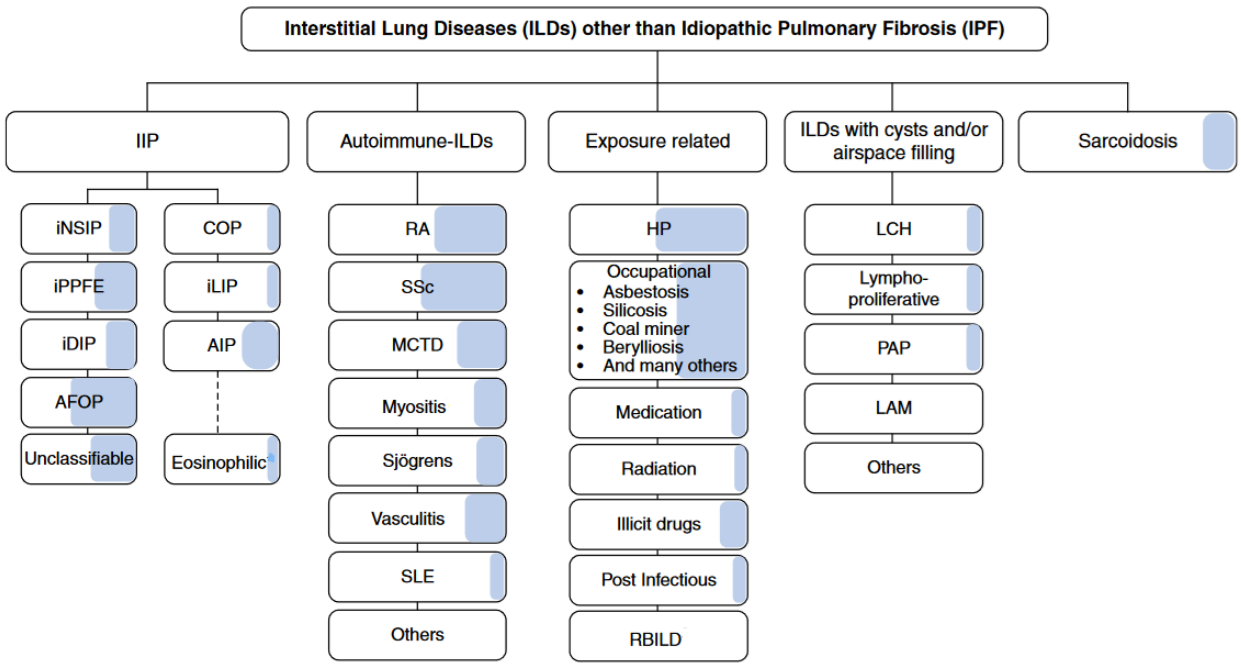

Figure 9: The shaded area represents the estimated proportion of patients with various types of ILD who manifest PPF. Modified from: Raghu, G.; Remy-Jardin, M.; Richeldi, L. et al. Idiopathic Pulmonary Fibrosis (an Update) and Progressive Pulmonary Fibrosis in Adults: An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline. *Am J Respir Crit Care Med*. **2022** May 1;205(9):e18-e47. doi: 10.1164/rccm.202202-0399ST.

Therapy

Lung fibrosis is a heterogeneous condition so, as many other similar heterogeneous diseases, therapeutic approach is obviously complex.

Until few years ago it was a real conundrum for pulmonologists to treat ILDs, since the discovery of specific antifibrotic drugs this clinical problem has been partially relieved. Nowadays, there are two important antifibrotic medications: pirfenidone and nintedanib. The first one is an oral antifibrotic drug with pleiotropic effects, like regulating crucial profibrotic and proinflammatory cytokine cascades while reducing fibroblast proliferation and collagen synthesis. The latter is an intracellular inhibitor of several tyrosine kinases that targets multiple growth factor receptors, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet derived growth factor (PDGF) (130). We should underline that until 2022 update both agents were available only for IPF, excluding all the other ILDs. Treating lung fibrosis other than IPF often is less straightforward, with a potential benefit of immunosuppression in some conditions characterized by inflammation. However, there is still uncertainty regarding the optimal use of anti-inflammatory therapies in various ILDs (122). Organising pneumonia, especially COP, often needs chronic steroid therapy as relapse prevention (131).

Nowadays, internationally adopted scientific documents suggest prescription of antifibrotic drug in the clinical picture of a PPF, even if the two broadly used compounds, nintedanib and pirfenidone, are not exactly equivalent. Indeed, only the first one is the molecule suggested by ERS and ATS, if the standard management of the specific ILD has already

failed. On the contrary, evidence about the potential role of pirfenidone is judged insufficient to make recommendation for or against its use (126). Anyway, a recent expert consensus statement underlines that a “free-for-all” approach in the prescription of antifibrotic therapy for all patients with fibrosing ILD at presentation should be avoided. Indeed, it fails to consider that conventional therapies meet the needs of a majority of non-IPF patients with lung fibrosis. So, it’s important to differentiate between PPF at first presentation (with worsening symptoms and, where available, worsening imaging features) and PPF occurring despite management: a comprehensive and complete diagnosis still remains crucial (132). We should also remember that pirfenidone and nintedanib are both really expensive, so an appropriate management would be beneficial (133).

Concerning the phenomenon of acute exacerbation (AE) in IPF, defined as an acute, clinically significant respiratory deterioration characterized by evidence of new widespread alveolar abnormality (134) there aren’t many therapeutic options. Many pulmonologists use steroid therapy, often at high dosage, even if there aren’t consistent proofs in favour of this strategy. A recent trial showed lack of usefulness for immunosuppressive therapy with cyclophosphamide (135,136).

Multiple nonpharmacological treatments are adopted to treat patients with fibrosing ILDs. Supplemental oxygen is pivotal in correcting hypoxia. Pulmonary rehabilitation, psychological support, symptom relief, and end-of-life care are commonly suggested. Comorbidities should be addressed and treated properly, such as gastroesophageal reflux, obstructive sleep apnoea, and lung cancer. Lung transplantation is a possibility for selected patients (122).

Pulmonary fibrosis and COVID-19

The community of pulmonologists all around the world is well aware that COVID-19 pandemic can leave us an increased burden of pulmonary fibrosis and its consequences (137). Bronchiectasis and fibrotic strands were described as one of the possible evolutions of COVID-19 in the first published case series (138). Considering the likeness of COVID-19 pneumonia and organising pneumonia, fibrosis development seemed a possible complication, so the importance of a radiological follow-up for the patients has been underlined since the start of the pandemic (139). The firsts bioptic records also reported fibrotic remodelling of the lung in some cases of COVID-19 (140).

Lung fibrosis can be a consequence of low tract respiratory infection or inflammation. Pneumonia caused by Influenza (Flu) virus can lead to persistent alterations of the lung, even after acute phase. In some case these abnormalities are consistent with parenchymal fibrosis, in some others are less specific. Xing and colleagues, in 2015, performed follow-up CT-scan to 24 patients 3 years after Influenza A H1N1 pneumonia and they find that 17 of them (70.8%) had radiologically evident lung sequelae. In particular, 10 patients (41.7%) showed lung fibrosis, ascertained by the presence of architectural distortion, traction bronchiectasis, or honeycombing. Fibrosis was associated also with other kinds of alterations; indeed, they had a higher rate of ground-glass opacity (100% vs. 35.7%), parenchymal bands (80% vs. 14.3%), air trapping (60% vs. 21.4%), and reticulation (70% vs. 7.1%) than patients without clear signs of fibrosis. It’s also interesting that manifestations like ground-glass opacities and air-trapping can persist even years after resolution of acute disease. Severity of acute disease seemed to be associated with higher risk of persistent sequelae (141). Another Chinese study established some years later included 232 patients with a diagnosis of H1N1 Influenza virus infection who met the diagnostic criteria for ARDS.

Of those 232 patients, 32 died, 16 refused to participate, and 144 survived to discharge. Of the 106 patients who remained alive at the 6 months follow-up, consent was obtained from 69: the final study population. Lung involvement assessed by chest CT-scan improved a lot during the 6 months after discharge and the presence of underlying diseases at hospitalization was the most important determinant of the CT scores. Interestingly, a longer ICU stay and duration of ventilator use (which theoretically can imply an higher severity) were associated with a higher CT score at 3 months but not at 6 months follow-up (142). Strains of Influenza different from H1N1 can also be a cause of chronic lung pathology. The extremely dangerous zoonosis H7N9 not only lead to death a significant proportion of the cases, but it also affected lungs of the patients years after the infection. A study revealed that radiological improvement substantially stopped 6 months after discharge and at the 12-month follow-up, up to more than 40% of the patients had fibrotic alterations and more than a half had parenchymal opacification including ground-glass opacities and reticular patterns. PFT detected various abnormalities even two years after discharge: for example, DLCO persisted decreased in more than three-quarters of the tested patients. Patients who had suffered from ARDS during acute phase showed a wider impairment of lung function at follow-up compared to the ones with less severe disease (143).

Moving from specific viral pneumonia to general inflammation conditions of the lung still shows the possibility of fibrosis development and chronic impairment. Acute Respiratory Distress Syndrome (ARDS) can have different aetiologies, and it is characterized by severe and diffuse inflammation of the lungs, being an important cause of endotracheal intubation and acute respiratory-related cause of death. Intensive management of ARDS has been improved in the past decades, decreasing mortality, anyway a significant proportion of ARDS survivors continue to suffer from reduced health-related quality of life for months to years after the disease. Generally, residual pulmonary impairment is largely discounted as a significant contributing factor to low quality of life comparing to depression and neuromuscular weakness, but clinicians should avoid this error. Studies conducted among ARDS survivors in which PFTs performed 6 months to years after intensive care unit (ICU) discharge were available, did not find global reduction of parameters like FVC, FEV1, total lung capacity (TLC) and DLCO, even if a significant proportion of the patients had impairment of some of them. Chest CT-scans of ARDS survivors often demonstrate reticular infiltrates that may persist for long time (months or years) after discharge even if studies that have included both PFT and chest CT-scans usually underline that persistent radiographical abnormalities are generally not severe (144).

Anyway, the risk of an overdiagnosis of lung fibrosis during COVID-19 follow-up, pointing out mild scars without clinical significance, is declared also in European Respiratory Society documents (145). Moreover, identifying subgroups of patients who develop clinically relevant fibrotic sequelae is difficult. Indeed, a significant proportion of people who suffer from COVID-19 tends to show sub-acute or chronic persistence of unspecific symptoms: this is the so-called “long-COVID” or “post-acute sequelae of COVID-19” (PASC); this illness has got a poorly understood aetiology. Nevertheless, some common symptoms of PASC (eg: fatigue, shortness of breath…) can overlap with typical manifestations of lung parenchymal chronic damage, as lung fibrosis, but only a minority of patients affected by PASC with these complaints has a fibrotic damage. Lung fibrosis is not the first cause of PASC (146). Etiopathogenesis of PASC is under investigation by many scientists. Probably it is sustained by different phenomena, involving immunity, chronic inflammation, reactivation of latent infections, imbalance of hypothalamic-pituitary axis and maybe others (147).

An interesting longitudinal study done by Huang and coll. screened more than one thousand patient who recovered from COVID-19 searching for long-term sequelae of the infection. Patients were also stratified according to the clinical severity at hospital admission. The authors observed a generally good recovery for the most part of the patients. Only a minority of the patients underwent to lung CT-scan (353) at 6-months follow-up, but more than half of them (186) showed some abnormalities and so were invited to repeat the CT-scan at 12-months follow. A general amelioration of the radiological abnormalities was observed, but especially among patients who had developed a severe form of COVID-19, some alterations were present. 76% of the 38 patients who suffered from severe disease still had ground-glass opacities in the lungs 12 months after infection and 11% of them showed interlobular septal thickening. Interestingly, none of these patients presented this radiological feature at 6 months follow-up, and that can indicate a sort of progressive fibrotic evolution of residual inflammation. Spirometry values and lung volume parameters of most patients, when collected, were within normal limits at 12-month visit, but the proportion of total lung capacity less than 80% of predicted was still 29% at 12 months in patients with severe disease and 7% in whom just required supplemental oxygen (148,149).

The exact mechanism of pulmonary fibrosis development in COVID-19 patients is not fully understood. There are two popular hypotheses that underline which factors influence the remodelling of lung tissue. The first is based on the ACE2-related profibrotic pathway being activated by SARS-CoV-2 infection. The second is related to a hyperinflammatory reaction due to the infection inducing lung fibrosis (150). Considering the first hypothesis, it is necessary to briefly sum up some concepts that we previously described: renin-angiotensin system (RAS) controls the volume of circulating blood and the concentration of sodium and potassium in body fluids and the key negative regulator of this system is ACE2. ACE2 is part of the ACE2/Ang(1-7)/MasR axis, and it is responsible for the hydrolysis of angiotensin II (Ang II) to Ang1-7. The activation of this axis has anti-inflammatory and antifibrotic effects. SARS-CoV-2 infection reduces ACE2 expression so causing increased levels of Ang II, which promotes inflammation and the fibrosis process (49,50): an effect demonstrated also for SARS-CoV (151). As a result of the decreased amount of Ang(1,7) peptide, the concentration of transforming growth factor β (TGF-β) increases and TGF-β is a well-known profibrotic stimulus, promoting the formation of myofibroblasts from fibroblasts and collagen synthesis (***Fig. 10***) (50).

Concerning the hypothesis that underlines a pivotal role of hyperinflammation in fibrosis development, it implies that the cytokine storm can obviously induce cellular lesions of airway epithelial and endothelial cells, severe lymphopenia, neutrophils recruitment, pulmonary cell infiltration, and finally, it can lead to lung tissue injury and ARDS. If this phenomenon is not suppressed, the progressive damage and regeneration processes can result in remodelling of the lung tissue (150,152).

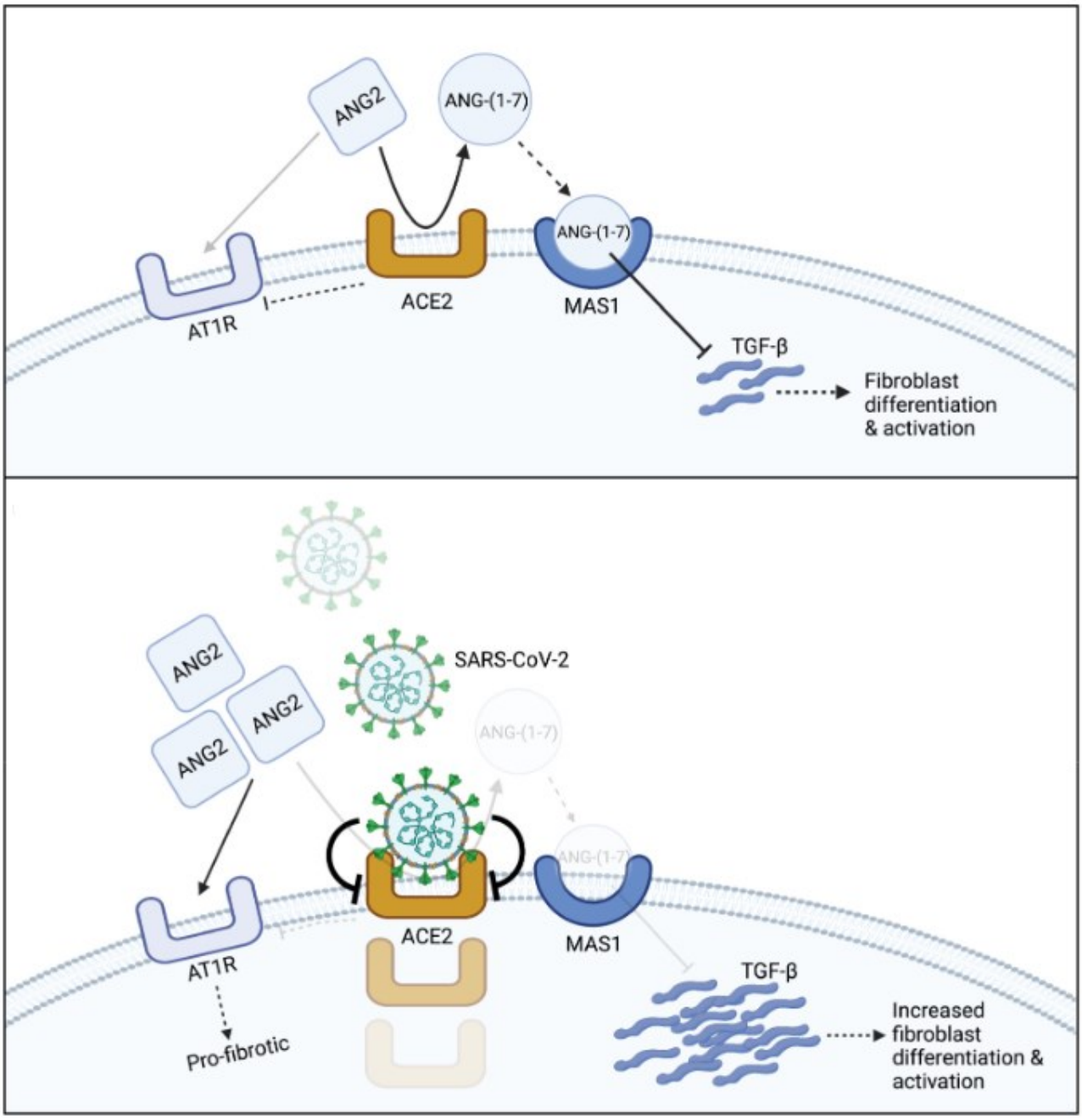

Figure 10: Scheme of the ACE2/Ang-(1–7)/MasR Axis. Up: Normal State. Down: Disease State (Inflammatory). In the last one, SARS-CoV2 binds ACE2, causing internalization and downregulation. Decreased levels of ACE II result in decreased ANG-(1-7), allowing TGF-B concentrations to rise. From: Morganstein, T.; Haidar, Z.; Trivlidis, J. et al. Involvement of the ACE2/Ang-(1-7)/MasR Axis in Pulmonary Fibrosis: Implications for COVID-19. *Int J Mol Sci*. **2021** Nov 30;22(23):12955. doi: 10.3390/ijms222312955.

Role of biomarkers

Definition

Biomarker (contraction of “biological marker”) is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Biomarkers may have the greatest value in early efficacy and safety evaluations, but they can also be useful as diagnostic tool for the identification of those patients with a disease or abnormal condition, tool for staging of disease or classification of the extent of disease, indicator of disease prognosis, for prediction and monitoring of clinical response to an intervention. Biomarkers can be surrogate endpoint in clinical practice. Surrogate endpoints can be defined as biomarkers that are intended to substitute for clinical endpoints. A surrogate endpoint is expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence (153).

The discovery of new biomarkers is generally based on two different approaches. The hypothesis-driven method selects new candidate biomarkers a priori based on previous evidence about the disease. On the contrary, the unbiased approach utilizes methods from systems biology to screen many possible candidate molecules for their association with the disease (154).

COVID-19

The importance of laboratory data collection to stratify patients with COVID-19 has been noticed immediately. Zhou and colleagues described a lower lymphocyte count in non-survivors; in survivors, lymphocyte count was lowest on day 7 after illness onset and improved during hospitalisation, whereas severe lymphopenia was observed until death in non-survivors. Many unspecific proinflammatory biomarkers (like lactate dehydrogenase, ferritin and procalcitonin) resulted higher in non-survivors, as like as cardiac damage indexes, such as high-sensitivity cardiac troponin I and creatine kinase; also interleukin-6 (IL-6) was clearly higher in patients with poor prognosis (71). Similar data were collected by different authors in other countries (155).

In general, during the cytokine storm, serum levels of IL-2R, IL-4, IL-6, TNF-α, IL-1RA, IL-1b, IFN-γ are elevated, in conjunction with increased levels of chemokines such as CCL2, CCL8, CXCL2, CXCL8, CXCL9, and CXCL16. Some of these cytokines (IL-6, IL-10) and chemokines (CXCL9, CXCL10) were found to be significantly higher in severe patients when compared to milder ones. It should be underlined that, among different cytokines involved, IL-6 has a pivotal role in driving the hyperinflammatory response, and it is an independent predictor of patient survival (46).

Nowadays, there is not much information regarding the role of biomarkers in long-COVID. A first systematic review literature looking for blood biomarkers potentially useful as indicators or therapeutic targets for long COVID was conducted in January 2023. Higher levels of IL-6, CRP, and TNF-α and lower levels of haemoglobin emerged as distinctive findings from a comparison between long-COVID patients and recovered COVID-19 patients. In addition, long-COVID patients showed increased levels of IL-6, TNF-α, IL-17, and CCL3 (C-C motif chemokine ligand 3) than healthy participants. The authors concluded that up-regulated IL-6, CRP, and TNF-α were a potential core set of biomarkers for long-COVID (***Fig. 11***) (156).

