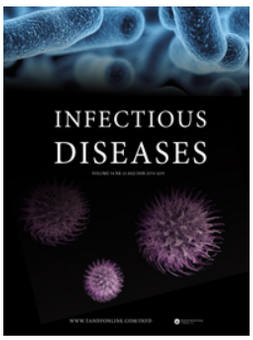

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BRIEF REPORT

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Markers of cellular senescence is associated with persistent pulmonary pathology after COVID-19 infection

Tove Lekva[a], Thor Ueland[a,b,c], Bente Halvorsen[a,b], Sarah Louise Murphy[a,b], Anne Ma Dyrhol-Riise[b,d], Anders Tveita[b,e], Ane-Kristine Finbråten[f], Alexander Mathiessen[g], Karl Erik Müller[h], Trond Mogens Aaløkken[b,i], Ole Henning Skjønsberg[b,j], Tøri Vigeland Lerum[b,j], Pål Aukrust[a,b,c,k] and Tuva Børresdatter Dahl[a,l]

[a]Research Institute of Internal Medicine, Oslo University Hospital, Rikshospitalet, Oslo, Norway; [b]Institute of Clinical Medicine, University of Oslo, Oslo, Norway; [c]K. G. Jebsen Thrombosis Research and Expertise Center, University of Tromsø, Tromsø, Norway; [d]Department of Infectious Diseases, Oslo University Hospital Ullevål, Oslo, Norway; [e]Department of Medicine, Baerum Hospital, Vestre Viken Hospital Trust, Drammen, Norway; [f]Department of Medicine, Lovisenberg Diaconal Hospital, Oslo, Norway; [g]Diakonhjemmet Hospital, Oslo, Norway; [h]Department of Internal Medicine, Drammen Hospital, Vestre Viken Hospital Trust, Drammen, Norway; [i]Department of Radiology and Nuclear Medicine, Oslo University Hospital Ullevål, Oslo, Norway; [j]Department of Pulmonary Medicine, Oslo University Hospital Ullevål, Oslo, Norway; [k]Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital, Rikshospitalet, Oslo, Norway; [l]Division of Critical Care and Emergencies, Oslo University Hospital, Rikshospitalet, Oslo, Norway

ABSTRACT

Background: The lungs are the organ most likely to sustain serious injury from coronavirus disease 2019 (COVID-19). However, the mechanisms for long-term complications are not clear. Patients with severe COVID-19 have shorter telomere lengths and higher levels of cellular senescence, and we hypothesized that circulating levels of the telomere-associated senescence markers chitotriosidase, β-galactosidase, cathelicidin antimicrobial peptide and stathmin 1 (STMN1) were elevated in hospitalized COVID-19 patients compared to controls and could be associated with pulmonary sequelae following hospitalization.

Methods: Ninety-seven hospitalized patients with COVID-19 who underwent assessment for pulmonary sequelae at three-month follow-up were included in the study. β-Galactosidase and chitotriosidase were analysed by fluorescence; stathmin 1 and cathelicidin antimicrobial peptide were analysed by enzyme immuno-assay in plasma samples from the acute phase and after three-months. In addition, the classical senescence markers cyclin-dependent kinase inhibitor 1A and 2A were analysed by enzyme immuno-assay in peripheral blood mononuclear cell lysate after three months.

Results: We found elevated plasma levels of the senescence markers chitotriosidase and stathmin 1 in patients three months after hospitalization with COVID-19, and these markers in addition to protein levels of cyclin-dependent kinase inhibitor 2A in cell lysate, were associated with pulmonary pathology. The elevated levels of these markers seem to reflect both age-dependent (chitotriosidase) and age-independent (stathmin 1, cyclin-dependent kinase inhibitor 2A) processes.

Conclusions: We suggest that accelerated ageing or senescence could be important for long-term pulmonary complications of COVID-19, and our findings may be relevant for future research exploring the pathophysiology and management of these patients.

KEYWORDS
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CONTACT
Tove Lekva
tove.lekva@ous-research.no
Research Institute of Internal Medicine, Oslo University Hospital, Rikshospitalet, Oslo, Norway

Introduction

Escalating immune activation with massive cytokine release seems to drive severe coronavirus disease 2019 (COVID-19). However, the mechanisms leading to acute and particular long-term sequela are still unclear. Korompoki et al. [1] recently reviewed long-term complications of COVID-19, identifying the lungs as the organ most likely to sustain serious injury. Causes of acute and long-term injury after COVID-19 seem to include hypoxia and mechanical ventilation-related damage, tissue destruction due to uncontrolled immune activation, micro-vascular/thrombotic disease and endothelial dysfunction, but these mechanisms are not clear.

