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Circulating markers of extracellular matrix remodelling in severe COVID-19 patients

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Background. Abnormal remodelling of the extracellular matrix (ECM) has generally been linked to pulmonary inflammation and fibrosis and may also play a role in the pathogenesis of severe COVID-19. To further elucidate the role of ECM remodelling and excessive fibrogenesis in severe COVID-19, we examined circulating levels of mediators involved in various aspects of these processes in COVID-19 patients.

Methods. Serial blood samples were obtained from two cohorts of hospitalised COVID-19 patients ($n = 414$). Circulating levels of ECM remodelling mediators were quantified by enzyme immunoassays in samples collected during hospitalisation and at 3-month follow-up. Samples were related to disease severity (respiratory failure and/or treatment at the intensive care unit), 60-day total mortality and pulmonary pathology after 3-months. We also evaluated the direct effect of inactivated SARS-CoV-2 on the release of the different ECM mediators in relevant cell lines.

Results. Several of the measured markers were associated with adverse outcomes, notably osteopontin (OPN), S100 calcium-binding protein A12 and YKL-40 were associated with disease severity and mortality. High levels of ECM mediators during hospitalisation were associated with computed tomography thorax pathology after 3-months. Some markers (i.e. growth differential factor 15, galectin 3 and matrix metalloproteinase 9) were released from various relevant cell lines (i.e. macrophages and lung cell lines) *in vitro* after exposure to inactivated SARS-CoV-2 suggesting a direct link between these mediators and the causal agent of COVID-19.

Conclusion. Our findings highlight changes to ECM remodelling and particularly a possible role of OPN, S100A12 and YKL-40 in the pathogenesis of severe COVID-19.

Keywords: COVID-19, extracellular matrix, inflammation, lung pathology, SARS-CoV-2

Introduction

Severe COVID-19 is characterised by a sustained and inappropriate immune activation involving a wide range of inflammatory mediators [1, 2]. The degree of immune activation is linked to prognosis, and some inflammatory mediators are potentially directly involved in the pathogenesis of severe COVID-19 [3, 4].

Abnormal extracellular matrix (ECM) remodelling plays an important role in the development of lung pathology during both acute and chronic inflammation [5]. Such processes also appear to be operating in COVID-19 not only within the lungs but also in other organs such as the cardiovascular system [6]. Thus, a potential interaction between ECM remodelling and inflammation, contributing to COVID-19 pathogenesis, could augment disease severity and in particular the development of respiratory failure (RF), at least partly involving mediators of ECM remodelling [7, 8]. Herein, we wanted to study markers that are known to be involved in ECM remodelling in various disorders, but data on COVID-19 are scarce. Thus, although YKL-40 (also known as Chitinase 3-like 1 [CHI3L1]) [9], S100A12 (S100 calcium-binding protein A12 also called ENRAGE) [10], osteopontin (OPN) [11, 12] and cystatin B (CYSB) [13] are all known to be up-regulated in various pulmonary disorders, playing a role in ECM remodelling, fibrogenesis and inflammation, data in COVID-19 patients are scarce or even lacking. For 'comparison', we also included markers that are well known to be involved in ECM remodelling and where there are several publications in COVID-19 disease (>25 publications in PubMed), that is matrix metalloproteinase 9 (MMP-9) [14–16], galectin 3 (GAL-3) [17–19] and growth differentiation factor 15 (GDF-15) [20–22].

To further elucidate the role of ECM remodelling and fibrogenesis in severe COVID-19, we examined serum levels of several mediators involved in these processes (see Table 1 for description and abbreviation) in two cohorts of hospitalised COVID-19 patients in Norway. Circulating levels of these mediators were related to disease severity (i.e. RF and/or the need for admission to the intensive care unit [ICU]) during hospitalisation and 60-day all-cause mortality. Furthermore, few studies have examined changes in mediators of ECM remodelling in relation to lung pathology beyond the acute phase; in addition, we therefore also examined the levels of these markers 3-months after hospital admission in relation to pulmonary pathology at this time point. Finally, we also tested the ability of inactivated SARS-CoV-2 to directly regulate the examined mediators in different cell types with relevance to ECM remodelling.

Table 1. *Abbreviation and description of examined extracellular matrix (ECM) markers.*

Abbreviation	Name and biological function
MMP-9	Metalloprotease 9 is a central regulator of ECM degrading and is also involved in cellular processes such as angiogenesis and inflammation [14]
CYSB	Cystatin B encoded by the *CSTB g*ene. Intracellular cysteine protease (such as papain and cathepsin) inhibitor and expression are increased upon cellular stress. Player in ECM and fibrogenesis [13]
GAL-3	Galectin 3. Encoded by the *LGALS3* gene. Involved in cell-cell adhesion, ECM remodelling, macrophage activation, angiogenesis and apoptosis [17]
OPN	Osteopontin. Encoded by the gene *SPP1*. Pro-fibrotic properties and involved in bone and ECM remodelling, immune regulation, inflammation and apoptosis [11, 12]
S100A12	S100 calcium-binding protein A12. Encoded by the gene *S100A12*. Role in ECM remodelling and inflammation and a potential biomarker for Idiopathic pulmonary fibrosis [10]
YKL-40	Also called Chitinase-3-like protein 1 (CHI3L1). Encoded by the CHI3L1 gene. Involved in ECM remodelling, angiogenesis and inflammation [9]
GDF-15	Growth differentiation factor 15. Member of the TGF-β superfamily involved in ECM remodelling and inflammation in various tissues including the lung and the myocardium [20]

Materials and methods

Study design and participants

In the present study, data from two prospective cohort studies were pooled as previously described [23]. Briefly, Cohort 1 was the NOR Solidarity trial (NCT04321616), a multicentre, open-label, adaptive, randomised controlled trial evaluating the effect of SoC alone or in combination with hydroxychloroquine (HCQ) or remdesivir in COVID-19 patients admitted to 23 Norwegian hospitals, as part of the WHO solidarity trial [24]. Study interventions in these trials did not show significant effects on chosen clinical outcomes or viral clearance [25]. Cohort 2 was the Norwegian SARS-CoV-2 study (NCT04381819), a prospective observational study of COVID-19 patients admitted to five Norwegian hospitals, conducted as part of an International Severe Acute Respiratory and Emerging Infection Consortium (ISARIC), WHO clinical characterization protocol study [26].