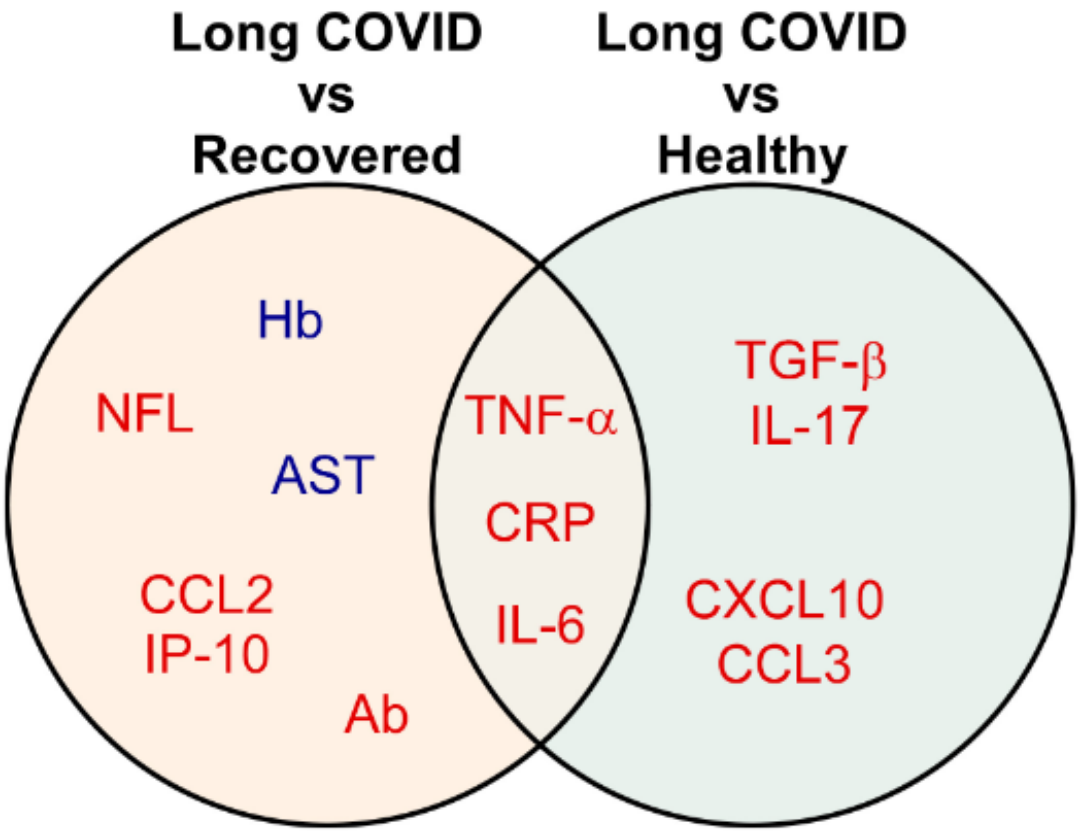

Figure 11: Biomarkers significantly associated with different comparison groups. Red ones are up-regulated, while blue refers to down-regulated biomarkers. From: Lai, YJ.; Liu, SH.; Manachevakul, S. et al. Biomarkers in long COVID-19: A systematic review. *Front Med (Lausanne)*. **2023** Jan 20;10:1085988. doi: 10.3389/fmed.2023.1085988.

Pulmonary fibrosis

Research activities about ILDs often concentrate on biomarkers. This approach agrees with the need to avoid invasive procedure to specifically classify any patients in the correct diagnostic picture, choosing the best therapeutic option available.

An interesting application field for biomarkers is the assessment of the so-called interstitial lung abnormalities (ILAs): the presence of non-dependent radiographic abnormalities on CT-scan occurring in an individual in whom interstitial lung disease is not suspected. A significant proportion of ILAs progress over the subsequent years, developing a classical ILD. Blood biomarkers have the potential to be a simple, minimally invasive way of identifying individuals at risk of developing ILA and for assessing likelihood of ILA progression. Increased levels of matrix metalloprotease-7 have been shown to predict progression of ILA. Also, surfactant protein B (SFTB) and WAP four-disulfide core domain protein-2 (WFDC2) together with growth differentiation factor-15 (GDF15) and cathepsin H (CTSH) have been associated with ILA progression. Other molecules have been studied or are still under study for their potential role as screening tools for ILAs (157,158).

Predisposition biomarkers reflects mechanism or biological pathways linked to disease predisposition. Variants of surfactant protein C (SP-C), surfactant protein A2 (SP-A2) and surfactant protein A1 (SP-A1) have been associated to familiar pulmonary fibrosis, but they are rare in sporadic IPF (154). A common single nucleotide polymorphism (SNP) in the

putative promoter region of the mucin 5B (MUC5B) gene (rs35705950) has been associated with familiar pulmonary fibrosis and sporadic pulmonary fibrosis, it has been found also in 9% of people with ILAs (159). Several variants of the telomerase complex and its regulatory proteins have been associated to pulmonary fibrosis, especially familiar forms. It is common in IPF patients compared with age-matched controls, but it is globally rare in sporadic IPF and not specific for this disease (154,160). Diagnostic biomarkers should reflect the mechanism or biological pathways that distinct IPF from the other ILDs. In some studies SP-A serum levels appear to be significantly higher in patients with IPF than in patients with non-IPF ILDs, pulmonary infection, and healthy controls; SP-B precursor, C-pro-SP-B, has been studied as a new biomarker being able to differentiate IPF patients from patients with all other pulmonary diseases (154,161). Metalloproteases (MMP) are another class of proteins widely studied for their role in in the aberrant fibrotic process, but their role is not completely understood. In particular, MMP-7, in association with other markers of fibrosis could help differentiate IPF from other ILDs (162). Krebs von den Lungen-6 (KL-6; often referred as mucin 1 or MUC1) is a serum high molecular weight glycoprotein. It is increased in many ILDs, including IPF and chronic hypersensitivity pneumonia and it is mainly produced by damaged alveolar type 2 pneumocytes. Its serum concentration is considered a biomarker of lung epithelial damage (163) and it has been correlated with 3-year mortality among patients with PPF (164). KL-6 can be useful in stratification of severity of the ILDs, even if it is not specific enough to distinguish IPF from the other ILDs (154). Prognostic biomarkers should contribute to quantitative assessment of mechanism or biological pathways relevant to disease progression; instead, therapeutic biomarkers should provide quantitative assessment or indicate the presence or absence of mechanisms or biological pathways targeted by therapy. Most of the previously described markers (like telomerase complex related proteins, surfactant proteins and MUC5B variant) have been studied as prognostic and therapeutics biomarkers in IPF, finding interesting evidences, but not definitive results (154). For example, KL-6 could be indicative of the response to nintedanib treatment; in particular, it seems that IPF patients treated with nintedanib who maintain stable FVC values also show stable KL-6 levels (***Fig. 12***) (165).

Another field of application for biomarkers research in lung fibrosis regards the phenomenon of acute exacerbation (AE) (134). Acute exacerbations are detrimental in the clinical course of ILDs (not only IPF) and early diagnosis before the onset of severe respiratory failure and early recognition of the patients who are more susceptible to AE would be important for therapeutic decisions in those patients. In case of a de novo diagnosed ILD presenting as an acute exacerbation, diagnostic tests that are currently used are autoantibodies, which can help to identify ILDs associated with collagen vascular diseases, and the precipitating antibodies supporting the diagnosis of hypersensitivity pneumonitis. In certain cases, BALF lymphocytosis might support an intensive immunosuppressive treatment for AE-ILD, contrary to a neutrophilic BALF. Despite many biomarkers being studied in AE-ILD, most of these studies are retrospective. Among biomarkers already cited KL-6 and surfactant proteins are ones more extensively studied in AE-ILD; inflammatory cytokines have also been assessed in acute exacerbations, like IL-6, IL-8 and IL-1b (166).

Biomarkers can be searched not only in blood, but also in bronchoalveolar lavage fluid (BALF). For example, neutrophil-to-lymphocyte ratio (NLR) in BALF and CD8+ T cells levels in the BALF have been associated with features of PPF compared with non-PPF interstitial lung disease patients (164). The importance of identifying serum biomarkers to point out patients at risk of PPF is stated also by ATS/ERS in their last publications (126).

Biomarker	Predisposition	Diagnosis	Prognosis	Therapy Monitoring
SP-C		Disease: ++		
SP-A	Disease: ++	Disease: +	Disease: ++	Disease: +
SP-D	Disease: ++	AE: ++	Disease: ++	Disease: +
C-pro-SP-B		Disease: ++		
MUC5B	Disease: +++		Disease: +/−	Disease: ++
Telomerase complex	Disease: +	Disease: ++	Disease: ++	
Toll-like receptors	Disease: +	Disease: +	Disease: ++	
ELMOD-2	Disease: +			
KL-6/MUC1		Disease: +	Disease: + AE: ++	Disease: ++
cCK18		Disease: ++	Disease: −	
metalloproteases		Diagnosis: ++	Disease: +++	Disease: +++
osteopontin		Disease: +	Disease: − AE: ++	
TOLLIP			Disease: ++	
α-defensins		Disease: + AE: ++		
Periostin			Disease: ++	

AE: acute exacerbation; SP surfactant protein; MUC5B mucin 5B; cCK18 Circulating caspase-cleaved cytokeratin-18

Figure 12: Examples of molecular biomarkers in IPF. Number of "+" suggest the potential usefulness in the specific field of application. Modified from: Stainer, A.; Faverio, P.; Busnelli, S. et al. Molecular Biomarkers in Idiopathic Pulmonary Fibrosis: State of the Art and Future Directions. *Int J Mol Sci*. **2021** Jun 10;22(12):6255. doi: 10.3390/ijms22126255.

AIM

In this context, the study focused on novel biomarkers to identify patients at risk of chronic lung fibrotic changes in patients affected by COVID19. In particular, we would like to understand the potential role for the recently described interleukin-32 (IL-32) in those patients. Subsequently, we evaluated the roles of these possible biomarkers in the follow-up of COVID-19 patients.

MATERIALS and METHODS

Study population

Our first study population comprised patients with positive nasopharyngeal swab for SARS-CoV-2 who required hospitalisation for clinical manifestations related to COVID-19. They were enrolled upon admission to Siena University Hospital. Period of enrolment was included between October 2020 and April 2021. Patients needing hospitalization for other than COVID- 19 issues with just a positive nasopharyngeal swab were not included. We excluded patients previously vaccinated against SARS-CoV-2 and patients submitted to previous home treatment with monoclonal antibodies specific for COVID-19.

We consecutively enrolled 64 COVID-19 patients (46 male, 65 (59–67) years). Upon hospitalization, patients were divided into three groups according to the severity of lung involvement: “mild” (patients treated with or without conventional oxygen support), “moderate” (patients requiring NIV and/or HFNC) and “severe” (patients requiring endotracheal intubation). Signs, symptoms, radiological data, immunological features and serum concentrations of inflammatory biomarkers were entered in a database. We also selected 27 healthy controls (9 male, 58 (36–78) years). Serum samples were all collected on the day of hospital admission, before any treatment or infusion of intravenous steroids or invasive ventilation. Serum aliquots were stored at -80 C° until assay. Clinical, demographical and laboratory data were collected for all patients. All patients gave their written informed consent to the study, previously approved by our local Ethics Committee (BIOBANCA-MIU-2010).

Subsequently, we considered a wider study population for a dual steps analysis. We included also subjects hospitalized at Grosseto Hospital, the time frame started in March 2020 and ended in June 2022. All COVID-19 cases were classified as mild, moderate, severe and critical according to WHO criteria, but we finally pooled mild and moderate cases into a single group and severe and critical into a second group for numerical reason. Lab tests and diagnostic procedures had been initially performed according to the clinical judgement of the emergency department staff, who also did SARS-CoV-2 nasopharyngeal swab. To be included in the study, all blood samplings had to be collected within the first 24h after admission to hospital, agreeing with the previous prospective study. Considering the wide heterogeneity of laboratory exams prescribed in this kind of setting, we decided to collect only the parameters fully available for all the patients (blood cell count, including haemoglobin, haematocrit, white blood cells count with percentages of neutrophils, lymphocytes, monocytes, eosinophils and basophils, platelets, and CRP). Final population comprised 108 participants (94 mild–moderate and 14 severe–critical patients); 8 patients died during hospitalization and 100 were discharged. After discharge from hospital, 56 patients participated in the post-COVID-19 follow-up protocol: the second step. These cases underwent medical examination, HRCT of the chest, blood tests and pulmonary function tests between 3 and 6 months after discharge from hospital. Main HRCT findings, like presence of air-trapping, fibrosis and ground glass opacities were recorded; 11 patients showed fibrosis and 45 did not. All data, including clinical, sociodemographic and survival,

as well as comorbidities and vaccinations were entered into a database. The study complied with the principles of the Declaration of Helsinki. The University Ethics Committee approved the study (CEAVSE PAN_HUB_2021, code number 17431_0_1). All patients gave their written informed consent to participate in the study and use their data.

After that, we chose another population of 89 follow-up adult patients previously hospitalized with COVID-19. Samples were collected in the period August 2021 to February 2023, during follow-up visits. To be included in the study, patients had to participate in the follow-up program organized by Siena University Hospital, which is intended for those who were previously hospitalized in the Siena COVID Unit. The follow-up protocol included medical examination, HRCT of the chest, blood tests and lung function tests. The clinical data and medical history, also concerning the acute phase of COVID-19, were available for 80/89 patients. The severity of COVID-19 during hospitalization was described according to WHO criteria (mild, moderate, severe). Visits have to be performed in the interval between weeks 12 and 24 after discharge from hospital. All data, including clinical, sociodemographic, comorbidities and the main HRCT findings (including air-trapping, fibrosis and ground glass opacities), were entered in an electronic database. Initially, patients were divided in two groups: with and without HRCT evidence of fibrotic lung alterations. In this case, we used a double supervised and unsupervised statistical approach. The study complied with the principles of the Declaration of Helsinki. The University Ethics Committee approved the study (CEAVSE PAN_HUB_2021, code number 17431_0_1). All patients gave their written informed consent to participate in the study and to use their data.

Pulmonary Function Tests

Lung function parameters (PFT) were recorded according to standard American Thoracic Society/European Respiratory Society (ATS/ERS) criteria using a Jaeger Body Plethysmograph with correction for temperature and barometric pressure. In particular, we recorded forced expiratory volume in the first second (FEV-1), forced vital capacity (FVC), carbon monoxide diffusing capacity (DLCO), and total lung capacity (TLC) (167,168). All parameters were expressed as percentages of predicted values, according to 2005 suggested strategies (169). Indeed, a part of the patients underwent to PFT before the publication of updated interpretative strategies of 2022 (170).

Laboratory Tests

Immunoassays

In the first prospective study (hospitalized patients) serum analyte concentrations of IL-1β, IL-10, IFN-γ, TNF-α and IL-6 were quantified by bead-based multiplex LEGENDplex™ analysis (LEGENDplex™ Custom Human Assay, Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. In the second study (extended population of hospitalized patients) we also determined with same devices and technique concentrations of IL-4, IL-2, CXCL10 (IP-10), CCL2 (MCP-1), IL-17A, IL-12p70 and TGF-β1 (free active form). In this latter case, we selected also five proteins related to different pathophysiological mechanisms (adiponectin, adipsin, RBP-4, leptin and resistin) to be assayed in the serum of a subset of 62 patients. Serum concentrations of the proteins were quantified in pg/mL by

bead-based multiplex LEGENDplex™ analysis (LEGENDplex™ Custom Human Assay, Biolegend) according to the manufacturer's instructions. Reactions were run in duplicate with a BD FACSLyric flow cytometer (BD-Biosciences San Jose, CA, USA). The adipokine concentrations were processed with Legendplex V8.0 software (Biolegend, San Diego, CA, USA).

For the third study (follow-up samples) we use the same devices and technique to assess serum analyte concentrations of IL-4, IL-2, CXCL10 (IP-10), IL-1β, TNF-α, CCL2 (MCP-1), IL-17A, IL-6, IL-10, IFN-γ, IL-12p70 and TGF-β1 (free active form): molecules already tested in the previous study involving hospitalized patients.

ELISA Kit

ELISA kit serum concentration of IL-8 and IL-32 were determined by enzyme-linked immunosorbent assay (ELISA) kits by Invitrogen (Waltham, MA, USA) and Mybiosource (San Diego, CA, USA) following the manufacturers' instructions. Concentrations were read at 450 nm with a Victor X4 fluorimeter (Perkin Elmer, Waltham, MA, USA) and expressed in pg/ml. The detection limit of IL-8 was ranging from 15.625 to 1000 pg/ml, while for IL-32 from 15.63 to 1000 pg/mL pg/ml.

Statistical Analysis

For the first analysis results were expressed as means and standard deviations (SD) or medians and 25th and 75th percentiles, as appropriate. One-way ANOVA non-parametric test (Kruskal-Wallis test) and Dunn test were performed for multiple comparisons among healthy controls group and mild, moderate and severe COVID-19 patients. The χ-squared test was used for categorical variables. A p value less than 0.05 was considered statistically significant. We also assessed the validity of variables used to distinguish COVID-19 severity groups by areas under the receiver operating characteristic curve (AUC ROC). Sensitivity, specificity and positive and negative predictive values (PPV and NPV, respectively) were calculated for cut-offs of the different variables. The Youden index (J = max [sensitivity + specificity-1]) was used to establish the best cut-offs for diagnosis. Statistical analysis and graphic representation of the data were performed by GraphPad Prism 4.0 software and BioVinci software (BioTuring Inc., San Diego, CA, USA).

The two other studies shared the same statistical approach. To compare cytokine levels in the two groups, non-parametric Mann–Whitney tests were used for continuous numerical variables. Same test was adopted to describe the study population of patients in terms of sociodemographic and clinical features. To compare the relative frequencies of different levels of nominal/categorical variables, the Fisher's exact and χ-squared tests were used. Correlations were determined by Spearman correlation coefficient. Probability values less than 0.05 were considered statistically significant. We also performed binomial logistic regression to identify the cytokines and comorbidities that most influenced HRCT evidence of fibrosis development. Statistical analysis was performed with GraphPad Prism 9.2 software and Jamovi free software version 2.3.26.

RESULTS

First population of hospitalized patients

Our first population showed a prevalence of males in the three groups of patients: 73%, 70% and 73% in the mild, moderate, and severe groups, respectively. Bilateral diffuse pneumonia was detected by chest X-rays in 65%, 57.2% and 52.6% of patients in the severe, mild, and moderate groups, respectively. Fever was the most common symptom. In the total population of 64, only 17 patients did not have specific medical or surgical comorbidities. Interestingly, among the blood parameters, neutrophil and lymphocyte counts were significantly lower in severe COVID-19 patients than in the other groups ($p = 0.002$ and $p = 0.03$ respectively). Conversely, C-reactive protein was significantly higher in severe patients than in other severity groups ($p = 0.02$). Data are summarized in ***Table 1***.

	HC (n = 27)	Mild (n = 32)	Moderate (n = 17)	Severe (n = 15)	P values
Sex (m/f)	12/15	23/9	12/5	11/4	ns
Age (years)	58 (36–78)	69 (60–83)	62 (57–71)	66 (60–70)	ns
Past medical history – no. (%)					
Cardiovascular Diseases	–	4 (12.5)	3 (17)	2 (13.3)	
Type 2 Diabetes	–	3 (9.3)	4 (23.5)	2 (13.3)	
Obesity (<30)	–	5 (15.6)	3 (17.6)	4 (26.6)	
Hypertension	–	10 (31)	6 (35)	4 (26.6)	
Other conditions*	–	9 (28)	4 (23.5)	7 (46)	
Blood Count	–				
Neutrophils (%) (n.r: 55–70)	–	78 (67.5–84)	78.2 (70–85)	60.5 (51–73)	0.002
Monocytes (%) (n.r: 1–13)	–	5.9 (4.7–9.8)	7.2 (5.3–8.4)	6.6 (5.3–8.4)	ns
Lymphocytes (%) (n.r: 25–48)	–	16.5 (10.1–24)	15.1 (9–19)	9.4 (6–13.4)	0.03
Eosinophils (%) (n.r: 1–5)	–	0.1 (0–0.7)	0 (0–0.2)	0 (0–0.1)	ns
Basophils (%) (n.r: 0–1,5)	–	0.3 (0.2–0.4)	0.2 (0.1–0.25)	0.2 (0.1–0.2)	ns
RBC (10^6 /mm^3) (n.r: 4,1–5,8)	–	4.7 (4.2–5)	4.7 (4–5)	4.6 (4.4–4.8)	ns
WBC (10^3 /mm^3) (n.r: 4–10)	–	6 (4.9–6)	6 (4.3–7.3)	6.2 (4.7–7.6)	ns
Hb (g/dl) (n.r: 12,8–18)	–	13.8 (11.7–14.5)	13.7 (12.7–14.5)	14.2 (13.3–14.5)	ns
HCT (%) (n.r: 4–52)	–	42.5 (35.5–43.4)	41.5 (37.7–42.3)	42.2 (40.2–43)	ns
PLT (10^3 /mm^3) (n.r: 150–400)	–	199 (167–242)	169 (124–210)	227 (178–351)	ns
CRP (mg/dl) (n.r: 0–0,5)	–	3.3 (1.8–6.2)	4 (2.2–12)	4.8 (3.9–10.9)	0.02

Table 1: Demographic data and blood parameters of the first study population.