In both cohorts, all patients ≥18 years admitted to the hospital with PCR-confirmed SARS-CoV-2 infection were eligible for inclusion. One–three sets of blood samples were obtained from each patient within 48 h of admission and up to 10 days during hospitalisation. Blood samples were also collected in a subset of patients at a 3-month follow-up.

Patients in Cohort 1 were included between March 28 and October 5, 2020, whereas patients in Cohort 2 between March 10, 2020 and September 1, 2021. Hence, the collected study period spanned the first three waves of the COVID-19 pandemic in Norway; as of February 2021, alpha was the dominating variant, replaced by the delta variant in July 2021.

In Cohort 1 ($n = 162$), participants were randomized and allocated to one of three treatment arms: (i) local SoC; (ii) SoC plus 800 mg of oral HCQ twice daily on day 1, then 400 mg twice daily up to 9 days; or (iii) SoC plus 200 mg of intravenous remdesivir on day 1, then 100 mg daily up to 9 days. All study treatments were terminated at hospital discharge or before. As the interventions did not affect the outcome (viral clearance or systemic inflammation) [25, 27], data from the different intervention arms were pooled together with samples from Cohort 2 ($n = 252$), in this sub-study.

Outcomes

Plasma/serum levels of ECM mediators were related to disease severity as assessed by (i) RF defined as pO_2/FiO_2–(P/F-ratio) <26.6 kPa (<200 mmHg) and/or the need for admission to the ICU during hospitalisation and (ii) 60-day post-admission all-cause mortality. In addition, plasma levels of these ECM markers during hospitalisation and after 3-months were related to pulmonary pathology at a 3-month follow-up.

Pulmonary testing

In Cohort 1, lung function tests ($n = 90$), consisting of spirometry and diffusion capacity of the lungs for carbon monoxide (DLCO), were performed as previously described [28]. DLCO was selected as a measure of pulmonary function as DLCO has been shown to be most frequently affected after hospitalisation for COVID-19. DLCO percentage of predicted value and the lower limit of normal (LLN) were calculated according to the Global Lung Function Initiative Network. Persistent respiratory dysfunction was defined as DLCO below LLN. Additionally, low-dose, thin-section chest computed tomography (CT) images were obtained ($n = 113$) as described in Ref. [28]. We assessed the prevalence of any ground-glass opacities >10% in at least one of the four lung zones, together with any mosaic pattern and grouped these findings together as 'potentially reversible changes'. Any consolidations, reticular patterns, parenchymal bands, interlobular septal thickening or any bronchiectasis were interpreted as 'potentially irreversible changes' and grouped together. Changes that were classified as potentially irreversible were interpreted to reflect fibrosis. Reversible changes were interpreted to reflect ongoing inflammation.

Ethics

The studies were approved by the Committee for Medical Research Ethics Region Southeast Norway (Cohort 1: approval no. 118684 (13.03.2020); Cohort 2: approval no. 106624 (13.02.2020)), and both studies were performed according to the Declaration of Helsinki. The study in Cohort 1 was also approved by the Norwegian Medicines Agency (20/04950-23). All participants gave informed consent prior to inclusion, either directly or through a legally authorised representative.

Blood sampling protocol and routine biochemical analyses

Peripheral venous blood was drawn into pyrogen-free blood collection tubes without any additives (serum, Cohort 2) or with EDTA anticoagulant (plasma, Cohort 1). Plasma was immediately immersed in melting ice and centrifuged at 2500*g* for 20 min within 30 min after sample collection to obtain platelet-poor plasma. Serum samples were allowed to clot at room temperature before centrifugation (1500*g* for 10 min). Serum and plasma were aliquoted and stored at −80°C until analysis and thawed <3 times.

Routine laboratory variables (i.e. C-reactive protein [CRP], ferritin and total leukocyte, neutrophil, lymphocyte and monocyte counts and creatinine/estimated glomerulus filtration rate [eGFR]) were measured at the biochemical laboratories at the participating hospitals.

Cell experiments. Alpha SARS-CoV-2 variant (SARS-CoV-2 strain 2019-nCoV/Italy-INMI1), originally obtained from the European Virus Archive, was used. High-titre virus stocks were made by infecting confluent Vero E6 cells (Cat. No. CRL-1586; American Type Culture Collection) with 0.01 multiplicity of infection (MOI) SARS-CoV-2 in Dulbecco's Modified Eagle Medium (Cat. no. D6429; Merck) with 2% foetal calf serum (FCS; Cat. no. F7524, Merck) 1% L-glutamine (Merck), and 1% Pen/Strep (Thermo Fischer) in 175 cm^2 cell culture flasks. The cells were monitored daily for cytopathic effect under an inverted microscope. Upon nearly complete cell lysis (96 h post-infection), the viral supernatant was harvested, aliquoted and stored at −80°C. The virus was titrated by calculating the 50% tissue culture infectious dose. The virus was heat inactivated at 65°C for 30 min [29].

In a representative functional assay, immortalized human alveolar epithelial (A549, ATTC CCL-185, ATCC), bronchial epithelial (BEAS-2B, ATTC CRL-9609, ATCC) cells and macrophages derived from phorbol-12-myristate-13-acetate (PMA) differentiated monocytes (THP-1, ATTC TIB-202, ATCC) were cultured in the presence of inactivated SARS-CoV-2 at 37°C, 5% CO_2 in RPMI 1640 medium without 2% FCS. Exposed cells were all exposed to one MOI inactivated SARS-CoV-2, implying that on average, there is a 1:1 relationship between viral particles and host cells. After 24 h, cell-free supernatants were harvested and stored at −80°C until further analyses.

Enzyme immunoassay (EIA)

Soluble levels of MMP-9, CYSB, YKL-40, GAL-3, OPN, GDF-15 and S100A12 were measured in duplicate by enzyme immunoassay (EIA) using commercially available antibodies (R&D Systems) in a 384-format using a combination of an SELMA pipetting robot and a BioTek dispenser/washer. Absorption was read at 450 nm with wavelength correction set to 540 nm using an EIA plate reader (BioTek). The intra-assay coefficient of variation based on data from our laboratory was <10%. For reference, the actual markers were also analysed in plasma from 24 age- and sex-matched healthy controls (mean age ± SD 55 ± 12). For all markers, excluding MMP-9, median levels in the population with plasma (Cohort 1) and serum (Cohort 2), were within 5% difference, and no strategy was utilized to harmonize these levels further. For MMP-9, which is abundant in blood platelets and released upon coagulation, levels were ∼100 times higher in serum. As the populations were demographically comparable (Table 2), and the results derived from the other markers were comparable, we hence harmonized levels by assuming that median levels would be similar. Accordingly, the levels in Cohort 2 were standardized to the levels in Cohort 1.

Publicly available datasets

To assess gene expression across cell types in COVID-19 lung tissue, we accessed publicly available single-cell RNA-seq atlases (GSE162911, $n = 9$ [30] and GSE171524, $n = 19$ [31]). Using the 'explore' function on their interactive website [32, 33], we were able to visualize gene expression of the queried genes of interest (MMP-9, CYSB, S100A12, OPN, GAL-3, GDF-15 and YKL-40).

Statistical and bioinformatic methods

Patient characteristics were compared using Student's *t*-test or Mann–Whitney *U*-test depending on the distribution or chi-square for continuous and categorical variables.

Circulating levels of ECM mediators were non-normally distributed and thus transformed using log10 for temporal comparisons between groups in relation to outcome with linear mixed model analysis. Linear mixed models were performed using

Table 2. *Demographic, clinical and biochemical characteristics in 414 patients hospitalised for COVID-19, stratified by two large multi-centre cohorts in Norway.*

Parameter	Cohort 1 ($n = 162$)	Cohort 2 ($n = 252$)	Combined ($n = 414$)
Age, years	59.7 ± 15.4	57.0 ± 15.3	58.0 ± 15.4
Male gender (%)	103 (64)	159 (63)	262 (63)
Body mass index, kg/m^2	28.2 ± 4.6	28.8 ± 5.2	28.5 ± 4.9
Treatment group			
Standard of care (SOC) (%)	81 (50)	254 (100)	335 (80)
SOC + hydroxychloroquine (%)	43 (27)	0 (0)	43 (10)
SOC + remdesivir (%)	38 (24)	0 (0)	38 (9)
Vaccinated ≥1 dose (%)	0 (0)	6 (2)	6 (1)
Dexamethasone (%)	2 (1)	134 (53)*	136 (33)
Oxygen therapy (%)	91 (56)	194 (77)*	285 (69)
Comorbidities			
Chronic cardiac disease (%)	24 (15)	47 (19)	71 (17)
Hypertension (%)	51 (32)	84 (35)	135 (34)
Chronic pulmonary disease (%)	31 (20)	67 (27)	98 (24)
Obesity (%)	43 (29)	70 (28)	113 (28)
Diabetes (%)	27 (17)	58 (25)	85 (22)
Current smoker (%)	5 (4)	16 (7)	21 (6)
HIV (%)	8 (5)	2 (1)	10 (3)
Outcomes			
ICU admission (%)	31 (19)	79 (31)*	110 (27)
Respiratory failure (%)	50 (31)	75 (30)	125 (31)
60-Day death (%)	8 (5)	23 (9)	31 (8)
P/F-ratio at admission (kPa)	42.4 (32.4,49.6)	40.0 (28.1,48.3)	41.3 (30.0,49.3)
Haemoglobin (g/dL)	13.2 ± 1.5	12.9 ± 1.8	13.0 ± 1.7
C-reactive protein (mg/L)	70 (35,136)	53 (24,117)	62 (29,125)
Ferritin (μg/L)	612 (358,1111)	617 (297,1146)	615 (322,1127)
White blood cell count ($\times 10^9$/L)	6.5 ± 2.8	6.9 ± 3.2	6.7 ± 3.1
Neutrophils ($\times 10^9$/L)	4.8 ± 2.7	5.3 ± 3.1	5.1 ± 3.0
Lymphocytes ($\times 10^9$/L)	1.2 ± 0.53	1.1 ± 0.5	1.1 ± 0.5
eGFR	87 ± 25	90 ± 29	89 ± 2

Abbreviations: eGFR, estimated glomerular filtration rate; ICU, intensive care unit; P/F-ratio, PaO_2/FiO_2-ratio; SOC, standard of care.

outcome (i.e. RF/ICU or death) as the dependent variable and subject as a random effect. Time and RF or ICU admission in addition to the adjustment variables (RF/ICU admission: age, sex, obesity, COVID-19 wave, dexamethasone treatment, randomised treatment, vaccination status and CRP levels) were set as covariates. Similar models were used for evaluating the effects of randomised treatment and DLCO and chest CT in Cohort 1 (adjusting for randomised treatment, age, sex and neutrophil counts), and 'COVID-19 wave' or dexamethasone was used in Cohort 2 (adjusting for age and sex) as adjustment variables.

The association between admission levels of ECM mediators and 60-day all-cause mortality was first assessed by receiver operating characteristic (ROC) analysis. The association with 60-day all-cause mortality was assessed by Kaplan–Meier analysis according to a cut-off identified by Youden's index and Cox regression analysis. For analysis of 60-day mortality in an adjusted Cox-regression model, we made propensity scores due to lower outcome numbers ($n = 37$) based on demographics and biochemistry in Table 2, right part: Model 1 (M1) included age, sex, obesity, COVID-19 wave, treatment modalities, vaccination status, oxygen