Cytokine levels in relation to severity of disease

Serum concentrations of the cytokines considered in our study are reported in ***Table 2*** and ***Figure 13.*** IL-8 concentrations were significantly higher in COVID-19 patients than in healthy controls. On the contrary, IL-32 concentrations were significantly higher in controls than in COVID-19 patients (p = 0.02). IL-6 concentrations were higher only in the group of severe COVID-19 patients compared than the other groups (mild, moderate and control groups; p = 0.0002). Higher concentrations of IL-1β were found in the severe group than in mild and moderate COVID-19 patients (p = 0.048; p = 0.042) Finally, lower concentrations of IL-10 were detected in severe COVID-19 patients than in other groups (p < 0.05). There were no differences in concentrations of TNF-α and INF-γ between COVID-19 severity groups and controls.

	HC (n=27)	**Mild (n=32)**	**Moderate (n=17)**	**Severe (n=15)**	**p-values**
IL-8 (pg/ml)	32 (21-49)	127 (37.8-359)	297.3 (202-422)	269.8 (197-513)	p<0.01
IL-32 (pg/ml)	22.7 (13.2-30)	7.2 (1-23)	18 (2-32)	1.3 (0.6-21)	p=0.02
IL-1β (pg/ml)	0 (0-3)	0 (0-0)	0 (0-0)	0 (0-7)	p=0.04
IL-6 (pg/ml)	0 (0-0)	0 (0-20)	10 (0-30)	30 (7.5-210)	p=0.0002
INF-γ (pg/ml)	50 (20-112,5)	50 (40-90)	60 (30-70)	65 (50-105)	ns
TNF-α (pg/ml)	0 (0-2)	0 (0-1.5)	0 (0-1)	0 (0-0)	ns
IL-10 (pg/ml)	2 (0-7)	0 (0-10)	0 (0-8)	0(0-0)	p=0.048

Table 2: Cytokines concentrations in the different groups.

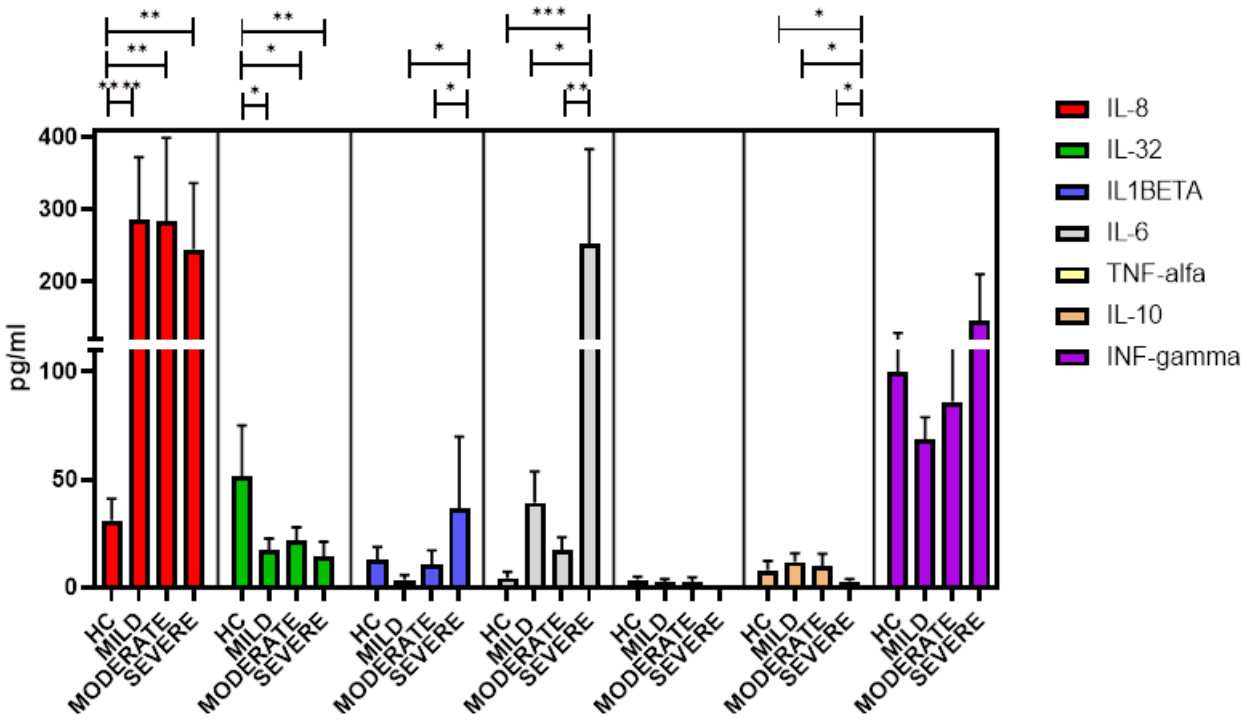

Figure 13: Cytokines concentrations comparison among healthy controls and mild, moderate and severe COVID-19 patients. The histograms report mean (center bar) ± SEM (upper and lower bars). If not indicated, p value is not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Cytokine profiles in controls and COVID-19 patients

ROC curves were plotted to assess the discriminatory value of the parameters and to determine cut-off values with sufficient sensitivity and specificity. We found that IL-8 and IL-32 were the best discriminatory markers to distinguish controls from COVID-19 patients. IL-8 showed AUC = 0.88 (95%CI: 0.81–0.96; $p < 0.001$), while IL-32 showed AUC = 0.71 (95%CI: 0.64–0.77; $p = 0.01$). IL-6 also had good discriminatory potential, showing AUC = 0.70 (95%CI: 0.57–0.85; $p = 0.007$). IL-8 values below a cut-off of 343.5 pg/ml had 66% sensitivity and 96% specificity in discriminating COVID-19 patients from controls. IL-32 below a cut-off of 54 pg/dl showed 59% sensitivity and 71% specificity in discriminating COVID-19 patients from controls. Regarding IL-6, values below a cut-off of 5 pg/ml showed 59% sensitivity and 88% specificity in discriminating patients from controls. ROC curve did not show statistical significance for other cytokines. Anyway, applying logistic regression with *COVID-19 patient* tested as dependent variable and all cytokines as independent variables, ROC curve analysis of model performance showed AUC= 0.91 (95%CI: 0.84–0.98; NPP(%): 92.5, PPP(%): 73.3; $p < 0.0001$).

Cytokine profiles in COVID-19 patients in relation to severity

As described above, we plotted ROC curves to assess the discriminatory value of the parameters and to identify cut-off values with sufficient sensitivity and specificity. IL-6 was confirmed to be the cytokine that best distinguished COVID-19 severity groups, showing an AUC = 0.69 (95%CI: 0.53–0.86; $p = 0.02$). IL-6 values below a cut-off of 15 pg/ml had 65%

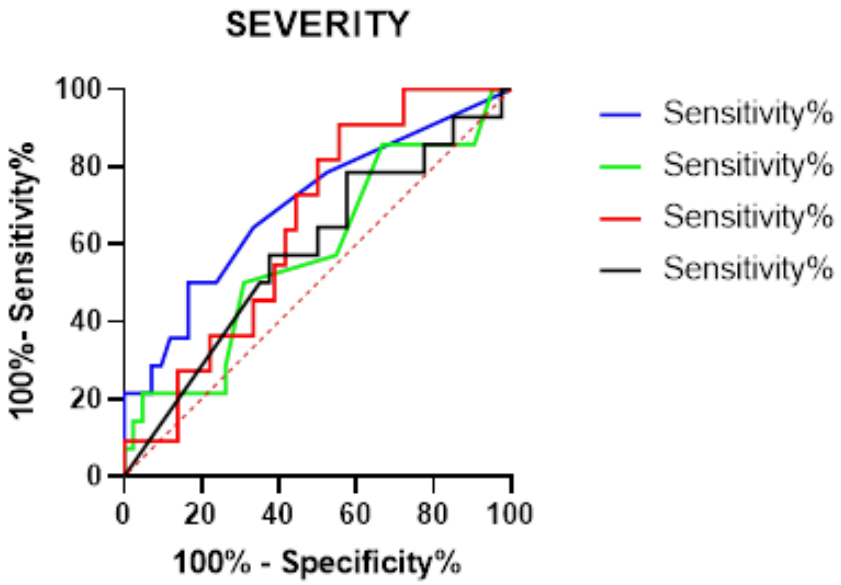

Figure 14: ROC curve analysis of IL-6 (blue), CRP (red), INF-γ (green), and IL-32 (black).

sensitivity and 67% specificity in discriminating severe from non-severe COVID-19. The logistic regression model was applied in order to identify the variables that best distinguished the group of severe COVID-19 patients. So, the severe COVID-19 group was considered the dependent variable, and the cytokines independent variables. The best model performance was obtained by the combination of IL-32, IL-6 and IFN-γ. This model showed AUC = 0.80 (95%CI: 0.67–0.92; NPP(%): 81.2, PPP(%): 60; p = 0.0015) (***Fig. 14***). Interestingly, when we tried to add blood parameters to this model, only serum concentrations of CRP increased model performance, showing AUC = 0.83 (95%CI: 0.68–0.97; NPP(%): 81.2, PPP(%): 60; p = 0.0029) (***Fig. 15***).

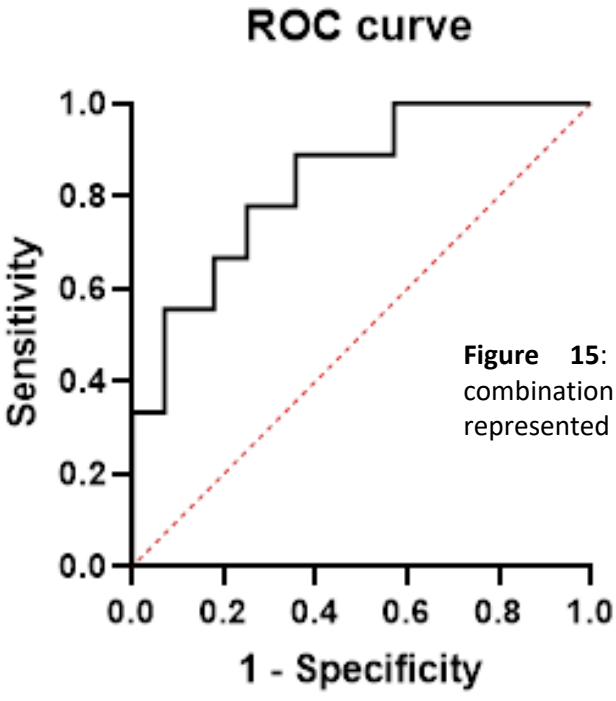

Figure 15: ROC curve resulted from the combination of the parameters distinctly represented in Fig. 14: IL-6, CRP, INF-γ and IL-32.

Survival curve analysis

We performed a log-rank (Mantel-Cox) test for COVID- 19 patients, stratified by IL-6 concentrations. The severity groups of COVID-19 patients showed a significant difference in survival rate (p = 0.008, hazard ratio: 0.25 (95 %CI 0.0003–27)) for a cut-off of 15 pg/ml in IL-6 median values (***Fig. 16***).

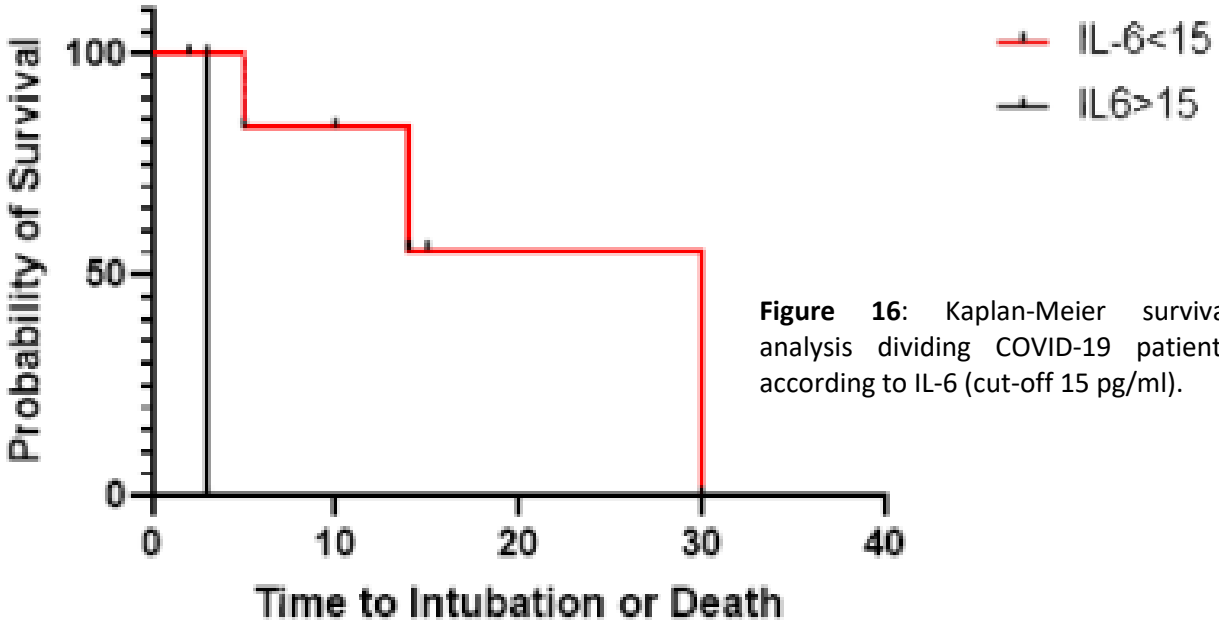

Figure 16: Kaplan-Meier survival analysis dividing COVID-19 patients according to IL-6 (cut-off 15 pg/ml).

Extended population of hospitalized patients

Males were the majority in the two groups of patients: 64% in the group with mild–moderate disease and 57% in the severe group. All patients underwent a chest X-ray in the first two days of hospitalization, showing a bilateral diffuse pneumonia in 71% (10/14) of severe patients and 61% (58/94) of mild–moderate patients (p = 0.04). Regarding symptoms, 98% of patients showed two or more symptoms at onset, with a prevalence of fever. In the total population, 27% of mild–moderate patients and 58% of severe patients were without specific medical or surgical comorbidities, among the others cardiomyopathy was the most common (p = 0.01). Considering blood parameters, severe COVID-19 patients showed significantly lower lymphocytes and higher monocytes percentage than mild–moderate group. CRP was significantly higher in severe patients than the other ones (p = 0.03). ***Table 3*** summarizes demographic data, clinical data, and immunological findings.

	Mild to Moderate COVID-19 (n=94)	Severe COVID-19 (n=14)	p-values
Gender (m/f) (%m)	61/33 (64)	8/6 (57)	**ns**
Age (M±SD)	69±14.4	73±11.8	**ns**
Comorbidities (yes/no) (%yes)	69/34 (73)	6/8 (42)	**0.01**
- Respiratory diseases	15/79	1/13	
- Diabetes	30/64	1/13	
- Cardiomyopathies	35/59	3/11	
- Kidney failure	19/75	0/14	
- Cancer	22/72	1/13	
Symptoms at admission:			
- Fever	68	10	**ns**
- Dyspnoea	50	11	**ns**
- Cough	25	4	**ns**
- Phlegm	8	3	**ns**
- Gastrointestinal sympt.	34	7	**ns**
- Chest pain	5	1	**ns**
- Other	40	8	**ns**
Chest X-rays at admission:			
- Bilateral pneumonia	58	10	**0.04**
- Monolateral pneumonia	20	3	
- No pneumonia	16	1	
Blood analysis (M±SD):			
- Monocytes (%)	76±12.6	70±8.3	**ns**
- Neutrophils (%)	5.2±6.5	9.6±7.6	**0.04**
- Lymphocytes (%)	14±7.3	8.3±4.2	**0.005**
- Eosinophils (%)	0.3±0.2	0.8±0.4	**ns**
- Basophils (%)	0.1±0.1	0.3±0.2	**ns**
CRP (mg/dl) (M±SD)	6.3±2.8	12.5±5.8	**0.03**

Table 3: Demographic data, clinical data, and immunological findings.

Cytokine Levels in Relation to the Severity of COVID-19

The logistic regression model with severity groups as the dependent variable showed that all the analytes helped discriminate the two groups. We obtained a ROC curve with an AUC of 0.83 ($p < 0.0001$) with good sensitivity (1.0) and specificity (0.66) (***Fig. 17***).

Predictor	Estimate	SE	Z	p
Intercept	3.20e+15	2.38e+7	1.35e+8	< .001
IL1B	−4.13e−11	9581	−4.31e−7	< .001
IL10	2.98e+14	5.33e+6	5.58e+7	< .001
IL6	−1.23e−13	772324	−1.60e−7	< .001
TNF	3.45e+13	1.16e+6	2.96e+7	< .001
IFN	1.00e+14	1.22e+6	8.20e+7	< .001
IL4	−7.39e−13	1.60e+6	−4.63e−7	< .001
IL2	1.80e+15	2.61e+7	6.91e+7	< .001
IP10	−2.59e−11	4397	−5.90e−7	< .001
MCP1	−1.58e−10	7742	−2.05e−6	< .001
IL17A	3.07e+14	1.75e+7	1.76e+7	< .001
IL12P70	−6.83e−15	1.04e+8	−6.60e−7	< .001
TGF	−2.24e−13	257908	−8.69e−7	< .001
IL8	−7.50e−11	61327	−1.22e−7	< .001
IL32	−1.66e−14	2.43e+6	−6.82e−7	< .001

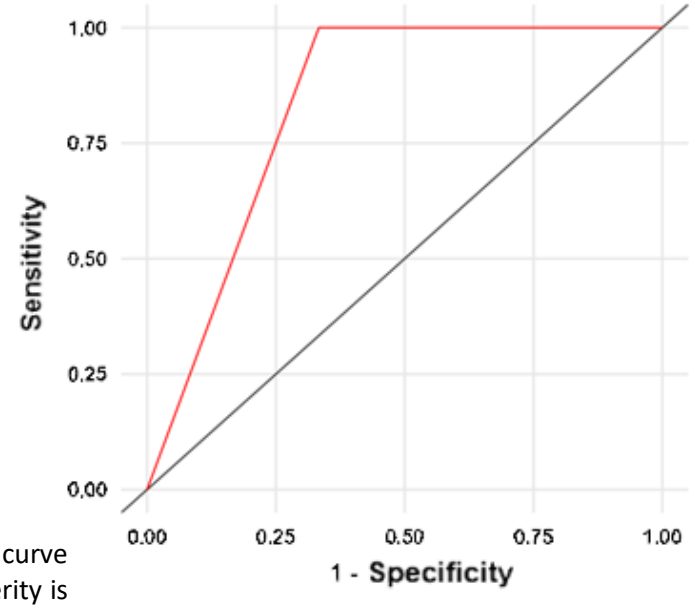

Figure 17: Logistic regression model and ROC curve with serum cytokines evaluated; disease severity is the dependent variable.