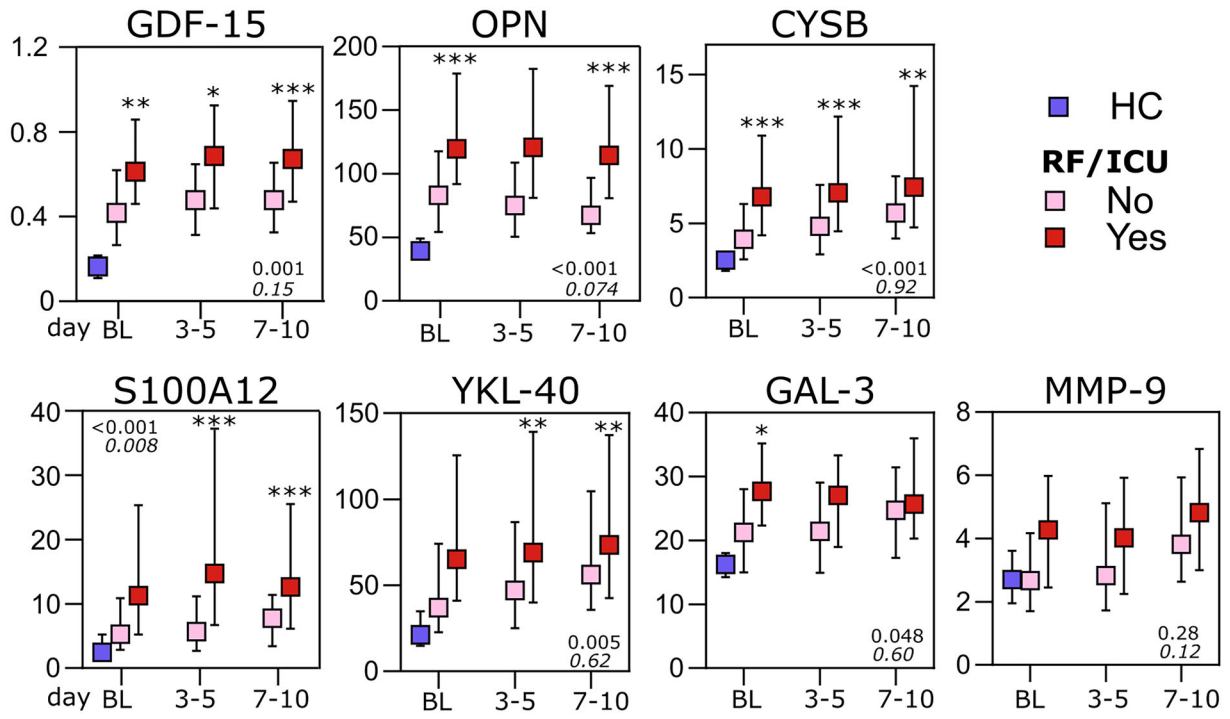

Fig. 1 *Circulating extracellular matrix (ECM) markers are increased in COVID-19. The temporal profile of circulating ECM mediators relates to a combined outcome of respiratory failure and intensive care unit (ICU) admission. Data are shown as median and 25th and 75th percentiles. The p-values reflect the group (outcome) effect, and group*time (italic) from the linear mixed models adjusted for age, sex, obesity, COVID wave, dexamethasone treatment, randomised treatment, vaccination status and C-reactive protein (CRP) levels. *p < 0.05, **p < 0.01, ***p < 0.001 between groups. The x-axis represents the different time points throughout the study. Concentrations are presented as ng/mL on the y-axis. BL, baseline; CYSB; cystatin B; GAL-3, galectin 3; GDF-15, growth differential factor 15; HC, healthy controls; MMP-9, matrix metalloproteinase 9; OPN, osteopontin; S100A12, S100 calcium-binding protein A12.*

therapy and comorbidities (chronic pulmonary and cardiac disease). M2 includes M1 + CRP, neutrophil counts and eGFR. A similar adjustment was applied for assessing the temporal profile of markers according to survival status in mixed models.

Statistics on clinical data were performed using SPSS version 29.0.0.0, whereas *in vitro* data were analysed with GraphPad Prism 8.3.0, which was in combination with Inkscape 0.92.4 used for the presentation of genes involved in ECM remodelling and regulation.

Results

Baseline characteristics of the study population

Demographics and clinical characteristics of the two cohorts were mostly comparable (Table 2), apart from the use of antiviral agents in Cohort 1, and dexamethasone used in Cohort 2, which was introduced as SoC in the management of severe COVID-19 in autumn 2020. In the combined cohort, 125 of 414 (31%) patients developed RF, whereas 110 (27%) were admitted to an ICU (Table 2). Sixty-six patients (22%) with RF did not receive treatment in an ICU. Thus, a total of 176 patients (45%) were classified as severe, as they were admitted to an ICU and/or developed RF during hospitalisation.

Temporal profile of ECM remodelling mediators in relation to disease severity during hospitalisation

Figure 1 shows the temporal profile of circulating ECM mediators in relation to the combined endpoint of RF and/or ICU admission after adjustment for age, sex, obesity, COVID-19 wave, dexamethasone treatment, randomised treatment, vaccination status and CRP levels. Except for GAL-3 and MMP-9, mediators of ECM remodelling

(i.e. GDF-15, OPN, CYSB, YKL-40 and S100A12) were significantly higher at two or more time points in those with the most severe disease (ICU admission and/or RF), as compared with those with moderate disease. However, although GDF-15, OPN and CYSB showed high levels at baseline with only minor changes throughout hospitalisation, YKL-40 and S100A12 showed increasing levels during hospitalisation.

In general, there were no differences according to treatment modalities in Cohort 1 (SoC with or without HCQ and remdesivir, Table S1). In contrast, patients who received dexamethasone in Cohort 2 had higher levels of GAL-3, OPN, S100A12 and MMP-9 compared to those who did not receive dexamethasone during hospitalisation, potentially reflecting more severe disease in these patients.

High levels of ECM mediators are associated with 60-day total mortality

In the combined cohort, 31 of 414 patients died within 60 days of hospital admission (Table 2). The ROC analysis of admission levels of ECM mediators (Fig. 2a) and Kaplan–Meier according to dichotomized admission levels of ECM mediators (Fig. 2b) showed that all measured markers of ECM remodelling were associated with 60-day total mortality. Cox regression model of admission levels (Fig. 2c) and of temporal circulating ECM remodelling mediators' profile during the first 10 days of hospitalisation (Fig. 2d) according to 60-day mortality show that baseline levels of all examined markers were associated with 60-day total mortality. However, when considering the temporal profile, only OPN, S100A12 and YKL-40 remained significantly increased in patients that experienced total 60-day mortality after accounting for comorbidities (chronic pulmonary and cardiac disease), and other factors potentially affecting the secretion of circulating markers (age, sex, obesity, COVID-19 wave, treatment modalities, vaccination status, oxygen therapy, CRP, neutrophil counts and eGFR), in a fully adjusted model. Numerical values for the hazard ratios and 95% confidence intervals are shown in Table S2.

Infected lung tissue shows increased ECM remodelling

To examine the expression levels of the analysed plasma circulating markers of ECM remodelling in relation to cell types in pulmonary tissue during ongoing SARS-CoV-2 infection, we accessed the publicly available single-cell lung atlas from COVID-19 patients (GSE162911, $n = 9$ [30] and GSE171524, $n = 19$ [31]) (Fig. 3). Differential expression of several ECM mediators was observed. First, GAL-3 (*LGALS3*), OPN (*SSP1*) and CYSB (*CSTB*) were strongly expressed in macrophages and to a lesser degree in monocytes. Second, GAL-3 was also expressed in fibroblasts, as well as in types 1 and 2 alveolar cells. A similar expression pattern in alveolar cells was seen for CYSB. Third, GDF-15 was expressed in types 1 and 2 alveolar cells and to a lesser degree in fibroblasts. Fourth, YKL-40 (*CHI3L1*) was moderately expressed in type 2 alveolar cells. Finally, MMP-9 and S100A12 did not show any noteworthy expression levels in any of the cell subsets assayed. In general, the same expression pattern was seen in both cohorts (i.e. GSE162911 and GSE171524).