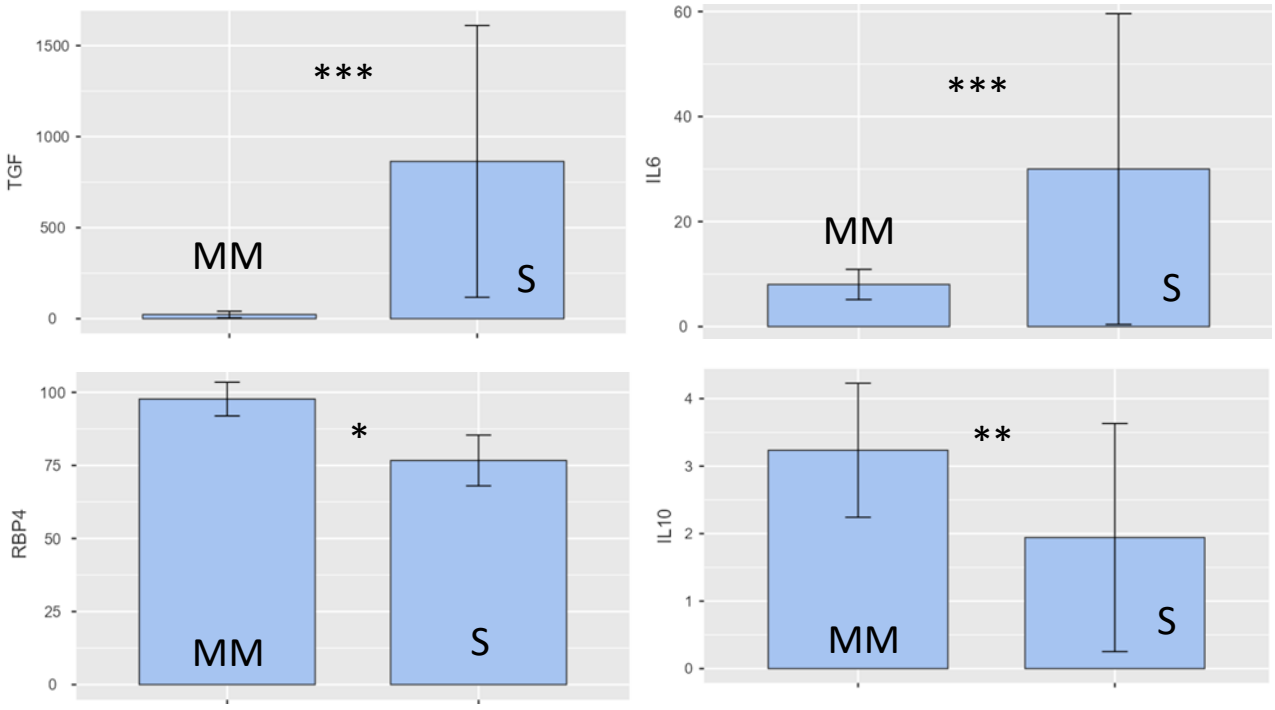

Figure 18: Comparison of serum TGF-β, IL-6, RBP4 and IL-10 concentrations between patients with mild to moderate (MM) and severe (S). *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$

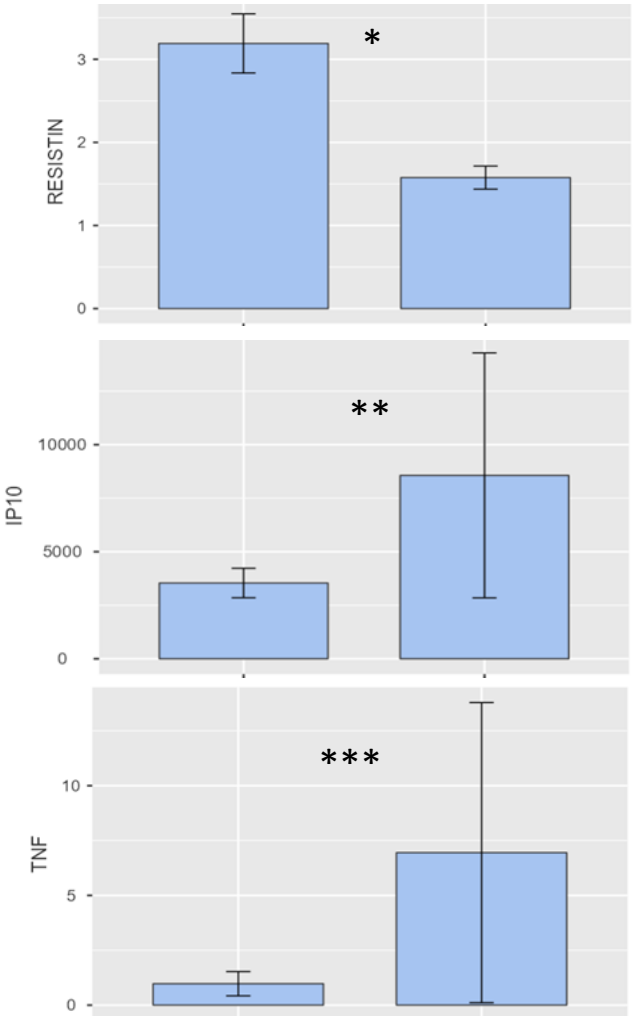

Figure 19: Comparison of serum resistin, IP-10 and TNF-α concentrations between survivors (on the left) and non survivors (on the right). *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$

Comparative analysis of these proteins evidenced that higher levels of TGF-β and IL-6 and lower levels of RBP-4 and IL-10 were mainly associated with the severe group (***Fig. 18***).

Resistin, IP-10 and TNF-α Concentrations Were Associated with Survival

Unfortunately, nine patients (8.3%) died in hospital. Resistin, IP-10 and TNF-α concentrations resulted associated with survival; more specifically, higher IP-10 and TNF-α values were found in dead patients while resistin showed an inverse trend (***Fig. 19***).

Vaccinated patients showed higher levels of MCP-1 and IL-10, instead there were not association between comorbidities and altered cytokines levels (***Fig. 20***).

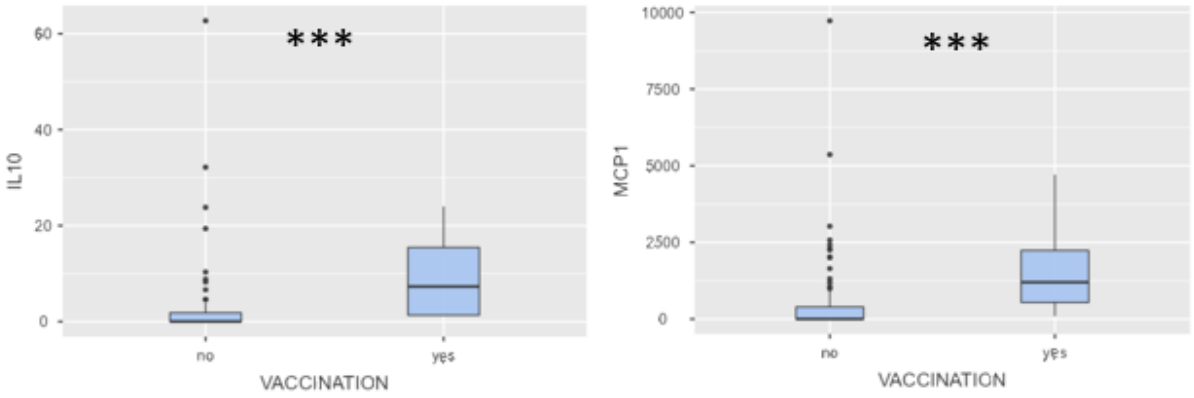

Figure 20: Levels of IL-10 and MCP-1 according to vaccination status. ***: $p < 0.0001$

Altered Levels of Cytokines Associated with the Development of Fibrosis 3–6 Months after Discharge

Potential associations between some of these biomarkers and lung sequelae after hospitalization for COVID-19 were evaluated. The clinical and radiological data with functional parameters (including FEV1, FVC and DLCO expressed as percentages of the predicted value), were collected for the follow-up patients. 11 patients, mean age 57 ± 37.4 years, prevalently males (10/11), showed HRCT evidence of fibrotic alterations; on the contrary the other 45 did not. Of these eleven patients, nine (81%) showed also air-trapping and ten (90%) had ground-glass opacities. Twenty-nine of the other forty-five patients (64%) showed evidence of air-trapping and thirty (66%) exhibited ground-glass opacities.

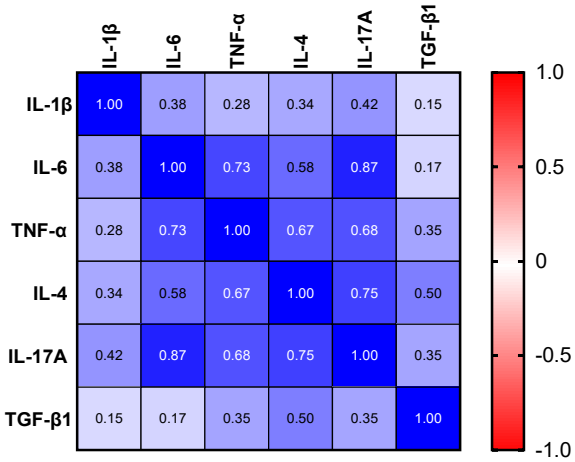

Figure 22: Correlation matrix among serum biomarkers.

Suffering from severe COVID-19 during hospitalization put at risk to develop fibrotic

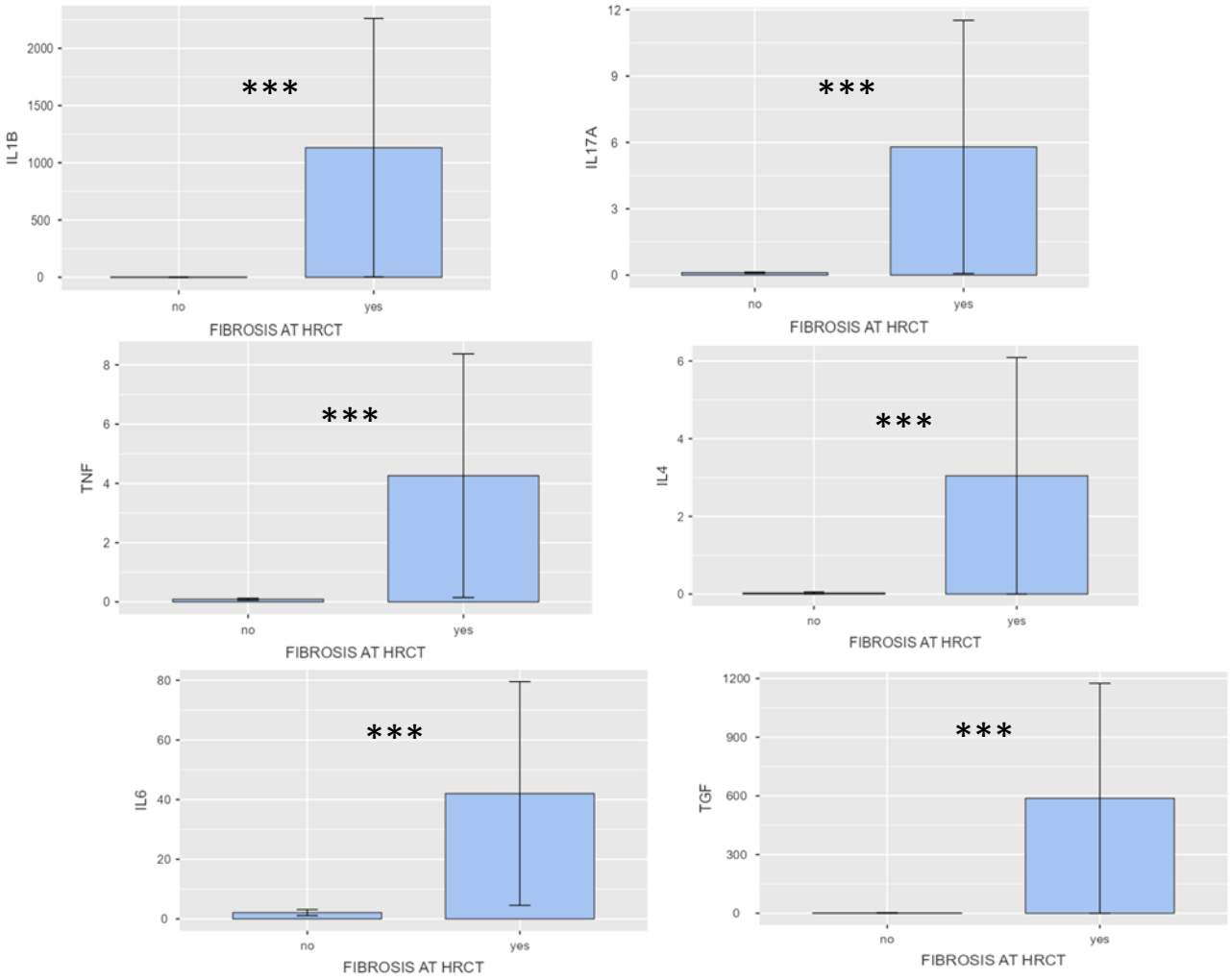

Figure 21: Comparison of serum IL-1β, IL17A, TNF-α, IL-4, IL-6, and TGF-β between patients with and without fibrotic alterations of the lungs. ***: $p < 0.0001$

sequelae in follow-up HRCT, in contrast to mild-moderate disease ($\chi 2 = 6.06$ *p* value = 0.048). We found that patients who showed HRCT evidence of fibrotic interstitial alterations at follow-up, also had showed significantly higher levels of some serum biomarkers on hospital admission, in particular IL-1β, IL17A, TNF-α, TGF-β, IL-4 and IL-6 (***Fig. 21***). These biomarkers appeared to be closely correlated with each other, exhibiting significantly strong bond between IL-6 and TNF-α (r = 0.73 p < 0.0001), IL-6 and IL-17a (r = 0.87 p < 0.0001) and between IL-4 and IL-17a (r = 0.75 p < 0.0001) (***Fig. 22***).

Concerning lung function parameters at follow-up, FVC% correlated significantly with TNF-α (r = -0.42, p = 0.01) and IL-32 (r = 0.34, p = 0.01), as shown in ***Figure 23***.

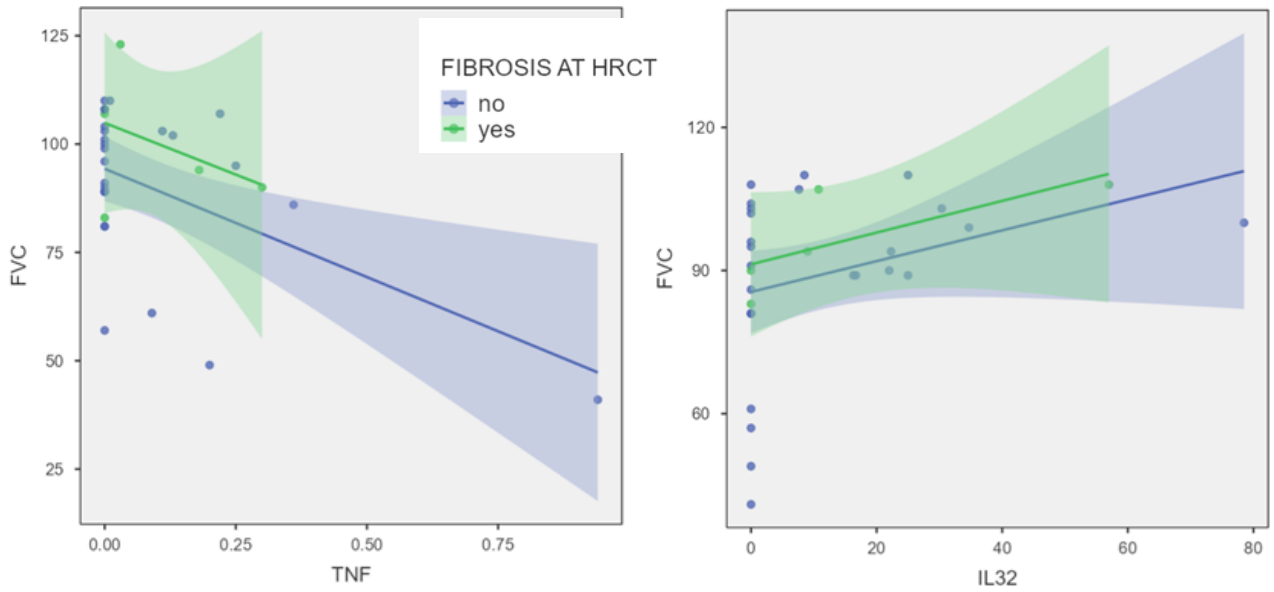

Figure 23: correlation plot between FVC expressed as percentage of predicted value and TNF-α and IL-32.

Follow-up patients

Main clinical and anamnestic features of our cohort of samples are summarized in ***Table 4.*** Patients with HRCT evidence of fibrotic abnormalities distinguish from the others because they were older. There were no differences in distribution of comorbidities.

Table 4: Clinical and anamnestic data of our study population.

	Fibrosis at HRCT		
	no (N=27)	**yes (N=53)**	**p-values**
GENDER			0.225
M	20.0 (74.1%)	32.0 (60.4%)	
F	7.0 (25.9%)	21.0 (39.6%)	
AGE			**0.005**
Mean (SD)	66.0 (12.0)	73.0 (8.6)	
Range	34.0 - 86.0	46.0 - 87.0	
SMOKING			0.162
former	11.0 (57.9%)	10.0 (32.3%)	
never	7.0 (36.8%)	20.0 (64.5%)	
current	1.0 (5.3%)	1.0 (3.2%)	
Comorbidities			
RESPIRATORY DISEASES			0.134
no	27.0 (100.0%)	47.0 (88.6%)	
yes	0.0 (0.0%)	6.0 (11.3%)	
NEOPLASIA			0.123
no	23.0 (85.1%)	50.0 (94.3%)	
yes	4.0 (14.9%)	3.0 (5.6%)	
DIABETES			0.788
yes	6.0 (22.3%)	12.0 (22.6%)	
no	21.0 (77.7%)	41.0 (77.3%)	
CARDIOMIOPATHY			0.892
no	21.0 (77.7%)	39.0 (73.5%)	
yes	6.0 (22.3%)	14.0 (26.4%)	
RENAL FAILURE			0.508
no	25.0 (92.3%)	49.0 (92.4%)	
yes	2.0 (7.7%)	4.0 (7.5%)	

In ***Table 5*** we provide main functional features of our population: patients with fibrotic alterations of the lungs showed significantly lower DLCO percentages, while there were not differences between the two groups in term of spirometry parameters.

Table 5: Functional data of the population.

	Fibrosis at HRCT		
	no (N=27)	**yes (N=53)**	**p-values**
GENDER			0.225
M	20.0 (74.1%)	32.0 (60.4%)	
F	7.0 (25.9%)	21.0 (39.6%)	
AGE			**0.005**
Mean (SD)	66.0 (12.0)	73.0 (8.6)	
Range	34.0 - 86.0	46.0 - 87.0	
Pulmonary function tests			
FEV1 (%)			0.820
Mean (SD)	101.2 (14.2)	100.1 (17.6)	
Range	71.0 - 123.0	44.0 - 144.0	
FVC (%)			0.845
Mean (SD)	95.6 (15.3)	94.7 (17.0)	
Range	61.0 - 122.0	34.0 - 135.0	
FEV1/FVC ratio			**0.002**
Mean (SD)	91.7 (15.1)	103.3 (11.6)	
Range	63.0 - 118.0	76.0 - 128.0	
TLC (%)			0.289
Mean (SD)	94.7 (3.5)	82.2 (18.4)	
Range	91.0 - 98.0	44.0 - 102.0	
DLCO (%)			**0.065**
Mean (SD)	81.5 (21.4)	72.4 (14.7)	
Range	48.0 - 122.0	41.0 - 106.0	

Patients with lung sequelae were distinguished by different cytokine levels

We stratified our population by presence or absence of lung fibrotic sequelae. Direct comparison of cytokine levels in the two groups exhibited increased levels of IL-32 and decreased levels of IL-8 in patients with lung involvement whereas IL-10 concentrations were similar in the two groups, as all the other cytokines (***Tab. 6***). ***Figure 24*** summarizes the significant results.