In vitro *secretion of ECM mediators after exposure to inactivated SARS-COV-2 virus*

To explore the possibility of a direct effect of SARS-CoV-2 on secretion of the identified ECM mediators, we tested whether inactivated SARS-CoV-2 could impact the release of these proteins in relevant cell lines (Fig. 4). In the macrophage cell line (PMA differentiated THP-1 monocytes), the addition of SARS-CoV-2 increased the levels of GAL-3 and MMP-9 with a minor decrease in YKL-40 secretion. In the two lung cell lines (the alveolar cell line A549 and the bronchial cell line BEAS-2B), we found increased secretion of GDF-15 without any major changes in the other markers. In fact, the decreased secretion of YKL-40 and CYSB in the alveolar cell line (A549), but not in bronchial cells (BEAS-2B), was observed following stimulation with SARS-CoV-2. OPN secretion was not affected by inactivated SARS-CoV-2 in any of our cell experiments. No release of S100A12 was observed in any of the cell line experiments.

ECM markers during hospitalisation and at 3-month follow-up in relation to persistent pulmonary pathology

We next examined if plasma markers of ECM remodelling during hospitalisation were associated with pulmonary pathology as assessed by pulmonary functional testing (i.e. DLCO below LLN) and chest CT findings (i.e. potential reversible and irreversible CT pathology) 3-months after hospital admission (Fig. 5). During hospitalisation, high S100A12, YKL-40 and CYSB levels at two or more time points were associated with reversible CT changes. Moreover, patients with reversible changes at 3-months expressed

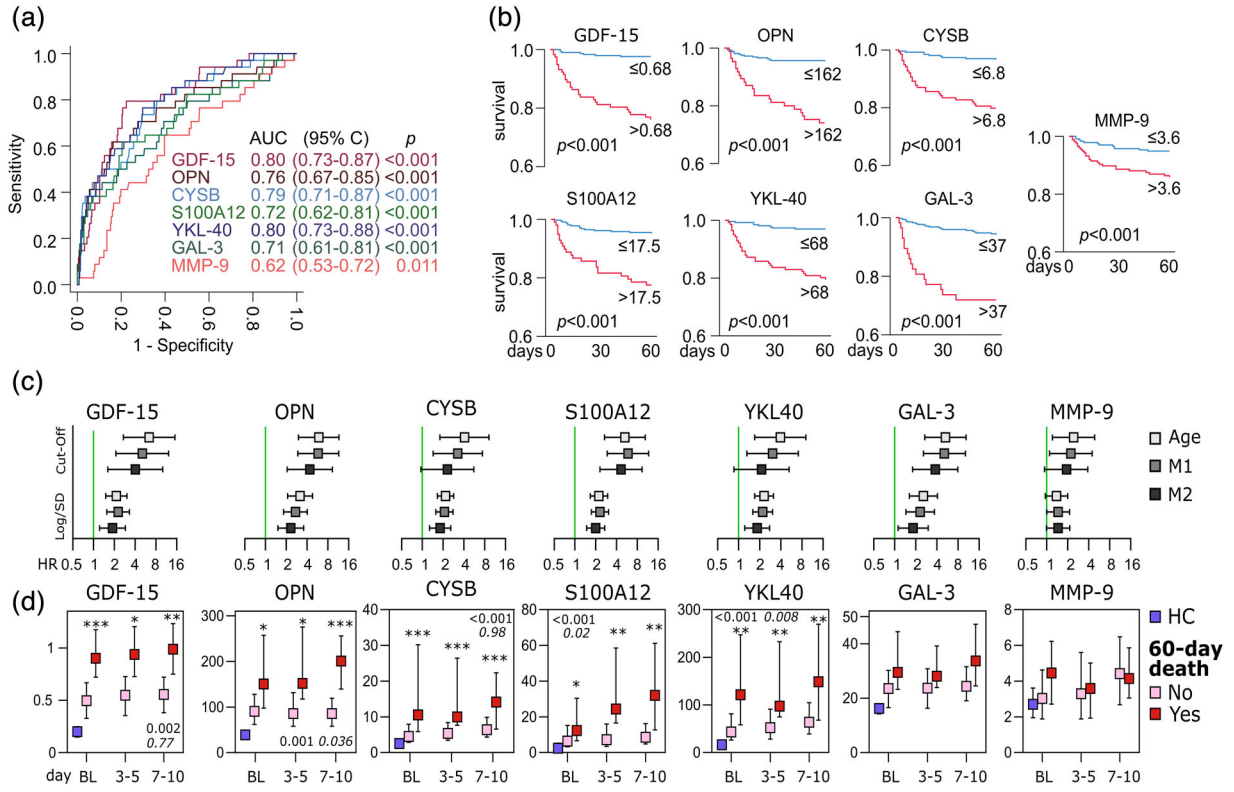

Fig. 2 *Increased 60-day mortality with increased extracellular matrix (ECM) remodelling. ECM mediators and 60-day mortality in severe COVID-19. (a) Receiver operating characteristic (ROC) analysis of admission levels of ECM markers in relation to 60-day mortality, (b) Kaplan–Meier analysis of 60-day mortality (n = 37) according to dichotomized admission levels of ECM markers (cut-offs determined by Youden's index). Log-rank p-values are shown (c). Cox regression of admission levels of ECM mediators (dichotomized according to Youden's index as in B (cut-off) and normalized log-values (Log/SD)) shown as hazard ratios and 95% confidence intervals (Table S1) in relation to 60-day mortality with different levels of adjustment (Model 1 [M1] includes age, sex, obesity, COVID-19 wave, treatment modalities, vaccination status, oxygen therapy, comorbidities [chronic pulmonary and cardiac disease]; M2 includes C-reactive protein [CRP], neutrophil counts and estimated glomerulus filtration rate [eGFR]). (d) Temporal profile of ECM mediators during the first 10 days after admission according to 60-day mortality shown as median and 25th and 75th percentile. The p-values reflect the group (60-day death) effect and group × time (italic) from the linear mixed models with similar adjustment as C. *p < 0.05, **p < 0.01, ***p < 0.001 between deceased groups. Concentrations are presented as ng/mL. BL, baseline; CYSB, cystatin B; GDF-15, GAL-3, galectin 3; growth differential factor 15; HC, healthy controls; MMP-9, matrix metalloproteinase 9; OPN, osteopontin; S100A12, S100 calcium-binding protein A12.*

significantly higher levels of GDF-15 and OPN at the tail of hospitalisation. None of the markers were associated with impaired DLCO.

Three months after discharge from the hospital (patient characterization Table S3), only CYSB was elevated compared to healthy controls. However, although CYSB levels tended to be higher in those with reduced DLCO and CT pathology, neither CYSB nor any of the other markers at 3-month follow-up were significantly related to pulmonary pathology (DLCO < LLN or pathological CT-findings) (Table S4).

Discussion

In the present study, we show that selected circulating mediators of ECM remodelling are increased during hospitalisation in patients with severe COVID-19. Several studies have highlighted ECM remodelling as an important part of COVID-19 pathogenesis, and in particular, MMP-9 has

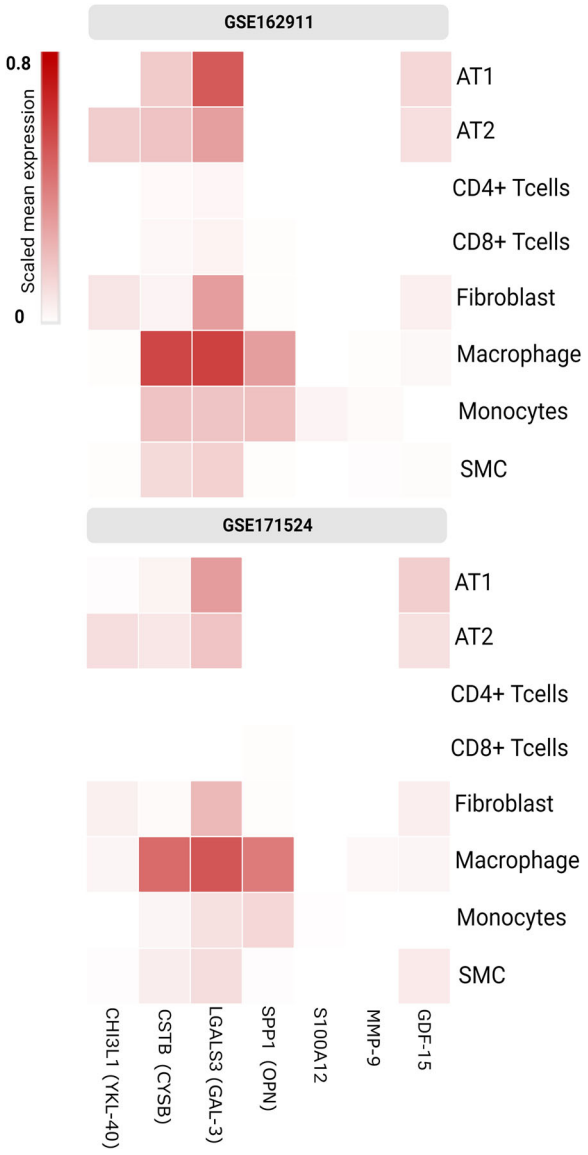

Fig. 3 *Extracellular matrix (ECM) remodelling in lung tissue and relevant cell types. Cell-specific gene expression of circulating ECM remodelling markers (matrix metalloproteinase 9 [MMP-9], growth differentiation factor 15 [GDF-15], S100 calcium-binding protein A12 [S100A12], cystatin B [CYSB] [CSTB], YKL-40 [CHI3L1], osteopontin [OPN] [SPP1], galectin 3 [GAL-3] [LGALS3]) in relevant lung cells are evaluated with two publicly available single-cell lung atlas (GSE171524 and GSE162911, total n = 28). Colour represents the scaled mean expression within a cell population (i.e. mean expression scaled by total gene expression). AT, alveolar type; SMC, smooth muscle cell.*

correlated with adverse outcomes [7, 8, 34]. However, although we found MMP-9 modestly associated with severe disease during hospitalisation, OPN, S100A12 and YKL-40 were associated with disease severity during hospitalisation (RF and/or treatment at ICU) and 60-day mortality, also in the fully adjusted model. High levels of these markers during hospitalisation were also associated with pathological findings on chest CT 3-months after hospital admission. Our findings underscore a role for mediators of ECM remodelling, in particular; OPN, S100A12 and YKL-40, in the pathogenesis of COVID-19 disease.

OPN, S100A12 and YKL-40 have been implicated in the pathogenesis of various pulmonary and vascular disorders [3, 35, 36]. In a recent study of 102 hospitalised COVID-19 patients, Schoneveld *et al.* found that YKL-40 levels were associated with the need for ICU treatment [37]. In addition, YKL-40 has recently been associated with mortality during hospitalisation in 89 COVID-19 patients [38]. Moreover, in a proteome analysis in bronchial alveolar lavage in five COVID-19 patients, YKL-40 and S100A12 were 2 of the 41 proteins that were up-regulated as compared with non-COVID-19 patients [35]. Most recently, Tonello *et al.* showed that high OPN levels at admission predicted adverse outcomes during hospitalisation in 189 COVID-19 patients [39], and Hayek *et al.* have previously shown that higher OPN levels are associated with increased odds of mechanical ventilation and death in 348 COVID-19 patients [40]. As for S100A12, data on serum/plasma levels in COVID-19 patients are scarce. However, in proteome analyses of pulmonary tissue in COVID-19 patients, S100A12 abundance was correlated and associated with inflammation severity [41]. Moreover, in RNA sequencing data from whole blood in 47 COVID-19 patients, S100A12 expression at hospital admission was robustly correlated with quantitative indexes of disease severity and outcome [42]. In the present study, we extend these findings in several ways. We included a larger number of patients than most previous studies and show associations not only with disease severity during hospitalisation but also with 60-day mortality, also after adjustment for multiple confounders. Moreover, high levels of OPN, S100A12 and YKL-40 during hospitalisation were associated with pathological findings on chest CT 3-months after hospital admission, suggesting induction of persistent pulmonary pathology. However, follow-up studies have shown a reduction in the