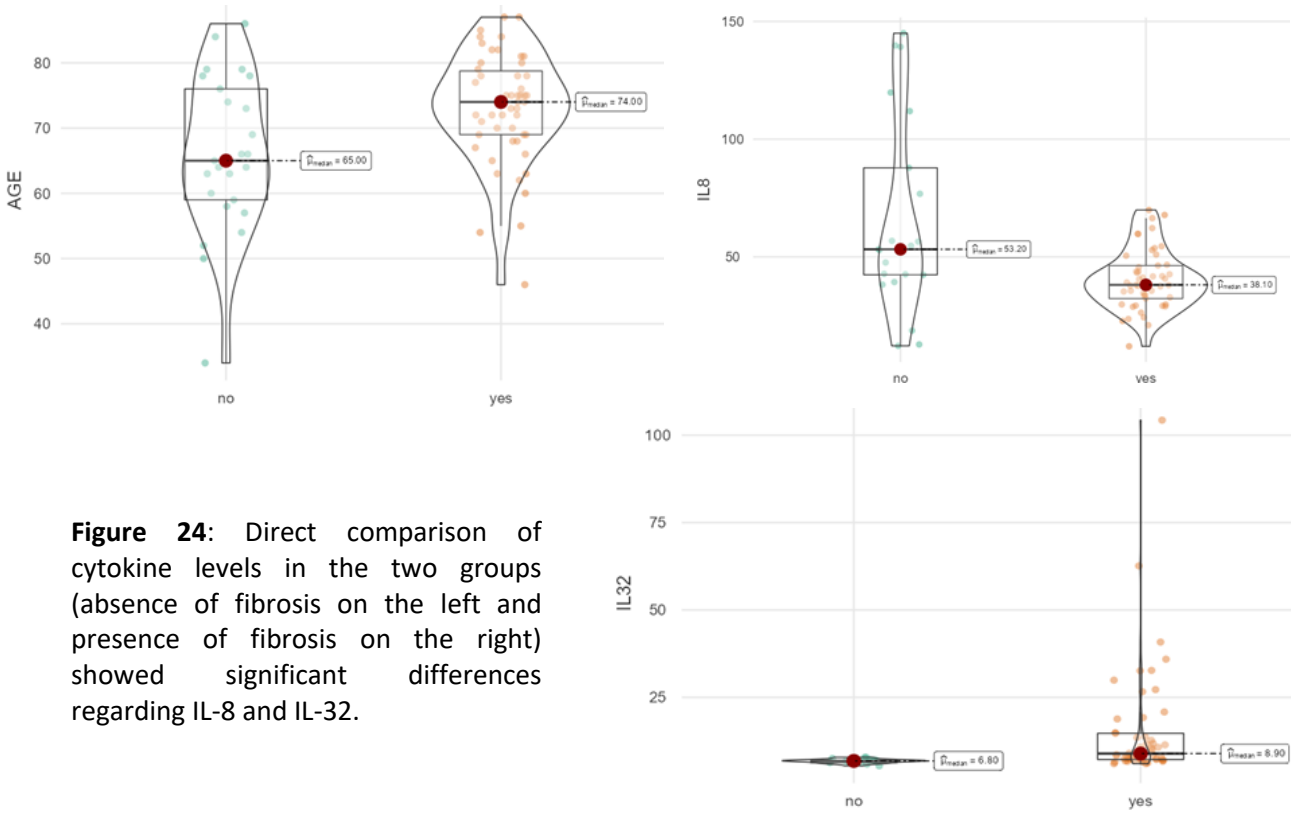

Figure 24: Direct comparison of cytokine levels in the two groups (absence of fibrosis on the left and presence of fibrosis on the right) showed significant differences regarding IL-8 and IL-32.

Table 6: Serum cytokines concentrations in the two groups of the study participants.			
	No fibrotic alterations (N=27)	**Presence of fibrotic alterations (N=53)**	**p-values**
AGE			**0.005**
Mean (SD)	66.0 (12.0)	73.0 (8.6)	
Range	34.0 - 86.0	46.0 - 87.0	
IL-8			**< 0.001**
N-Miss	6.0	4.0	
Mean (SD)	66.2 (41.7)	40.3 (12.7)	
Range	12.2 - 145.0	12.0 - 69.9	
IL-32			**0.021**
Mean (SD)	6.7 (0.6)	15.3 (16.6)	
Range	5.4 - 7.8	6.0 - 104.3	
IL-4			0.328
Mean (SD)	1.1 (4.6)	4.6 (17.4)	
Range	0.0 - 23.0	0.0 - 98.3	
IL-2			0.373
Mean (SD)	0.2 (0.2)	0.3 (0.8)	
Range	0.0 - 0.9	0.0 - 4.5	
IP-10			0.507
Mean (SD)	35.5 (50.4)	42.2 (35.7)	
Range	0.0 - 244.6	0.0 - 178.3	
IL1-β			0.198
Mean (SD)	0.2 (0.8)	0.1 (0.1)	
Range	0.0 - 3.8	0.0 - 0.9	
TNF-α			0.325
Mean (SD)	0.0 (0.0)	0.1 (0.4)	
Range	0.0 - 0.2	0.0 - 2.8	
MCP-1			0.474
Mean (SD)	41.5 (35.2)	52.1 (68.9)	
Range	0.0 - 137.9	0.0 - 409.4	
IL-17A			0.916
Mean (SD)	0.1 (0.2)	0.1 (0.3)	
Range	0.0 - 0.8	0.0 - 2.1	
IL-6			0.327
Mean (SD)	0.4 (0.4)	1.0 (2.9)	
Range	0.0 - 2.0	0.0 - 19.7	
IL-10			0.811
Mean (SD)	0.7 (3.2)	0.5 (3.2)	
Range	0.0 - 16.0	0.0 - 22.6	
IFN-α			0.659
Mean (SD)	13.0 (47.7)	8.4 (38.5)	
Range	0.0 - 221.8	0.0 - 208.6	
IL12P70			0.523
Mean (SD)	0.1 (0.2)	0.1 (0.6)	
Range	0.0 - 1.1	0.0 - 4.0	
TGF-β			0.908
Mean (SD)	10.2 (45.5)	11.7 (55.6)	
Range	0.0 - 223.0	0.0 - 372.0	

Variables impacting the development of lung sequelae: IL-8 and IL-32 are the best predictors

A logistic regression model was applied to explore whether comorbidities reported at hospitalization can affect the development of post-acute COVID-19 lung sequalae. We performed a binomial logistic regression using presence/absence of HRCT evidence of fibrosis as dependent variable and comorbidities as predictors. None of the comorbidities showed significant influence (***Tab. 7***).

Table 7: Binomial logistic regression model designed to explore if presence of comorbidities can affect the development of fibrotic lung sequelae. Presence/absence of HRCT evidence of fibrosis was the dependent variable and comorbidities were predictors.

Predictors	Estimates	SE	Z	p
Intercept	-14.272	1898.921	-0.00752	0.994
RESPIRATORY DISEASES:				
no – yes	17.400	1898.921	0.00916	0.993
NEOPLASIA:				
no – yes	-2.192	1.160	-1.89029	0.059
DIABETES:				
no – yes	-0.267	0.645	-0.41463	0.678
CARDIOPATHY:				
no – yes	-0.176	0.645	-0.27317	0.785
RENAL FAILURE:				
no – yes	-1.348	1.288	-1.04686	0.295

A second model was created using the two groups as coefficient and levels of any cytokine as variables. In this case, serum concentrations of IL-8 (z score: 0.381 p = 0.017) and IL-32 (z score: -1.674, p = 0.094) appeared to significantly influence the development of lung sequelae after COVID-19 (***Tab. 8***). ROC analysis from this model showed an AUC of 0.93, specificity 0.976 and sensitivity 0.824. A third model was designed from the two previous ones using only the significant results, therefore including values of both IL-8 and IL-32. ROC analysis of the model showed an AUC of 0.891, specificity 0.898 and sensitivity 0.571 (***Fig.25***).

Predictors	Estimates	SE	Z	p
Intercept	1.60154	4.6407	0.345	0.730
IL-8	0.06743	0.0283	2.381	**0.017**
IL-32	-0.99991	0.5972	-1.674	**0.094**
IL-4	0.22435	1.7323	0.130	0.897
IL-2	9.65058	9.6776	0.997	0.319
IP-10	-0.01730	0.0169	-1.023	0.306
IL1β	1.93836	3.6002	0.538	0.590
TNF-α	-15.76195	42.4147	-0.372	0.710
MCP-1	0.00194	0.0154	0.125	0.900
IL-17A	18.98555	13.0768	1.452	0.147
IL-6	0.79834	1.5918	0.502	0.616
IL-10	21.53196	35.7203	0.603	0.547
IFN-γ	-2.85723	4.0224	-0.710	0.478
IL12P70	-165.34689	147.3375	-1.122	0.262
TGF-β	-3.47228	4.8488	-0.716	0.474

Table 8: Binomial logistic regression using presence/absence of fibrosis as the dependent variable and serum cytokine concentrations as predictors.

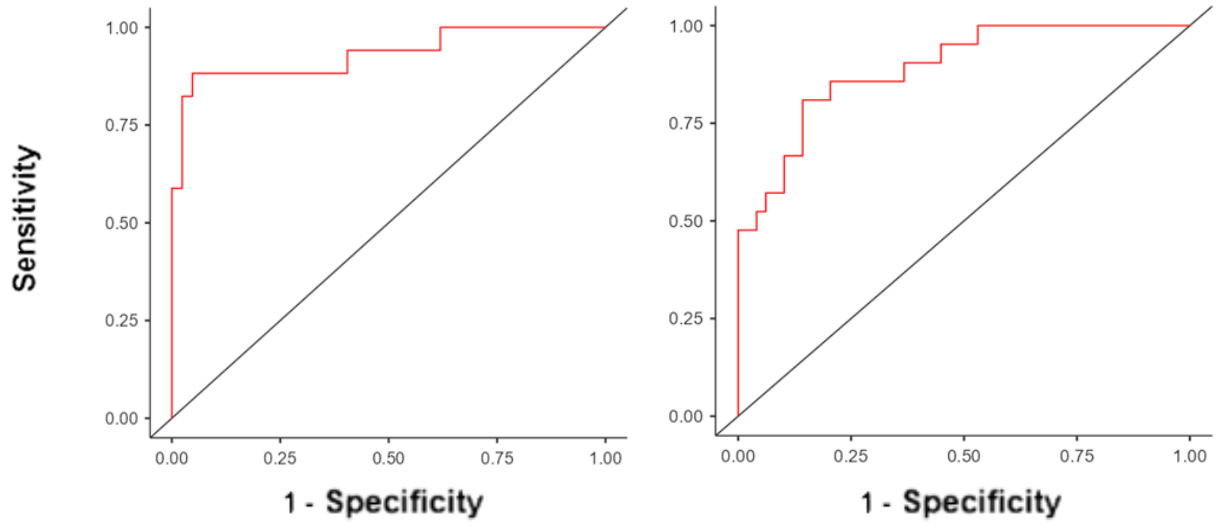

Figure 25: ROC curve analysis with all cytokines as variables (left) and only IL-8 and IL-32 (right).

Post-COVID-19 lung sequelae and relationship with severity of acute disease

We did not find a relationship between severity of COVID-19 and post-COVID-19 symptoms, although it was clear that the most part of moderate and severe patients developed lung sequelae detectable by HRCT. After further stratification of patients by severity (severe versus mild/moderate), IL-10 emerged as the only cytokine showing decreased levels in severe patients (***Fig.26***).

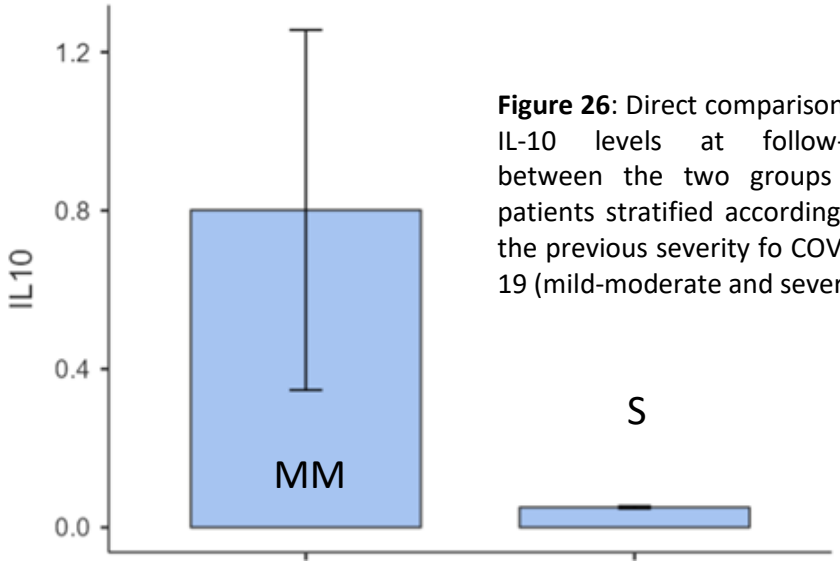

Figure 26: Direct comparison of IL-10 levels at follow-up between the two groups of patients stratified according to the previous severity fo COVID-19 (mild-moderate and severe).

DISCUSSION

COVID-19, as Influenza pneumonia and ARDS, can leave chronic respiratory signs to the patients. In the introduction we summarized some of the findings of Huang and colleagues in one of the first studies about COVID-19 follow-up: many of the patients who suffered from severe disease still had ground-glass opacities in the lungs one year after discharge and some of them (11%) showed interlobular septal thickening. Almost 29% of these patients showed a decreased total lung capacity at 12 months follow-up, instead among less severe patients (who only had required supplemental oxygen) this proportion was 7% (148). In an Italian study the persistence of radiological abnormalities was also extremely common: 80% of the patients who had required intubation, almost two thirds of the ones treated with CPAP and almost half (46%) of the ones who just had required oxygen supplementation. The ground-glass opacities were the most common alterations, reticular abnormalities were present in almost half of the previously intubated subjects, but also milder patients could be affected, in line with the present data. Even here, functional impairment was less evident. TLC was reduced in 18% of severe patients and in around 10% of the milder ones; only a minority (7%), with no differences between groups, showed a restrictive pattern defined as normal FEV1/FVC and FVC less than 80% predicted. DLCO impairment was more common. Interestingly, improvement between 6 months follow-up and one year follow-up was absent or limited for all the parameters. However, functional defects, even if detectable, were usually mild (171). Similar proportions of functional alterations were also found in studies specifically focused on PFT, even if these tend to evidence a better degree of improvement between 6 months and 12 months follow-up and a more frequent decrease of spirometry volumes in severe cases. Generally, persistence of symptoms over time is associated with PFT abnormalities (172,173).

Our prospective study over hospitalized population evidenced, among patients for whom CT-scans at follow-up was available, a significant proportion of fibrotic alterations (11 over a total population of 56). Presence of other abnormalities at imaging, like ground-glass opacities and air-trapping, was even more common, affecting the vast majority of whom had fibrotic scars but also two-third of the others. These proportions are not irreconcilable with data reported by Faverio and colleagues (171), even if our study was not designed to specifically investigate the burden of radiological signs at follow-up. Focusing on a population of follow-up patients, we evidenced that, even if chest CT-scan is often pathological, functional impairment is modest. Patients with sign of fibrotic alterations did not show a significative difference from the others in term of spirometry (FEV1 and FVC) or plethysmography (TLC) values, which were, on average, within normal range. About DLCO, the difference of average values between fibrotic and non-fibrotic patients was borderline significant ($p= 0.065$). Moreover, the first ones had a mean value of 72.4% of predicted, which is around the lower limit of normal (LLN) (170).

However, as clearly evidenced by Bazdyrev and colleagues, the issue of post-COVID-19 pulmonary sequelae is not simply functional. We cannot just consider the degree of functional impairment to establish the relevance of the disease. Indeed, the morbidity burden caused by pulmonary PASC (post-acute sequelae of COVID-19) can be considered a sort of pandemic of organising pneumonia (OP). This disease is characterized by a chronic and sometimes relapsing inflammation of the lungs in which pulmonary fibrosis is one of the worst possible outcomes. Bazdyrev and colleagues also tried to estimate the number of patients involved for some countries and globally: results are shocking consisting in hundreds of thousands of people. Obviously, it is difficult to establish the impact of

vaccination and viral mutations over time. It's also extremely hard to understand if pulmonary PASC behave like common OP in term of probability of relapse (174); moreover, it would be interesting to understand if relapses were idiopathic or linked to reinfection by SARS-CoV-2 or other pathogens. Summing up, understanding of immunopathology of chronic sequelae of COVID-19 (and of COVID-19 in general) is crucial to understand their similarity to better known diseases and their effective impact in term of clinic and public health. Additionally, as brilliantly described by Jha and colleagues, effective detection methods to point out subgroup of patients at risk for these complications would be useful to optimize the clinical management. Cited authors contributed to the issue building a machine learning tools to quickly select patients at high risk of post-COVID-19 lung fibrosis (175).

Our studies explored the role of some cytokines in COVID-19. Specifically, we focused on the less known IL-32, but we also tried to describe cytokines' dynamic over time and the possible correlations with development of fibrotic lung sequelae.

IL-32, IL-8 and risk of lung fibrosis

Initially, for the first time, serum concentrations of IL-32 in a cohort of COVID-19 patients, along with IL-8 and other better-known biomarkers were evaluated (IL-6, TNF-α, INF- γ, IL-10 and IL-1β). IL-32 resulted lower in COVID-19 compared to controls, conversely IL-8 was significantly elevated in COVID-19 patients and was the marker that best discriminated patients from healthy controls, in agree with available literature (176). Extending study population, we found that higher concentrations of IL-32 were present in survivors. This effect was not detectable in the first smaller population but strengthened by the positive correlation revealed by IL-32 level at hospitalization and FVC percentage of predicted value at follow-up.

These two cytokines' concentrations showed opposite trends. Previous studies do not clarify the exact relationship between the two molecules: prevailing results suggest a counterbalance role of IL-32 in regard to physiopathology of IL-8 (177,178), but some authors reported that overexpression of some isoforms of IL- 32 results in enhanced expression of IL-8 (179). Regarding viral infections IL-32 is usually described as pro-inflammatory, even if it shows important negative feedback properties (180,181).

Moving toward patients at follow-up (our third study), we need a real effort to understand the meaning of our founding. In blood samples collected at follow-up, increased levels of IL-32 and decreased levels of IL-8 were observed among patients with lung sequelae. IL-32 is involved in several processes such as regulation of apoptosis, accentuation of inflammation, and angiogenesis. Our observation that IL-32 was significantly higher in patients with post-COVID-19 sequelae may be explained by the evidence that IL-32 promotes the epithelial to mesenchymal transition in lung alveolar epithelial cells by triggering oxidative stress, although IL-32 pathway mechanisms in such patients have not been established (182).

Unfortunately, if the IL-8/IL-32 axis has a pathogenetic role in fibrosis development, it cannot be simply used as a predictive biomarker. Concerning serum biomarkers evaluated on admission to hospital, the ones significantly higher in patients who showed HRCT evidence of fibrotic interstitial alterations at follow-up were IL-1β, IL17A, TNF-α, TGF-β, IL-4 and IL-6. As described below most of them resulted to be markers of severity (especially IL-6), so we can conclude that at present situation severity of disease in acute phase should be considered an important element in identifying patients at higher risk of morphological

alteration during follow-up. This consideration is supported by comparison with the other viral infections and by many epidemiological publications (141,143,183,184). In our previous paper we also observed this reasonable correlation, suggesting a possible predictive role for the well-known biomarker KL-6. Indeed, patients with fibrotic alterations at follow-up had showed higher level of this biomarker at hospitalization, but KL-6 was also higher at hospitalization in severe patients, compared to the other ones (185). However, role of KL-6 in COVID-19 is still controversial: many authors reported its utility as severity biomarkers in acute phase of COVID-19 (186–188) or predictor of poor outcome (189,190), but this opinion is not universally accepted by scientific community (191,192). Role of KL-6 as possible predictor of chronic fibrotic sequelae is intriguing and some authors suspected it (191,193,194) finding also a correlation with lung function test at follow-up (195), but a clear application for this biomarker is still pending.

Unfortunately, our third study did not identify a clear correlation between clinical severity recorded during hospitalization and signs at follow-up, but we have to consider that patients were enrolled at follow-up and severity was retrospectively investigated. Moreover, population was quite small and characterized by high burden of radiological signs. Interestingly, the low levels of IL-10 evidenced in severe cases at hospitalization, still were detectable in follow-up population of previously severe cases. Some authors reported reduced markers of T-cell-mediated immunity in COVID-19. This could be explained by a sort of “immune exhaustion” involving failure to heal of tissues injured during acute infection (196,197). Williams et al. found heavy reductions in circulating levels of IFN-γ and IL-8 in long-COVID patients, agreeing with the hypothesis of an immune exhaustion driven long-COVID. In line with this hypothesis, we noticed that patients with post-COVID-19 fibrotic lung sequelae had depressed serum concentrations of IL-8: a possible alert of defecting healing after acute infection (197). Literature reported also lower percentage of Treg cells in post-COVID-19 patients (198) and IL-10 is considered one of the main cytokines released by Treg cells. Our results could suggest that Treg cells contribute to prolonged or failed recovery of the immune system in severe cases, even if they apparently do not impact over healing and fibrogenic processes.

The influence of age on the clinical course of COVID-19 is strong (199). The decline in immune function may affect cytokine responses and disease severity during viral infections but also progression and complications of COVID-19 (200,201). We described an influence of age in the development of post-COVID-19 chronic respiratory impairment, as pulmonary fibrosis is: this element is supported by available literature, even if an accurately description on linkage between age or other risk factors and fibrosis in the context of COVID-19 is still missing (202).

Severity stratification and risk of mortality

Our analyses over hospitalized patients confirm previous results over direct correlations between IL-6 levels and severity of COVID-19 (71,155,203) and opposite relation between this one and IL-10 levels. We also tested panels of biomarkers to point out the best combination of cytokines for stratification of COVID-19 severity. We found that the combination of all the cytokines measured (IL-1β, IL-10, IFN-γ, TNF-α, IL-8, IL-32 and IL-6) provided good discrimination between COVID-19 patients and controls, along with remarkable sensitivity and specificity. Anyway, the combination of IL-6, IL-32, INF-γ and CRP proved to be the best one in discriminating severe forms of COVID-19. This result

agreed with another study, where the best combination markers in a small cohort of patients was evaluated, confirming IL-6 and CRP as reliable biomarkers of disease severity (204). Similar findings derived from the extension of study population, where the logistic regression showed that all cytokines (IL-4, IL-2, IP-10, IL-1β, TNF-α, MCP-1, IL-17A, IL-6, IL-10, IFN-γ, IL-12p70 and free active form of TGF-β1) displayed good accuracy for discriminating severe patients from mild to moderate cases of COVID-19. Our studies confirmed that higher serum concentrations of pro-inflammatory cytokines and, subsequently, lower values of anti-inflammatory/immunoregulatory molecules are related with COVID-19 severity.
Importance of IL-6 in COVID-19 is widely described (205) and one of the preferred immunomodulators in treating severe COVID-19 is an IL-6 inhibitor (103). Increasing the population, we confirmed data about IL-6 and its ability to detect severe COVID-19 cases, a feature possibly shared by TGF-β. The last one is involved in epithelial–mesenchymal transition: a known pathway to lung fibrosis development (183). So, our finding of increased TGF-β in most severe cases can support the linkage between severity of acute infection and risk of fibrosis development. Increased immuno-expression of TGF-β1 in patients death for COVID-19 has also been described by Busatta Vaz de Paula and colleagues (206).

IL-10 is a known powerful anti-inflammatory cytokine, displaying multiple effects, including limitation of host immune response to infection (207). Interestingly, we also noted higher concentrations of that in patients who were vaccinated before contracting the disease and requiring hospitalization. We know that IL-10-producing T-reg cells can protect against tissue damage (208). Indeed, during acute viral infections, IL-10 released from innate immune cells and effector T-cells balances immune damage and defence (209). The potential of IL-10-producing virus-specific T-regs as treatment option has been recently discussed, even in human coronavirus disease, albeit concerning specific virus-induced demyelination (210). Correlation between vaccination and biomarker emerged also for another molecule: MCP-1. While physiopathological actions of IL-10 are quite known, we do not have clear views regarding MCP-1 specific role in COVID-19 (211). In this context, high levels of MCP-1 have been associated with the recruitment and activation of monocytes, which can contribute to the dangerous inflammatory response of COVID-19 (212). Anyway, MCP-1 was also described to be linked to pathogenesis of mild COVID-19 disease more than sever one (211). In our population, we did not evidence for MCP-1 a usefulness for stratifying patients on the basis of severity; inclusion of a high number of vaccinated patients may have influenced its values. The lower level of RBP-4 in severe cases seemed to agree with existing literature. This can be due to its anti-inflammatory properties or maybe it reflects alterations of vitamin A metabolism in severe infection or inflammation (213).

Severity stratification, that in our studies is led by IL-6 along with IL-10, could have a significant impact in terms of early mortality. In our first study, a cut-off of 15 pg/ml in IL-6 median values showed a significant difference in survival rate.

Conclusion

The most severe COVID19 patients have been associated with chronic HRCT alterations. The scar tissue repairing, resulting in a sort of lung fibrosis, can lead to a reduction in lung volumes and diffusion capacity. The association between COVID-19 and lung fibrosis is still far from being accurately depicted, even though similar molecular, genetic and immunological patterns with lung fibrosis of unknown origin (such as idiopathic pulmonary fibrosis) have been reported (214,215). Actually, we should consider these manifestations milder than other ILDs in term of physical and functional impairment, nevertheless clinically

relevant. Lung fibrosis is a treatable condition and even if the risk derived from COVID-19 infection is maybe not high as initially dreaded, accounting for the large numbers of people involved and the high cost of specific antifibrotic therapy, any tools for a precise characterization of the patients are useful.

Moreover, chronic involvement of the lungs by COVID-19 for the persistence of inflammatory infiltrations, like ground-glass opacities and consolidations, should be evaluated even if it's not immediately correlated with measurable parameters (PFT). Due to empirically usefulness of steroid therapy for this kind of conditions, considering the challenges evocated by its chronic administration (174,216), any effort to better identify reliable biomarkers for disease risk is important.

The scenario of chronic consequences of COVID-19 is problematic. Factors involved can vary moving from "simple" PASC (long-COVID) that is not characterized by specific pulmonary signs, to lung-related specific conditions. The last ones can also be differentiated in chronic inflammation of the lungs, like a sort of organising pneumonia (well depicted by Bazdyrev and colleagues) and rarer pulmonary fibrosis. There are also other possible chronic complications of COVID-19 involving the lungs, like development of bullae (pneumatoceles) and even abscesses (217–221).

Our results provided an emerging role of IL-32, suggesting multiple and complex functions in viral infection and a possible cross-talk with IL-8. In particular, a sort of imbalance in the IL-8/IL-32 appeared to be important during the acute phase of the disease, conversely an increase of IL-32 over time could identify chronic fibrosing processes.

BIBLIOGRAPHY

1. Petrosillo N, Viceconte G, Ergonul O, Ippolito G, Petersen E. COVID-19, SARS and MERS: are they closely related? Clinical Microbiology and Infection. 2020 Jun;26(6):729–34.

2. Su S, Wong G, Shi W, Liu J, Lai ACK, Zhou J, et al. Epidemiology, Genetic Recombination, and Pathogenesis of Coronaviruses. Trends in Microbiology. 2016 Jun;24(6):490–502.

3. Masters PS. The Molecular Biology of Coronaviruses. In: Advances in Virus Research [Internet]. Elsevier; 2006 [cited 2023 Feb 23]. p. 193–292. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0065352706660053

4. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med. 2020 Feb 20;382(8):727–33.

5. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM. Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. N Engl J Med. 2012 Nov 8;367(19):1814–20.

6. Hui DS, I Azhar E, Madani TA, Ntoumi F, Kock R, Dar O, et al. The continuing 2019-nCoV epidemic threat of novel coronaviruses to global health — The latest 2019 novel coronavirus outbreak in Wuhan, China. International Journal of Infectious Diseases. 2020 Feb;91:264–6.

7. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, Gorbalenya AE, Baker SC, Baric RS, de Groot RJ, Drosten C, et al. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. Nat Microbiol. 2020 Mar 2;5(4):536–44.

8. Wahba L, Jain N, Fire AZ, Shoura MJ, Artiles KL, McCoy MJ, et al. An Extensive Meta-Metagenomic Search Identifies SARS-CoV-2-Homologous Sequences in Pangolin Lung Viromes. Imperiale MJ, editor. mSphere. 2020 Jun 24;5(3):e00160-20.

9. WHO Director. WHO Director-General's remarks at the media briefing on 2019-nCoV on 11 February 2020 [Internet]. Available from: https://www.who.int/director-general/speeches/detail/who-director-general-s-remarks-at-the-media-briefing-on-2019-ncov-on-11-february-2020

10. WHO Director. WHO Director-General's opening remarks at the media briefing on COVID-19 - 11 March 2020 [Internet]. Available from: https://www.who.int/director-general/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---11-march-2020

11. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020 Mar 12;579(7798):270–3.

12. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. Addendum: A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020 Dec 3;588(7836):E6–E6.

13. Chen Y, Liu Q, Guo D. Emerging coronaviruses: Genome structure, replication, and pathogenesis. J Med Virol. 2020 Apr;92(4):418–23.

14. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor Recognition by the Novel Coronavirus from Wuhan: an Analysis Based on Decade-Long Structural Studies of SARS Coronavirus. Gallagher T, editor. J Virol. 2020 Mar 17;94(7):e00127-20.

15. Harmer D, Gilbert M, Borman R, Clark KL. Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme. FEBS Letters. 2002 Dec 4;532(1–2):107–10.

16. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature. 2003 Nov;426(6965):450–4.

17. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. Science. 2020 Mar 13;367(6483):1260-1263. doi: 10.1126/science.abb2507. Epub 2020 Feb 19. Available from: https://www.science.org/doi/full/10.1126/science.abb2507?rfr_dat=cr_pub++0pubmed&url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org

18. Matsuyama S, Nagata N, Shirato K, Kawase M, Takeda M, Taguchi F. Efficient Activation of the Severe Acute Respiratory Syndrome Coronavirus Spike Protein by the Transmembrane Protease TMPRSS2. J Virol. 2010 Dec 15;84(24):12658–64.

19. Glowacka I, Bertram S, Müller MA, Allen P, Soilleux E, Pfefferle S, et al. Evidence that TMPRSS2 Activates the Severe Acute Respiratory Syndrome Coronavirus Spike Protein for Membrane Fusion and Reduces Viral Control by the Humoral Immune Response. J Virol. 2011 May;85(9):4122–34.

20. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell. 2020 Apr;181(2):271-280.e8.

21. Chan JFW, Yuan S, Kok KH, To KKW, Chu H, Yang J, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. The Lancet. 2020 Feb;395(10223):514–23.

22. Schünemann HJ, Khabsa J, Solo K, Khamis AM, Brignardello-Petersen R, El-Harakeh A, et al. Ventilation Techniques and Risk for Transmission of Coronavirus Disease, Including COVID-19: A Living Systematic Review of Multiple Streams of Evidence. Annals of Internal Medicine. 2020 Aug 4;173(3):204–16.

23. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, et al. Detection of SARS-CoV-2 in Different Types of Clinical Specimens. JAMA [Internet]. 2020 Mar 11 [cited 2023 Feb 26]; Available from: https://jamanetwork.com/journals/jama/fullarticle/2762997

24. Cheung KS, Hung IFN, Chan PPY, Lung KC, Tso E, Liu R, et al. Gastrointestinal Manifestations of SARS-CoV-2 Infection and Virus Load in Fecal Samples From a Hong Kong Cohort: Systematic Review and Meta-analysis. Gastroenterology. 2020 Jul;159(1):81–95.

25. Li D, Jin M, Bao P, Zhao W, Zhang S. Clinical Characteristics and Results of Semen Tests Among Men With Coronavirus Disease 2019. JAMA Netw Open. 2020 May 7;3(5):e208292.

26. Colavita F, Lapa D, Carletti F, Lalle E, Bordi L, Marsella P, et al. SARS-CoV-2 Isolation From Ocular Secretions of a Patient With COVID-19 in Italy With Prolonged Viral RNA Detection. Annals of Internal Medicine. 2020 Aug 4;173(3):242–3.

27. Zeng G, Xie SY, Li Q, Ou JM. Infectivity of Severe Acute Respiratory Syndrome during Its Incubation Period. Biomedical and Environmental Sciences. 2009 Dec;22(6):502–10.

28. Hu Z, Song C, Xu C, Jin G, Chen Y, Xu X, et al. Clinical characteristics of 24 asymptomatic infections with COVID-19 screened among close contacts in Nanjing, China. Sci China Life Sci. 2020 May;63(5):706–11.

29. Liu Z, Chu R, Gong L, Su B, Wu J. The assessment of transmission efficiency and latent infection period in asymptomatic carriers of SARS-CoV-2 infection. International Journal of Infectious Diseases. 2020 Oct;99:325–7.

30. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus–Infected Pneumonia. N Engl J Med. 2020 Mar 26;382(13):1199–207.

31. Guan W jie, Ni Z yi, Hu Y, Liang W hua, Ou C quan, He J xing, et al. Clinical Characteristics of Coronavirus Disease 2019 in China. N Engl J Med. 2020 Apr 30;382(18):1708–20.

32. Poudel S, Ishak A, Perez-Fernandez J, Garcia E, León-Figueroa DA, Romaní L, et al. Highly mutated SARS-CoV-2 Omicron variant sparks significant concern among global experts – What is known so far? Travel Medicine and Infectious Disease. 2022 Jan;45:102234.

33. WHO. Statement on the update of WHO's working definitions and tracking system for SARS-CoV-2 variants of concern and variants of interest [Internet]. Available from: https://www.who.int/news/item/16-03-2023-statement-on-the-update-of-who-s-working-definitions-and-tracking-system-for-sars-cov-2-variants-of-concern-and-variants-of-interest

34. Long B, Carius BM, Chavez S, Liang SY, Brady WJ, Koyfman A, et al. Clinical update on COVID-19 for the emergency clinician: Presentation and evaluation. The American Journal of Emergency Medicine. 2022 Apr;54:46–57.

35. Tian S, Hu W, Niu L, Liu H, Xu H, Xiao SY. Pulmonary Pathology of Early-Phase 2019 Novel Coronavirus (COVID-19) Pneumonia in Two Patients With Lung Cancer. Journal of Thoracic Oncology. 2020 May;15(5):700–4.

36. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. The Lancet Respiratory Medicine. 2020 Apr;8(4):420–2.

37. Carsana L, Sonzogni A, Nasr A, Rossi RS, Pellegrinelli A, Zerbi P, et al. Pulmonary post-mortem findings in a series of COVID-19 cases from northern Italy: a two-centre descriptive study. The Lancet Infectious Diseases. 2020 Oct;20(10):1135–40.

38. Gonzalez-Garcia P, Fiorillo Moreno O, Zarate Peñata E, Calderon-Villalba A, Pacheco Lugo L, Acosta Hoyos A, et al. From Cell to Symptoms: The Role of SARS-CoV-2 Cytopathic Effects in the Pathogenesis of COVID-19 and Long COVID. IJMS. 2023 May 5;24(9):8290.

39. Rosa-Fernandes L, Lazari LC, da Silva JM, de Morais Gomes V, Machado RRG, dos Santos AF, et al. SARS-CoV-2 activates ER stress and Unfolded protein response [Internet]. Biochemistry; 2021 Jun [cited 2023 Sep 17]. Available from: http://biorxiv.org/lookup/doi/10.1101/2021.06.21.449284

40. Sureda A, Alizadeh J, Nabavi SF, Berindan-Neagoe I, Cismaru CA, Jeandet P, et al. Endoplasmic reticulum as a potential therapeutic target for covid-19 infection management? European Journal of Pharmacology. 2020 Sep;882:173288.

41. Cortese M, Lee JY, Cerikan B, Neufeldt CJ, Oorschot VMJ, Köhrer S, et al. Integrative Imaging Reveals SARS-CoV-2-Induced Reshaping of Subcellular Morphologies. Cell Host & Microbe. 2020 Dec;28(6):853-866.e5.

42. Zhang J, Kennedy A, Xing L, Bui S, Reid W, Joppich J, et al. SARS-CoV-2 triggers Golgi fragmentation via down-regulation of GRASP55 to facilitate viral trafficking [Internet]. Cell Biology; 2022 Mar [cited 2023 Sep 17]. Available from: http://biorxiv.org/lookup/doi/10.1101/2022.03.04.483074

43. Onnis A, Andreano E, Cassioli C, Finetti F, Della Bella C, Staufer O, et al. SARS-CoV-2 Spike protein suppresses CTL-mediated killing by inhibiting immune synapse assembly. Journal of Experimental Medicine. 2023 Feb 6;220(2):e20220906.

44. Remy KE, Mazer M, Striker DA, Ellebedy AH, Walton AH, Unsinger J, et al. Severe immunosuppression and not a cytokine storm characterizes COVID-19 infections. JCI Insight. 2020 Sep 3;5(17):e140329.

45. Karki R, Kanneganti TD. The 'cytokine storm': molecular mechanisms and therapeutic prospects. Trends in Immunology. 2021 Aug;42(8):681–705.

46. Rovito R, Augello M, Ben-Haim A, Bono V, d'Arminio Monforte A, Marchetti G. Hallmarks of Severe COVID-19 Pathogenesis: A Pas de Deux Between Viral and Host Factors. Front Immunol. 2022 Jun 10;13:912336.

47. Christgen S, Zheng M, Kesavardhana S, Karki R, Malireddi RKS, Banoth B, et al. Identification of the PANoptosome: A Molecular Platform Triggering Pyroptosis, Apoptosis, and Necroptosis (PANoptosis). Front Cell Infect Microbiol. 2020 May 29;10:237.

48. Baldari CT, Onnis A, Andreano E, Del Giudice G, Rappuoli R. Emerging roles of SARS-CoV-2 Spike-ACE2 in immune evasion and pathogenesis. Trends in Immunology. 2023 Jun;44(6):424–34.

49. Mahmudpour M, Roozbeh J, Keshavarz M, Farrokhi S, Nabipour I. COVID-19 cytokine storm: The anger of inflammation. Cytokine. 2020 Sep;133:155151.

50. Morganstein T, Haidar Z, Trivlidis J, Azuelos I, Huang MJ, Eidelman DH, et al. Involvement of the ACE2/Ang-(1–7)/MasR Axis in Pulmonary Fibrosis: Implications for COVID-19. IJMS. 2021 Nov 30;22(23):12955.

51. Aminpour M, Hameroff S, Tuszynski JA. How COVID-19 Hijacks the Cytoskeleton: Therapeutic Implications. Life. 2022 May 30;12(6):814.

52. Jain A, Pasare C. Innate Control of Adaptive Immunity: Beyond the Three-Signal Paradigm. The Journal of Immunology. 2017 May 15;198(10):3791–800.

53. Netea MG, Quintin J, van der Meer JWM. Trained Immunity: A Memory for Innate Host Defense. Cell Host & Microbe. 2011 May;9(5):355–61.

54. Netea MG, Latz E, Mills KHG, O'Neill LAJ. Innate immune memory: a paradigm shift in understanding host defense. Nat Immunol. 2015 Jul;16(7):675–9.

55. Hamada A, Torre C, Drancourt M, Ghigo E. Trained Immunity Carried by Non-immune Cells. Front Microbiol. 2019 Jan 14;9:3225.

56. Zhang Q, Bastard P, Liu Z, Le Pen J, Moncada-Velez M, Chen J, et al. Inborn errors of type I IFN immunity in patients with life-threatening COVID-19. Science. 2020 Oct 23;370(6515):eabd4570.

57. Mantovani S, Daga S, Fallerini C, Baldassarri M, Benetti E, Picchiotti N, et al. Rare variants in Toll-like receptor 7 results in functional impairment and downregulation of cytokine-mediated signaling in COVID-19 patients. Genes Immun. 2022 Feb;23(1):51–6.

58. Baldassarri M, Fava F, Fallerini C, Daga S, Benetti E, Zguro K, et al. Severe COVID-19 in Hospitalized Carriers of Single CFTR Pathogenic Variants. JPM. 2021 Jun 15;11(6):558.

59. Fallerini C, Daga S, Benetti E, Picchiotti N, Zguro K, Catapano F, et al. SELP Asp603Asn and severe thrombosis in COVID-19 males. J Hematol Oncol. 2021 Dec;14(1):123.

60. COVID-19 Host Genetics Initiative, COVID-19 Host Genetics Initiative Leadership, Niemi MEK, Karjalainen J, Liao RG, Neale BM, et al. Mapping the human genetic architecture of COVID-19. Nature. 2021 Dec 16;600(7889):472–7.

61. COVID-19 Host Genetics Initiative, COVID-19 Host Genetics Initiative Leadership, Pathak GA, Karjalainen J, Stevens C, et al. A first update on mapping the human genetic architecture of COVID-19. Nature. 2022 Aug 4;608(7921):E1–10.

62. Fallerini C, Picchiotti N, Baldassarri M, Zguro K, Daga S, Fava F, et al. Common, low-frequency, rare, and ultra-rare coding variants contribute to COVID-19 severity. Hum Genet. 2022 Jan;141(1):147–73.

63. Onoja A, Picchiotti N, Fallerini C, Baldassarri M, Fava F, GEN-COVID Multicenter Study, et al. An explainable model of host genetic interactions linked to COVID-19 severity. Commun Biol. 2022 Oct 26;5(1):1133.