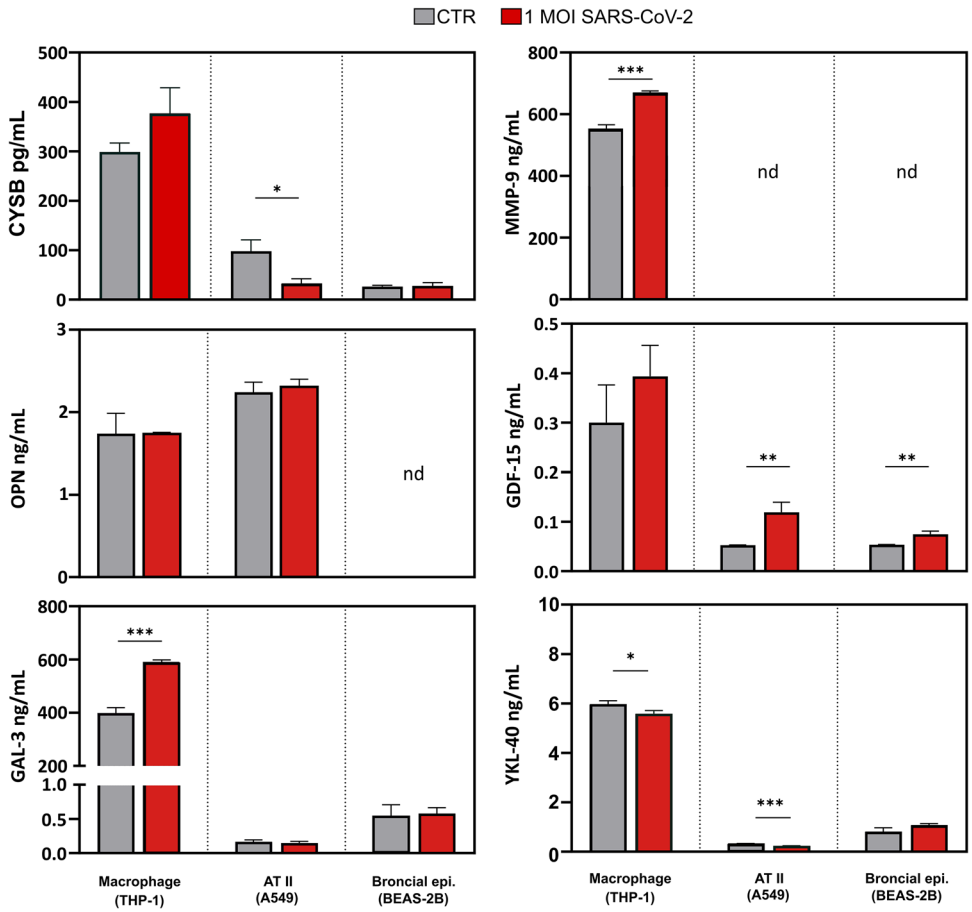

Fig. 4 *SARS-CoV-2 induced secretion of extracellular matrix (ECM) mediators and possible immune activation. Secretion of ECM mediators in macrophages (THP-1), alveolar type II cells (A549) and bronchial epithelial cells (BEAS-2B) after exposure to 1 multiplicity of infection (MOI) inactivated SARS-CoV-2 virus, n = 3. AT II, alveolar type 2; CYSB, cystatin B; CTR, unexposed controls; GAL-3, galectin 3; MMP-9, matrix metalloproteinase 9; Nd, not detectable; OPN, osteopontin; S100A12, S100 calcium-binding protein A12.*

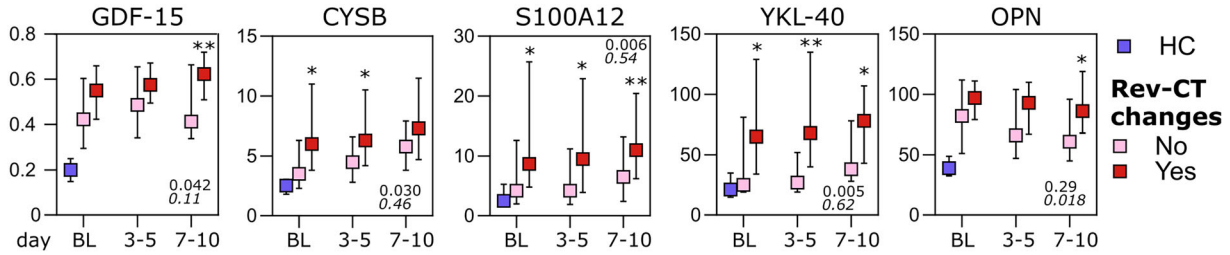

Fig. 5 *Extracellular matrix (ECM) markers in relation to pulmonary pathology at follow-up. Temporal profile of ECM markers during the first 10 days after admission according to reversible computed tomography (CT) changes at 3-month follow-up. The p-values reflect the group (reversible CT changes) effect and group*time interaction (italic) from the linear mixed models adjusting for randomized treatment, age, sex, randomized treatment and neutrophil counts. BL, baseline and HC, healthy controls. *p < 0.05, **p < 0.01, between groups. The x-axis represents the different time points throughout the study. Concentrations are presented as ng/mL on the y-axis.*

prevalence of such findings on chest CT from 3 to 12-months after COVID-19 [43]. Interestingly, YKL-40 (CHI3L1) have been shown to stimulate the expression of angiotensin-converting enzyme 2 and host proteases implicated in SARS-CoV-2 infectivity [44], and YKL-40 inhibition augmented SARS-CoV-2 infection in epithelial cells, including infection with omicron variants [45]. OPN expression is closely linked to fibrosis, and its expression in macrophages is indicative of a pro-fibrotic profile [31, 46]. With relevance to severe COVID-19 disease [40], OPN has also been related to pulmonary arterial hypertension, at least partly involving its effects on vascular remodelling [47]. In relation to S100A12, transcriptional reprogramming of infiltrating S100A12-expressing mature neutrophils seems to promote persistent and self-sustaining pulmonary neutrophilia with features of acute respiratory distress syndrome despite low viral load in the airways in COVID-19 patients [48]. Thus, although our findings suggest that OPN, S100A2 and YKL-40 are independently associated with disease severity in hospitalised COVID-19 patients, these molecules could potentially also be involved in the pathogenesis of severe COVID-19 by promoting persistent pulmonary pathology and may thus represent novel therapeutic targets.