64. Peirlinck M, Linka K, Sahli Costabal F, Bhattacharya J, Bendavid E, Ioannidis JPA, et al. Visualizing the invisible: The effect of asymptomatic transmission on the outbreak dynamics of COVID-19. Computer Methods in Applied Mechanics and Engineering. 2020 Dec;372:113410.

65. Mizumoto K, Kagaya K, Zarebski A, Chowell G. Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. Eurosurveillance [Internet]. 2020 Mar 12 [cited 2023 Sep 28];25(10). Available from: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2020.25.10.2000180

66. Eurosurveillance editorial team. Author's correction for Euro Surveill. 2020;25(10).

67. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. JAMA.

68. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. The Lancet. 2020 Feb;395(10223):497–506.

69. Mehta OP, Bhandari P, Raut A, Kacimi SEO, Huy NT. Coronavirus Disease (COVID-19): Comprehensive Review of Clinical Presentation. Front Public Health. 2021 Jan 15;8:582932.

70. Krishnakumar HN, Momtaz DA, Sherwani A, Mhapankar A, Gonuguntla RK, Maleki A, et al. Pathogenesis and progression of anosmia and dysgeusia during the COVID-19 pandemic. Eur Arch Otorhinolaryngol. 2023 Feb;280(2):505–9.

71. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. The Lancet. 2020 Mar;395(10229):1054–62.

72. WHO. Clinical management of COVID-19 Living guideline 18 August 2023 [Internet]. Available from: https://www.who.int/teams/health-care-readiness/covid-19

73. Bikdeli B, Madhavan MV, Jimenez D, Chuich T, Dreyfus I, Driggin E, et al. COVID-19 and Thrombotic or Thromboembolic Disease: Implications for Prevention, Antithrombotic Therapy, and Follow-Up. Journal of the American College of Cardiology. 2020 Jun;75(23):2950–73.

74. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. Journal of Thrombosis and Haemostasis. 2020 Apr;18(4):844–7.

75. Han H, Yang L, Liu R, Liu F, Wu K lang, Li J, et al. Prominent changes in blood coagulation of patients with SARS-CoV-2 infection. Clinical Chemistry and Laboratory Medicine (CCLM). 2020 Jun 25;58(7):1116–20.

76. Sposato B, Croci L, Di Tomassi M, Puttini C, Olivieri C, Alessandri M, et al. Spontaneous abdominal bleeding associated with SARS-CoV-2 infection: causality or coincidence? Abdominal bleeding and SARS-CoV-2. Acta Biomedica Atenei Parmensis. 2021 May 12;92(2):e2021199.

77. Lippi G, Plebani M. Laboratory abnormalities in patients with COVID-2019 infection. Clinical Chemistry and Laboratory Medicine (CCLM). 2020 Jun 25;58(7):1131–4.

78. Zhang W, Li L, Liu J, Chen L, Zhou F, Jin T, et al. The characteristics and predictive role of lymphocyte subsets in COVID-19 patients. International Journal of Infectious Diseases. 2020 Oct;99:92–9.

79. on behalf of Siena COVID Unit, d'Alessandro M, Bennett D, Montagnani F, Cameli P, Perrone A, et al. Peripheral lymphocyte subset monitoring in COVID-19 Italian patients. Minerva Med [Internet]. 2021 May [cited 2023 Oct 19];112(3). Available from: https://www.minervamedica.it/index2.php?show=R10Y2021N03A0423

80. d'Alessandro M, Bergantini L, Cameli P, Curatola G, Remediani L, Sestini P, et al. Peripheral biomarkers' panel for severe COVID-19 patient.

81. Luo M, Liu J, Jiang W, Yue S, Liu H, Wei S. IL-6 and CD8+ T cell counts combined are an early predictor of in-hospital mortality of patients with COVID-19. JCI Insight. 2020 Jul 9;5(13):e139024.

82. Urra JM, Cabrera CM, Porras L, Ródenas I. Selective CD8 cell reduction by SARS-CoV-2 is associated with a worse prognosis and systemic inflammation in COVID-19 patients. Clinical Immunology. 2020 Aug;217:108486.

83. Liu J, Li S, Liu J, Liang B, Wang X, Wang H, et al. Longitudinal characteristics of lymphocyte responses and cytokine profiles in the peripheral blood of SARS-CoV-2 infected patients. EBioMedicine. 2020 May;55:102763.

84. Bermejo-Martin JF, Almansa R, Menéndez R, Mendez R, Kelvin DJ, Torres A. Lymphopenic community acquired pneumonia as signature of severe COVID-19 infection. Journal of Infection. 2020 May;80(5):e23–4.

85. Pan F, Ye T, Sun P, Gui S, Liang B, Li L, et al. Time Course of Lung Changes at Chest CT during Recovery from Coronavirus Disease 2019 (COVID-19). Radiology. 2020 Jun;295(3):715–21.

86. Wu J, Wu X, Zeng W, Guo D, Fang Z, Chen L, et al. Chest CT Findings in Patients With Coronavirus Disease 2019 and Its Relationship With Clinical Features. Invest Radiol. 2020 May;55(5):257–61.

87. Bennett D, De Vita E, Mezzasalma F, Lanzarone N, Cameli P, Bianchi F, et al. Portable Pocket-Sized Ultrasound Scanner for the Evaluation of Lung Involvement in Coronavirus Disease 2019 Patients. Ultrasound in Medicine & Biology. 2021 Jan;47(1):19–24.

88. Chinese Critical Care Ultrasound Study Group (CCUSG), Peng QY, Wang XT, Zhang LN. Findings of lung ultrasonography of novel corona virus pneumonia during the 2019–2020 epidemic. Intensive Care Med. 2020 May;46(5):849–50.

89. Msemburi W, Karlinsky A, Knutson V, Aleshin-Guendel S, Chatterji S, Wakefield J. The WHO estimates of excess mortality associated with the COVID-19 pandemic. Nature. 2023 Jan 5;613(7942):130–7.

90. Al Wahaibi A, Al-Maani A, Alyaquobi F, Al Harthy K, Al-Jardani A, Al Rawahi B, et al. Effects of COVID-19 on mortality: A 5-year population-based study in Oman. International Journal of Infectious Diseases. 2021 Mar;104:102–7.

91. Yang L, Zhong J, Wang W, Zhou F, Tong Z, Zheng Y, et al. Clinical features of Omicron variant infection in 445 patients with coronavirus 19 disease. Ann Saudi Med. 2023 May;43(3):161–5.

92. Bouzid D, Visseaux B, Kassasseya C, Daoud A, Fémy F, Hermand C, et al. Comparison of Patients Infected With Delta Versus Omicron COVID-19 Variants Presenting to Paris Emergency Departments: A Retrospective Cohort Study. Ann Intern Med. 2022 Jun;175(6):831–7.

93. Núñez-Gil IJ, Fernández-Pérez C, Estrada V, Becerra-Muñoz VM, El-Battrawy I, Uribarri A, et al. Mortality risk assessment in Spain and Italy, insights of the HOPE COVID-19 registry. Intern Emerg Med. 2021 Jun;16(4):957–66.

94. Tartof SY, Qian L, Hong V, Wei R, Nadjafi RF, Fischer H, et al. Obesity and Mortality Among Patients Diagnosed With COVID-19: Results From an Integrated Health Care Organization. Ann Intern Med. 2020 Nov 17;173(10):773–81.

95. Task Force COVID-19 del Dipartimento Malattie Infettive e Servizio di Informatica, Istituto Superiore di Sanità. Epidemia COVID-19. Aggiornamento Nazionale: 25 Gennaio 2023 [Internet]. [cited 2023 Feb 3]. Available from: https://www.epicentro.iss.it/coronavirus/bollettino/ Bollettino-sorveglianza-integrata-COVID-19_25-gennaio-2023.pdf

96. Nunziata F, Salomone S, Catzola A, Poeta M, Pagano F, Punzi L, et al. Clinical Presentation and Severity of SARS-CoV-2 Infection Compared to Respiratory Syncytial Virus and Other Viral Respiratory Infections in Children Less than Two Years of Age. Viruses. 2023 Mar 9;15(3):717.

97. Riphagen S, Gomez X, Gonzalez-Martinez C, Wilkinson N, Theocharis P. Hyperinflammatory shock in children during COVID-19 pandemic. The Lancet. 2020 May;395(10237):1607–8.

98. Radia T, Williams N, Agrawal P, Harman K, Weale J, Cook J, et al. Multi-system inflammatory syndrome in children & adolescents (MIS-C): A systematic review of clinical features and presentation. Paediatric Respiratory Reviews. 2021 Jun;38:51–7.

99. Sperotto F, Gutiérrez-Sacristán A, Makwana S, Li X, Rofeberg VN, Cai T, et al. Clinical phenotypes and outcomes in children with multisystem inflammatory syndrome across SARS-CoV-2 variant eras: a multinational study from the 4CE consortium. eClinicalMedicine. 2023 Oct;64:102212.

100. Morris SB, Schwartz NG, Patel P, Abbo L, Beauchamps L, Balan S, et al. Case Series of Multisystem Inflammatory Syndrome in Adults Associated with SARS-CoV-2 Infection — United Kingdom and United States, March–August 2020. MMWR Morb Mortal Wkly Rep. 2020 Oct 9;69(40):1450–6.

101. Zasada AA, Darlińska A, Wiatrzyk A, Woźnica K, Formińska K, Czajka U, et al. COVID-19 Vaccines over Three Years after the Outbreak of the COVID-19 Epidemic. Viruses. 2023 Aug 23;15(9):1786.

102. Roche N, Crichton ML, Goeminne PC, Cao B, Humbert M, Shteinberg M, et al. Update June 2022: management of hospitalised adults with coronavirus disease 2019 (COVID-19): a European Respiratory Society living guideline. Eur Respir J. 2022 Aug;60(2):2200803.

103. COVID-19 Treatment Guidelines Panel, National Institutes of Health. Coronavirus Disease 2019 (COVID-19) Treatment Guidelines.

104. CHMP Committee for Medicinal Products for Human Use (EMA). Parere negativo sull'autorizzazione all'immissione in commercio di Lagevrio (molnupiravir). EMA/82948/2023 EMEA/H/C/005789. [cited 2023 Nov 12]. Available from: https://www.aifa.gov.it/documents/20142/1616529/Questions_answers_lagevrio-molnupiravir_it.pdf

105. Gazzetta Ufficiale. Determina n. DG/85/2023; GU Serie Generale n.62 del 14-03-2023. Gazzetta Ufficiale Mar 14, 2023. Available from: https://www.gazzettaufficiale.it/eli/id/2023/03/14/23A01684/SG

106. Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, et al. Remdesivir for the Treatment of Covid-19 — Final Report. N Engl J Med. 2020 Nov 5;383(19):1813–26.

107. Gottlieb RL, Vaca CE, Paredes R, Mera J, Webb BJ, Perez G, et al. Early Remdesivir to Prevent Progression to Severe Covid-19 in Outpatients. N Engl J Med. 2022 Jan 27;386(4):305–15.

108. Mahase E. Covid-19: Pfizer's paxlovid is 89% effective in patients at risk of serious illness, company reports. BMJ. 2021 Nov 8;n2713.

109. Merck and Ridgeback. Merck and Ridgeback's Investigational Oral Antiviral Molnupiravir Reduced the Risk of Hospitalization or Death by Approximately 50 Percent Compared to Placebo for Patients with Mild or Moderate COVID-19 in Positive Interim Analysis of Phase 3 Study. 2021 Jan. [cited 2023 Nov 14]. Available from: https://www.merck.com/news/merck-and-ridgebacks-investigational-oral-antiviral-molnupiravir-reduced-the-risk-of-hospitalization-or-death-by-approximately-50-percent-compared-to-placebo-for-patients-with-mild-or-moderat/

110. Pillaiyar T, Manickam M, Namasivayam V, Hayashi Y, Jung SH. An Overview of Severe Acute Respiratory Syndrome–Coronavirus (SARS-CoV) 3CL Protease Inhibitors: Peptidomimetics and Small Molecule Chemotherapy. J Med Chem. 2016 Jul 28;59(14):6595–628.

111. Gandhi S, Klein J, Robertson AJ, Peña-Hernández MA, Lin MJ, Roychoudhury P, et al. De novo emergence of a remdesivir resistance mutation during treatment of persistent SARS-CoV-2 infection in an immunocompromised patient: a case report. Nat Commun. 2022 Mar 17;13(1):1547.

112. Martinot M, Jary A, Fafi-Kremer S, Leducq V, Delagreverie H, Garnier M, et al. Emerging RNA-Dependent RNA Polymerase Mutation in a Remdesivir-Treated B-cell Immunodeficient Patient With Protracted Coronavirus Disease 2019. Clinical Infectious Diseases. 2021 Oct 5;73(7):e1762–5.

113. Fischer WA, Eron JJ, Holman W, Cohen MS, Fang L, Szewczyk LJ, et al. A phase 2a clinical trial of molnupiravir in patients with COVID-19 shows accelerated SARS-CoV-2 RNA clearance and elimination of infectious virus. Sci Transl Med. 2022 Jan 19;14(628):eabl7430.

114. Kaka AS, MacDonald R, Linskens EJ, Langsetmo L, Vela K, Duan-Porter W, et al. Major Update 2: Remdesivir for Adults With COVID-19: A Living Systematic Review and Meta-analysis for the American College of Physicians Practice Points. Ann Intern Med. 2022 May;175(5):701–9.

115. Nigro M, Chalmers JD, Aliberti S. A patient-tailored approach for corticosteroid treatment in COVID-19: still not there yet.

116. Devasenapathy N, Ye Z, Loeb M, Fang F, Najafabadi BT, Xiao Y, et al. Efficacy and safety of convalescent plasma for severe COVID-19 based on evidence in other severe respiratory viral infections: a systematic review and meta-analysis. CMAJ. 2020 Jul 6;192(27):E745–55.

117. Franchini M, Focosi D, Percivalle E, Beccaria M, Garuti M, Arar O, et al. Variant of Concern-Matched COVID-19 Convalescent Plasma Usage in Seronegative Hospitalized Patients. Viruses. 2022 Jun 30;14(7):1443.

118. Bhimraj A, Shumaker AH, Baden LR, Cheng VCC, Edwards KM, Gallagher JC, et al. Infectious Diseases Society of America Guidelines on the Treatment and Management of Patients With Coronavirus Disease 2019 (COVID-19). Clin Infect Dis. 2022 Sep 5:ciac724. doi: 10.1093/cid/ciac724. Epub ahead of print.

119. AIFA. TRATTAMENTI UTILIZZABILI NEI PAZIENTI COVID-19 NEL SETTING OSPEDALIERO. Vers. 2021 Oct 04. [cited 2023 Nov 15]. Available from: https://www.aifa.gov.it/documents/20142/1269602/SOC_ospedaliera_04.10.2021.pdf

120. AIFA. RACCOMANDAZIONI AIFA SUI FARMACI per la gestione domiciliare di COVID-19 Vers. 10. 2023 Mar 10. [cited 2023 Nov 15]. Available from: https://www.aifa.gov.it/documents/20142/1616529/IT_Raccomandazioni_AIFA_gestione_domiciliare_COVID-19_Vers10_10.03.2023.pdf

121. Tzou PL, Tao K, Pond SLK, Shafer RW. Coronavirus Resistance Database (CoV-RDB): SARS-CoV-2 susceptibility to monoclonal antibodies, convalescent plasma, and plasma from vaccinated persons. Bhattacharya J, editor. PLoS ONE. 2022 Mar 9;17(3):e0261045.

122. Koudstaal T, Funke-Chambour M, Kreuter M, Molyneaux PL, Wijsenbeek MS. Pulmonary fibrosis: from pathogenesis to clinical decision-making. Trends in Molecular Medicine. 2023 Dec;29(12):1076–87.

123. Adegunsoye A, Ryerson CJ. Diagnostic Classification of Interstitial Lung Disease in Clinical Practice. Clin Chest Med. 2021 Jun;42(2):251–61.

124. Travis WD, King TE. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. Am J Respir Crit Care Med. 2002 Jan 15;165(2):277–304.

125. Travis WD, Costabel U, Hansell DM, King TE, Lynch DA, Nicholson AG, et al. An Official American Thoracic Society/European Respiratory Society Statement: Update of the International Multidisciplinary Classification of the Idiopathic Interstitial Pneumonias. Am J Respir Crit Care Med. 2013 Sep 15;188(6):733–48.

126. Raghu G, Remy-Jardin M, Richeldi L, Thomson CC, Inoue Y, Johkoh T, et al. Idiopathic Pulmonary Fibrosis (an Update) and Progressive Pulmonary Fibrosis in Adults: An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline. Am J Respir Crit Care Med. 2022 May 1;205(9):e18–47.

127. Drakopanagiotakis F, Paschalaki K, Abu-Hijleh M, Aswad B, Karagianidis N, Kastanakis E, et al. Cryptogenic and secondary organizing pneumonia: clinical presentation, radiographic findings, treatment response, and prognosis. Chest. 2011 Apr;139(4):893–900.

128. Ujita M, Renzoni EA, Veeraraghavan S, Wells AU, Hansell DM. Organizing Pneumonia: Perilobular Pattern at Thin-Section CT. Radiology. 2004 Sep;232(3):757–61.

129. Baque-Juston M, Pellegrin A, Leroy S, Marquette CH, Padovani B. Pneumopathie organisée : qu'est-ce que c'est ? Sémiologie conceptuelle et revue iconographique. Journal de Radiologie Diagnostique et Interventionnelle. 2014 Sep;95(9):766–73.

130. Raghu G, Rochwerg B, Zhang Y, Garcia CAC, Azuma A, Behr J, et al. An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline: Treatment of Idiopathic Pulmonary Fibrosis. An Update of the 2011 Clinical Practice Guideline. Am J Respir Crit Care Med. 2015 Jul 15;192(2):e3–19.

131. Atsumi K, Hisakane K, Mikami E, Suzuki T, Matsuki S, Seike M, et al. Minimal effective dose of maintenance steroid therapy for relapse of cryptogenic organizing pneumonia. Respiratory Medicine. 2023 Nov;218:107390.

132. Rajan SK, Cottin V, Dhar R, Danoff S, Flaherty KR, Brown KK, et al. Progressive pulmonary fibrosis: an expert group consensus statement. Eur Respir J. 2023 Mar;61(3):2103187.

133. AIFA. Elenchi farmaci di classe A e H [Internet]. 2023. [accessed 2023 Dec 02]. Available from: https://www.aifa.gov.it/liste-farmaci-a-h

134. Collard HR, Ryerson CJ, Corte TJ, Jenkins G, Kondoh Y, Lederer DJ, et al. Acute Exacerbation of Idiopathic Pulmonary Fibrosis. An International Working Group Report. Am J Respir Crit Care Med. 2016 Aug 1;194(3):265–75.

135. Kreuter M, Polke M, Walsh SLF, Krisam J, Collard HR, Chaudhuri N, et al. Acute exacerbation of idiopathic pulmonary fibrosis: international survey and call for harmonisation. Eur Respir J. 2020 Apr;55(4):1901760.

136. Naccache JM, Jouneau S, Didier M, Borie R, Cachanado M, Bourdin A, et al. Cyclophosphamide added to glucocorticoids in acute exacerbation of idiopathic pulmonary fibrosis (EXAFIP): a randomised, double-blind, placebo-controlled, phase 3 trial. The Lancet Respiratory Medicine. 2022 Jan;10(1):26–34.

137. Vasarmidi E, Tsitoura E, Spandidos D, Tzanakis N, Antoniou K. Pulmonary fibrosis in the aftermath of the Covid-19 era (Review). Exp Ther Med [Internet]. 2020 Jul 9 [cited 2023 Mar 1]; Available from: http://www.spandidos-publications.com/10.3892/etm.2020.8980

138. Han X, Cao Y, Jiang N, Chen Y, Alwalid O, Zhang X, et al. Novel Coronavirus Disease 2019 (COVID-19) Pneumonia Progression Course in 17 Discharged Patients: Comparison of Clinical and Thin-Section Computed Tomography Features During Recovery. Clin Infect Dis. 2020 Jul 28;71(15):723-731. doi: 10.1093/cid/ciaa271.

139. Wang Y, Dong C, Hu Y, Li C, Ren Q, Zhang X, et al. Temporal Changes of CT Findings in 90 Patients with COVID-19 Pneumonia: A Longitudinal Study. Radiology. 2020 Aug;296(2):E55–64.

140. Grillo F, Barisione E, Ball L, Mastracci L, Fiocca R. Lung fibrosis: an undervalued finding in COVID-19 pathological series. The Lancet Infectious Diseases. 2021 Apr;21(4):e72.

141. Xing ZH, Sun X, Xu L, Wu Q, Li L, Wu XJ, et al. Thin-section Computed Tomography Detects Long-term Pulmonary Sequelae 3 Years after Novel Influenza A Virus-associated Pneumonia. Chinese Medical Journal. 2015 Apr 5;128(7):902–8.

142. Gao J, Chu W, Duan J, Li J, Ma W, Hu C, et al. Six-Month Outcomes of Post-ARDS Pulmonary Fibrosis in Patients With H1N1 Pneumonia. Front Mol Biosci. 2021 Jun 8;8:640763.

143. Chen J, Wu J, Hao S, Yang M, Lu X, Chen X, et al. Long term outcomes in survivors of epidemic Influenza A (H7N9) virus infection. Sci Rep. 2017 Dec 8;7(1):17275.

144. Burnham EL, Janssen WJ, Riches DWH, Moss M, Downey GP. The fibroproliferative response in acute respiratory distress syndrome: mechanisms and clinical significance. European Respiratory Journal. 2014 Jan 1;43(1):276–85.

145. Antoniou KM, Vasarmidi E, Russell AM, Andrejak C, Crestani B, Delcroix M, et al. European Respiratory Society statement on long COVID follow-up. Eur Respir J. 2022 Aug;60(2):2102174.

146. Haunhorst S, Bloch W, Wagner H, Ellert C, Krüger K, Vilser DC, et al. Long COVID: a narrative review of the clinical aftermaths of COVID-19 with a focus on the putative pathophysiology and aspects of physical activity. Oxford Open Immunology. 2022 Jun 11;3(1):iqac006.

147. Klein J, Wood J, Jaycox JR, Dhodapkar RM, Lu P, Gehlhausen JR, et al. Distinguishing features of long COVID identified through immune profiling. Nature. 2023 Nov 2;623(7985):139–48.

148. Huang L, Yao Q, Gu X, Wang Q, Ren L, Wang Y, et al. 1-year outcomes in hospital survivors with COVID-19: a longitudinal cohort study. The Lancet. 2021 Aug;398(10302):747–58.

149. The Lancet Editors. Expression of concern: 6-month consequences of COVID-19 in patients discharged from hospital: a cohort study. The Lancet. 2023 Jan;401(10371):90.

150. Krygier A, Szmajda-Krygier D, Świechowski R, Pietrzak J, Wosiak A, Wodziński D, et al. Molecular Pathogenesis of Fibrosis, Thrombosis and Surfactant Dysfunction in the Lungs of Severe COVID-19 Patients. Biomolecules. 2022 Dec 10;12(12):1845.

151. Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus–induced lung injury. Nat Med. 2005 Aug;11(8):875–9.

152. Colarusso C, Maglio A, Terlizzi M, Vitale C, Molino A, Pinto A, et al. Post-COVID-19 Patients Who Develop Lung Fibrotic-like Changes Have Lower Circulating Levels of IFN-β but Higher Levels of IL-1α and TGF-β. Biomedicines. 2021 Dec 17;9(12):1931.

153. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. Clinical Pharmacology & Therapeutics. 2001 Mar;69(3):89–95.

154. Stainer A, Faverio P, Busnelli S, Catalano M, Della Zoppa M, Marruchella A, et al. Molecular Biomarkers in Idiopathic Pulmonary Fibrosis: State of the Art and Future Directions. IJMS. 2021 Jun 10;22(12):6255.

155. Rapti I, Asimakopoulos A, Liontos A, Kosmidou M, Christaki E, Biros D, et al. Association of patient characteristics with clinical outcomes in a cohort of hospitalised patients with SARS-CoV-2 infection in a Greek referral centre for COVID-19. Epidemiol Infect. 2022;150:e160.

156. Lai YJ, Liu SH, Manachevakul S, Lee TA, Kuo CT, Bello D. Biomarkers in long COVID-19: A systematic review. Front Med. 2023 Jan 20;10:1085988.

157. Axelsson GT, Gudmundsson G, Pratte KA, Aspelund T, Putman RK, Sanders JL, et al. The Proteomic Profile of Interstitial Lung Abnormalities. Am J Respir Crit Care Med. 2022 Aug 1;206(3):337–46.

158. Maher TM. Biomarkers for Interstitial Lung Abnormalities: A Stepping-stone Toward Idiopathic Pulmonary Fibrosis Prevention? Am J Respir Crit Care Med.

159. Hunninghake GM, Hatabu H, Okajima Y, Gao W, Dupuis J, Latourelle JC, et al. *MUC5B* Promoter Polymorphism and Interstitial Lung Abnormalities. N Engl J Med. 2013 Jun 6;368(23):2192–200.

160. Calado RT, Young NS. Telomere Diseases. N Engl J Med. 2009 Dec 10;361(24):2353–65.

161. Kahn N, Rossler AK, Hornemann K, Muley T, Grünig E, Schmidt W, et al. C-proSP-B: A Possible Biomarker for Pulmonary Diseases? Respiration. 2018;96(2):117–26.

162. Morais A, Beltrão M, Sokhatska O, Costa D, Melo N, Mota P, et al. Serum metalloproteinases 1 and 7 in the diagnosis of idiopathic pulmonary fibrosis and other interstitial pneumonias. Respiratory Medicine. 2015 Aug;109(8):1063–8.

163. d'Alessandro M, Bergantini L, Cameli P, Vietri L, Lanzarone N, Alonzi V, et al. Krebs von den Lungen-6 as a biomarker for disease severity assessment in interstitial lung disease: a comprehensive review. Biomarkers in Medicine. 2020 Jun;14(8):665–74.

164. Watase M, Mochimaru T, Kawase H, Shinohara H, Sagawa S, Ikeda T, et al. Diagnostic and prognostic biomarkers for progressive fibrosing interstitial lung disease. Subbian S, editor. PLoS ONE. 2023 Mar 17;18(3):e0283288.

165. Bergantini L, Bargagli E, Cameli P, Cekorja B, Lanzarone N, Pianigiani L, et al. Serial KL-6 analysis in patients with idiopathic pulmonary fibrosis treated with nintedanib. Respiratory Investigation. 2019 May;57(3):290–1.

166. Drakopanagiotakis F, Markart P, Steiropoulos P. Acute Exacerbations of Interstitial Lung Diseases: Focus on Biomarkers. IJMS. 2023 Jun 15;24(12):10196.

167. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R. Standardisation of spirometry. European Respiratory Journal. 2005 Aug 1;26(2):319–38.

168. MacIntyre N. Standardisation of the single-breath determination of carbon monoxide uptake in the lung. European Respiratory Journal. 2005 Oct 1;26(4):720–35.

169. Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R, et al. Interpretative strategies for lung function tests. Eur Respir J. 2005 Nov;26(5):948-68. doi: 10.1183/09031936.05.00035205.

170. Stanojevic S, Kaminsky DA, Miller MR, Thompson B, Aliverti A, Barjaktarevic I, et al. ERS/ATS technical standard on interpretive strategies for routine lung function tests. Eur Respir J. 2022 Jul;60(1):2101499.

171. Faverio P, Luppi F, Rebora P, D'Andrea G, Stainer A, Busnelli S, et al. One-year pulmonary impairment after severe COVID-19: a prospective, multicenter follow-up study. Respir Res. 2022 Dec;23(1):65.

172. Van Willigen HDG, Wynberg E, Verveen A, Dijkstra M, Verkaik BJ, Figaroa OJA, et al. One-fourth of COVID-19 patients have an impaired pulmonary function after 12 months of disease onset. Yon DK, editor. PLoS ONE. 2023 Sep 11;18(9):e0290893.

173. Steinbeis F, Thibeault C, Doellinger F, Ring RM, Mittermaier M, Ruwwe-Glösenkamp C, et al. Severity of respiratory failure and computed chest tomography in acute COVID-19 correlates with pulmonary function and respiratory symptoms after infection with SARS-CoV-2: An observational longitudinal study over 12 months. Respiratory Medicine. 2022 Jan;191:106709.

174. Bazdyrev E, Panova M, Zherebtsova V, Burdenkova A, Grishagin I, Novikov F, et al. The Hidden Pandemic of COVID-19-Induced Organizing Pneumonia. Pharmaceuticals. 2022 Dec 16;15(12):1574.

175. Jha M, Gupta R, Saxena R. A Precise Method to Detect Post-COVID-19 Pulmonary Fibrosis Through Extreme Gradient Boosting. SN COMPUT SCI. 2022 Dec 13;4(1):89.

176. Chi Y, Ge Y, Wu B, Zhang W, Wu T, Wen T, et al. Serum Cytokine and Chemokine Profile in Relation to the Severity of Coronavirus Disease 2019 in China. The Journal of Infectious Diseases. 2020 Aug 4;222(5):746–54.

177. Imaeda H, Andoh A, Aomatsu T. A new isoform of interleukin-32 suppresses IL-8 mrna expression in the intestinal epithelial cell line ht-29. Mol Med Rep [Internet]. 2011 Feb 18 [cited 2023 Dec 27]; Available from: http://www.spandidos-publications.com/10.3892/mmr.2011.442

178. Ouhara K, Kawai T, Silva MJB, Fujita T, Hayashida K, Karimbux NY, et al. Expression levels of novel cytokine IL-32 in periodontitis and its role in the suppression of IL-8 production by human gingival fibroblasts stimulated with *Porphyromonas gingivalis*. Journal of Oral Microbiology. 2012 Jan;4(1):14832.

179. Heinhuis B, Plantinga TS, Semango G, Küsters B, Netea MG, Dinarello CA, et al. Alternatively spliced isoforms of IL-32 differentially influence cell death pathways in cancer cell lines. CARCIN. 2016 Feb;37(2):197–205.

180. Rasool ST, Tang H, Wu J, Li W, Mukhtar MM, Zhang J, et al. Increased level of IL-32 during human immunodeficiency virus infection suppresses HIV replication. Immunology Letters. 2008 May;117(2):161–7.

181. Wang J, Wang Q, Han T, Li YK, Zhu SL, Ao F, et al. Soluble interleukin-6 receptor is elevated during influenza A virus infection and mediates the IL-6 and IL-32 inflammatory cytokine burst. Cell Mol Immunol. 2015 Sep;12(5):633–44.

182. Gong L, Liu G, Zhu H, Li C, Li P, Liu C, et al. IL-32 induces epithelial-mesenchymal transition by triggering endoplasmic reticulum stress in A549 cells. BMC Pulm Med. 2020 Dec;20(1):278.

183. Oatis D, Simon-Repolski E, Balta C, Mihu A, Pieretti G, Alfano R, et al. Cellular and Molecular Mechanism of Pulmonary Fibrosis Post-COVID-19: Focus on Galectin-1, -3, -8, -9. IJMS. 2022 Jul 26;23(15):8210.

184. Gujral HS, Sahasrabudhe TR, Nirmala MA. A Systematic Evaluation of Risk Predictors for COVID-19 Sequelae. Cureus [Internet]. 2023 Jun 21 [cited 2024 Jan 6]; Available from: https://www.cureus.com/articles/153778-a-systematic-evaluation-of-risk-predictors-for-covid-19-sequelae

185. d'Alessandro M, Bergantini L, Cameli P, Curatola G, Remediani L, Bennett D, et al. Serial KL-6 measurements in COVID-19 patients. Intern Emerg Med. 2021 Sep;16(6):1541–5.

186. Xue M, Zheng P, Bian X, Huang Z, Huang H, Zeng Y, et al. Exploration and correlation analysis of changes in Krebs von den Lungen-6 levels in COVID-19 patients with different types in China. BST. 2020 Aug 31;14(4):290–6.

187. Awano N, Inomata M, Kuse N, Tone M, Takada K, Muto Y, et al. Serum KL-6 level is a useful biomarker for evaluating the severity of coronavirus disease 2019. Respiratory Investigation. 2020 Nov;58(6):440–7.

188. Frix A, Schoneveld L, Ladang A, Henket M, Duysinx B, Vaillant F, et al. Could KL-6 levels in COVID-19 help to predict lung disease? Respir Res. 2020 Dec;21(1):309.

189. Azekawa S, Chubachi S, Asakura T, Namkoong H, Sato Y, Edahiro R, et al. Serum KL-6 levels predict clinical outcomes and are associated with *MUC1* polymorphism in Japanese patients with COVID-19. BMJ Open Resp Res. 2023 May;10(1):e001625.

190. Kattner S, Sutharsan S, Berger MM, Limmer A, Jehn LB, Herbstreit F, et al. Serum KL-6 as a Candidate Predictor of Outcome in Patients with SARS-CoV-2 Pneumonia. JCM. 2023 Oct 26;12(21):6772.

191. Arnold DT, Donald C, Lyon M, Hamilton FW, Morley AJ, Attwood M, et al. Krebs von den Lungen 6 (KL-6) as a marker for disease severity and persistent radiological abnormalities following COVID-19 infection at 12 weeks. Choi WI, editor. PLoS ONE. 2021 Apr 29;16(4):e0249607.

192. Castellví I, Castillo D, Corominas H, Mariscal A, Orozco S, Benito N, et al. Krebs von den Lungen-6 glycoprotein circulating levels are not useful as prognostic marker in COVID-19 pneumonia: A large prospective cohort study. Front Med. 2022 Aug 8;9:973918.

193. Peng DH, Luo Y, Huang LJ, Liao FL, Liu YY, Tang P, et al. Correlation of Krebs von den Lungen-6 and fibronectin with pulmonary fibrosis in coronavirus disease 2019. Clinica Chimica Acta. 2021 Jun;517:48–53.

194. Xue M, Zhang T, Chen H, Zeng Y, Lin R, Zhen Y, et al. Krebs Von den Lungen-6 as a predictive indicator for the risk of secondary pulmonary fibrosis and its reversibility in COVID-19 patients. Int J Biol Sci. 2021;17(6):1565–73.

195. Sánchez-Díez S, Gómez-Ollés C, Cruz MJ, De Homdedeu M, Espejo D, Ferrer J, et al. Biomarker Profiles Associated with COVID-19 Severity and Mortality. CIMB. 2023 Mar 1;45(3):1998–2012.

196. Davis HE, McCorkell L, Vogel JM, Topol EJ. Long COVID: major findings, mechanisms and recommendations. Nat Rev Microbiol. 2023 Mar;21(3):133–46.

197. Williams ES, Martins TB, Shah KS, Hill HR, Coiras M, Spivak AM, et al. Cytokine Deficiencies in Patients with Long-COVID. J Clin Cell Immunol. 2022;13(6):672. Epub 2022 Nov 18.

198. Silva BSDA, Pereira T, Minuzzi LG, Padilha CS, Figueiredo C, Olean-Oliveira T, et al. Mild to moderate post-COVID-19 alters markers of lymphocyte activation, exhaustion, and immunometabolic responses that can be partially associated by physical activity level— an observational sub-analysis fit- COVID study. Front Immunol. 2023 Sep 11;14:1212745.

199. CDC COVID-19 Response Team, Bialek S, Boundy E, Bowen V, Chow N, et al. Severe Outcomes Among Patients with Coronavirus Disease 2019 (COVID-19) — United States, February 12–March 16, 2020. MMWR Morb Mortal Wkly Rep. 2020 Mar 27;69(12):343–6.

200. Reeves J, Kooner JS, Zhang W. Accelerated ageing is associated with increased COVID-19 severity and differences across ethnic groups may exist. Front Public Health. 2022 Dec 13;10:1034227.

201. Romero Starke K, Reissig D, Petereit-Haack G, Schmauder S, Nienhaus A, Seidler A. The isolated effect of age on the risk of COVID-19 severe outcomes: a systematic review with meta-analysis. BMJ Glob Health. 2021 Dec;6(12):e006434.

202. Duong-Quy S, Vo-Pham-Minh T, Tran-Xuan Q, Huynh-Anh T, Vo-Van T, Vu-Tran-Thien Q, et al. Post-COVID-19 Pulmonary Fibrosis: Facts—Challenges and Futures: A Narrative Review. Pulm Ther. 2023 Sep;9(3):295–307.

203. Herold T, Jurinovic V, Arnreich C, Lipworth BJ, Hellmuth JC, Von Bergwelt-Baildon M, et al. Elevated levels of IL-6 and CRP predict the need for mechanical ventilation in COVID-19. Journal of Allergy and Clinical Immunology. 2020 Jul;146(1):128-136.e4.

204. Bergantini L, Bargagli E, d'Alessandro M, Refini RM, Cameli P, Galasso L, et al. Prognostic bioindicators in severe COVID-19 patients. Cytokine. 2021 May;141:155455.

205. Aziz M, Rawish F, Ragheb A. Elevated interleukin-6 and severe COVID-19: A meta-analysis. J Med Virol. 92(11):2283–5.

206. Vaz De Paula CB, Nagashima S, Liberalesso V, Collete M, Da Silva FPG, Oricil AGG, et al. COVID-19: Immunohistochemical Analysis of TGF-β Signaling Pathways in Pulmonary Fibrosis. IJMS. 2021 Dec 24;23(1):168.

207. Iyer SS, Cheng G. Role of Interleukin 10 Transcriptional Regulation in Inflammation and Autoimmune Disease. Crit Rev Immunol. 2012;32(1):23–63.

208. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, et al. Regulatory T Cell-Derived Interleukin-10 Limits Inflammation at Environmental Interfaces. Immunity. 2008 Apr;28(4):546–58.

209. Rojas JM, Avia M, Martín V, Sevilla N. IL-10: A Multifunctional Cytokine in Viral Infections. Journal of Immunology Research. 2017;2017:1–14.

210. Perlman S, Zhao J. Roles of regulatory T cells and IL-10 in virus-induced demyelination. Journal of Neuroimmunology. 2017 Jul;308:6–11.

211. Xi X, Guo Y, Zhu M, Wei Y, Li G, Du B, et al. Higher expression of monocyte chemotactic protein 1 in mild COVID-19 patients might be correlated with inhibition of Type I IFN signaling. Virol J. 2021 Dec;18(1):12.

212. Park J, Dean LS, Jiyarom B, Gangcuangco LM, Shah P, Awamura T, et al. Elevated circulating monocytes and monocyte activation in COVID-19 convalescent individuals. Front Immunol. 2023 Apr 3;14:1151780.

213. Vollenberg R, Tepasse PR, Fobker M, Hüsing-Kabar A. Significantly Reduced Retinol Binding Protein 4 (RBP4) Levels in Critically Ill COVID-19 Patients. Nutrients. 2022 May 10;14(10):2007.

214. Bergantini L, Mainardi A, d'Alessandro M, Cameli P, Bennett D, Bargagli E, et al. Common Molecular Pathways Between Post-COVID19 Syndrome and Lung Fibrosis: A Scoping Review. Front Pharmacol. 2022 Mar 4;13:748931.

215. Bergantini L, Baldassarri M, d'Alessandro M, Brunelli G, Fabbri G, Zguro K, et al. Ultra-rare RTEL1 gene variants associate with acute severity of COVID-19 and evolution to pulmonary fibrosis as a specific long COVID disorder. Respir Res. 2023 Jun 16;24(1):158.

216. Melani AS, Croce S, Cassai L, Montuori G, Fabbri G, Messina M, et al. Systemic Corticosteroids for Treating Respiratory Diseases: Less Is Better, but… When and How Is It Possible in Real Life? Pulm Ther. 2023 Sep;9(3):329–44.

217. Sun R, Liu H, Wang X. Mediastinal Emphysema, Giant Bulla, and Pneumothorax Developed during the Course of COVID-19 Pneumonia. Korean J Radiol. 2020;21(5):541.

218. Renaud-Picard B, Gallais F, Riou M, Zouzou A, Porzio M, Kessler R. Delayed pulmonary abscess following COVID-19 pneumonia: A case report. Respiratory Medicine and Research. 2020 Nov;78:100776.

219. Sanivarapu RR, Farraj K, Sayedy N, Anjum F. Rapidly developing large pneumatocele and spontaneous pneumothorax in SARS-CoV-2 infection. Respiratory Medicine Case Reports. 2020;31:101303.

220. Ershadi R, Rafieian S, Salehi M, Kazemizadeh H, Amini H, Sohrabi M, et al. COVID-19 and spontaneous pneumothorax: a survival analysis. J Cardiothorac Surg. 2023 Jul 4;18(1):211.

221. Rahil S, Naous A, Naja Z, Rajab M. Pneumatocele-induced pneumothorax after COVID-19 infection in a 45-day-old infant. Radiology Case Reports. 2024 Feb;19(2):737–40.