Existing data on CYSB in COVID-19 are, to the best of our knowledge, scarce or even lacking. Here, we show that CYSB was persistently elevated in patients with severe disease as compared with those with moderate disease during hospitalisation and is associated with 60-day total mortality, although not significantly in the fully adjusted model. Moreover, although not related to persistent CT or pulmonary pathology at the 3-month follow-up, CYSB was the only mediator that was elevated at this time point. CYSB is an endogenous cysteine cathepsin inhibitor localized in the cytosol, mitochondria and nucleus and has been implicated in the pathogenesis of various pulmonary disorders such as sarcoidosis [49, 50]. Further, in neuroinflammatory diseases, CYSB deficiency has been shown to enhance inflammatory responses [51]. However, whether the persistently elevated CYSB in COVID-19 patients reflects a compensatory mechanism or if it is involved in maladaptive pulmonary remodelling is at present not clear.

Through our current examination, it was revealed that inactivated SARS-CoV-2 exposure *in vitro* resulted in increased release of certain ECM remodelling markers. GDF-15 was increased in those with the most severe disease, as also suggested by others [52], and showed enhanced release from SARS-CoV-2 exposed alveolar and bronchial cell lines. Our findings suggest that some of the studied ECM markers could be directly induced by the virus and not only induced secondary to systemic inflammation and immune activation. Based on publicly available single-cell lung atlas from COVID-19 patients, we also showed the expression of several of these mediators within infected pulmonary tissue from COVID-19 patients further supporting such a notion. Somewhat surprisingly, SARS-CoV-2 down-regulated the release of YKL-40 and CYSB in the alveolar type 2 cell line. Although significant, the levels of YKL-40 in this cell line were in general very low. One could speculate that the early inflammatory response following SARS-CoV-2 infection *in vitro*, and potentially also *in vivo*, as assessed by CYSB and YKL-40 levels, could be decreased as compared with the late-stage in fulminant COVID-19 disease as in hospitalised patients. However, in general, we believe that the *in vitro* data on CYSB and in particular YKL-40 should be interpreted with caution.

There are certain limitations inherent in this study. Although we included samples from the first three waves of COVID-19 in Norway and found no association with vaccination status, we lack data on new virus variants that have dominated during the recent 1–2 years in a fully vaccinated population. Moreover, although we show the expression of some of molecules in pulmonary tissue from COVID-19 patients, the serum/plasma data may not necessarily reflect the situation within the infected, inflammatory and fibrotic lung tissue. In line with this, correlations do not necessarily prove any causal relationship in relation to pathogenic importance. Moreover, although the levels of the actual markers were mostly similar in serum and plasma (except for MMP-9), the use of different blood samples (serum vs. platelet-poor plasma) in the two different cohorts is also a limitation of the study.

Herein we show that markers of ECM remodelling and in particular, GDF-15, OPN, S100A12 and YKL-40, are associated with severe disease including 60-day all-cause mortality in hospitalised COVID-19 patients, as well as with CT thorax pathology 3-months after hospitalisation. This pattern seemed not to be modified by

dexamethasone indicating the need for more targeted therapy in these patients. However, larger and more mechanistic studies that also include samples from the site of infection are needed to fully understand the role of these ECM mediators in COVID-19.

Author contributions

Tuva Børresdatter Dahl, Sarah Louise Murphy, Bente Halvorsen, Thor Ueland, Pål Aukrust and Andreas Barratt-Due were responsible for the study conception and execution of the present sub-study and securing the financial support. Andreas Barratt-Due, Bente Halvorsen, Anne Ma Dyrholt-Riise, Pål Aukrust, Tuva Børresdatter Dahl, Camilla Huse, Hedda Hoel, Sarah Louise Murphy, Annika E Michelsen, Thor Ueland, Anders Tveita, Lars Heggelund and Marius Trøseid were responsible for the management, coordination, research activity planning and execution of the NOR-solidarity trial. Jan Cato Holter, Anne Ma Dyrholt-Riise, Lars Heggelund, Anders Benjamin Kildal, Kristian Tonby, Beathe Kiland Granerud, Susanne Dudman, Simen Bøe, Andreas Lind and Aleksander Rygh Holten were responsible for the management, coordination, research activity, planning and execution of the Norwegian SARS-CoV-2 study. Tøri Vigeland Lerum, Ole Henning Skjønsberg and Trond M Aaløkken were responsible for the 3-month follow-up protocol for pulmonary function and CT scan. Tuva Børresdatter Dahl, Andreas Barratt-Due, Bente Halvorsen, Pål Aukrust, Anne Ma Dyrholt-Riise, Hedda Hoel, Camilla Huse, Beathe Kiland Granerud and Jan Cato Holter coordinated the collection and storage of the biobank material. Tuva Børresdatter Dahl, Thor Ueland, Annika E Michelsen and Sarah Louise Murphy were responsible for the molecular – and/or bioinformatic analyses of ECM markers. Sarah Louise Murphy, Tuva Børresdatter Dahl; Bente Halvorsen, Pål Aukrust and Thor Ueland drafted the manuscript. All authors revised and approved the final version of the manuscript.

Conflict of interest statement

Within 36-months prior to this publication, A.R.H received payment for lectures on Acute Medicine by Pfizer. L.H has participated on a Data Safety Monitoring Board or Advisory Board received personal funds from Pharma Holdings AS and holds minor stocks in AlgiPharma AS. K.T. holds a role of unpaid presidency for the Norwegian Society of Infectious Diseases. J.C.H. received a philanthropic donation from Vivaldi Invest AS. The authors S.L.M., H.H., B.H., A.B.D., A.T., O.H.S., B.K.G., A.E.M., T.B.D., C.H., M.T., T.M.A., A.L., S.D., A.B.K., T.V.L., A.M.D.R., T.U. and S.B. declare no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1: Circulating levels of ECM markers presented in ng/mL during hospitalisation according to treatment modalities over time.
Table S2: Association between baseline levels of circulating ECM markers and 60-day survival in hospitalised COVID-19 patients.
Table S3: Admission demographic, clinical, and biochemical characteristics in 113 patients who assessed pulmonary function and/or chest CT at 3-month follow-up, stratified according if they had DLCO<LLN or reversible CT changes.
Table S4: Circulating levels of ECM markers (ng/mL) at 3 months according to pulmonary pathology at 3 months follow-up. ■