Recently, Mahmoodpoor et al. [2] reported that older COVID-19 patients with shorter telomeres and low lymphocyte counts were at increased risk for post-COVID pulmonary fibrosis. Indeed, short telomeres may result in chromosomal instability and loss of cell viability by inducing replicative senescence and apoptosis. Pulmonary alveolar cells with telomere dysfunction induce pulmonary fibrosis in mice, suggesting that dysfunctional telomeres in lungs could promote loss of viability and increased fibrosis. As patients with severe COVID-19 have shorter telomere lengths [3] and higher levels of cellular senescence [4], we hypothesized that circulating levels of the telomere-associated senescence markers chitotriosidase (CHIT1), β-galactosidase, cathelicidin antimicrobial peptide and stathmin 1 (STMN1) [5] could be elevated in COVID-19 patients compared to controls, and thus serve as markers of pulmonary sequelae following hospitalization for COVID-19.

Materials and methods

Plasma samples from 97 hospitalized patients with COVID-19 who had undergone assessment for pulmonary sequelae at three-month follow-up were included in the study. The pulmonary status was evaluated by the pulmonary function test (diffusion capacity of the lungs [DL_{CO}]) and a protocol for chest-computed tomography (CT) as previously described [6,7]. For comparison, plasma samples from 22 healthy age- and sex-matched controls (mean age ± SD: 55.5 ± 12.5, p=.47 vs. patients; 13 males (59%), p=.80 vs. patients) were also analysed. Demographics at admission and at three-month follow-up are shown in Table 1. At three months follow-up after hospital admission, inflammatory markers (C-reactive protein, ferritin) and blood cell counts had largely normalized, and 86% had antibodies against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [6,7]. The study was approved by the Committee for Medical Research Ethics Region South East Norway (REK 118684) and registered on ClinicalTrials.gov, 25 March 2020 (NCT04321616).

Plasma levels of cathelicidin antimicrobial peptide and STMN1 were measured in duplicate by enzyme immuno-assay with antibodies obtained from Mybiosource (San Diego, CA), and β-galactosidase and CHIT1 in duplicate by fluorescence [8]. We also investigated classical senescence markers in peripheral blood mononuclear cells in our available RNAseq data (acute phase) and in cell lysates (three months). Total RNA was isolated from peripheral blood mononuclear cells using the miRNeasy Kit (Qiagen, Hilden, Germany). Novogene

Table 1. Admission and three-month characteristics.

	Admission	3 months	Ref limits
Age, years	57.8 ± 13.9		
Male gender	60 (62%)		
Body mass index	28.3 ± 4.5		
Symptom duration (days)	8 (6, 11)		
Treatment group			
Standard of care	47 (49%)		
Hydroxychloroquine	36 (37%)		
Remdesivir	14 (14%)		
Any comorbidity	66 (68%)		
pO_2/F_iO_2 ratio, kPa	42 (32, 48)	58 (52, 66)	
Ferritin, μg/L	643 (328, 1266)	89 (48, 187)	W: 10–170; M: 30–400
C-reactive protein, mg/L	76 (40, 135)	2 (1, 5)	<4 mg/L
White blood cells, $\times 10^9$/L	6.2 (4.6, 8.5)	6.1 (5.0, 7.1)	3.5–10
Neutrophils, $\times 10^9$/L	4.0 (2.7, 6.5)	3.5 (2.8, 4.4)	1.5–7.3
Lymphocytes, $\times 10^9$/L	1.1 (0.8, 1.4)	2.0 (1.5, 2.4)	1.1–3.3
Viral load (log_{10}/1000)	1.8 (0.6, 2.8)	0 (0, 0)	
Anti-SARS-CoV-2 Ab[a]			
Receptor binding domain ≥5	52 (56%)	83 (86%)	
Nucleocapsid ≥10	50 (54%)	83 (86%)	

[a]Anti-SARS-CoV-2 (antibodies against severe acute respiratory syndrome coronavirus 2) antibody available in all 97 patients at 3 months, 93 at baseline. For antibody detection, see [6].

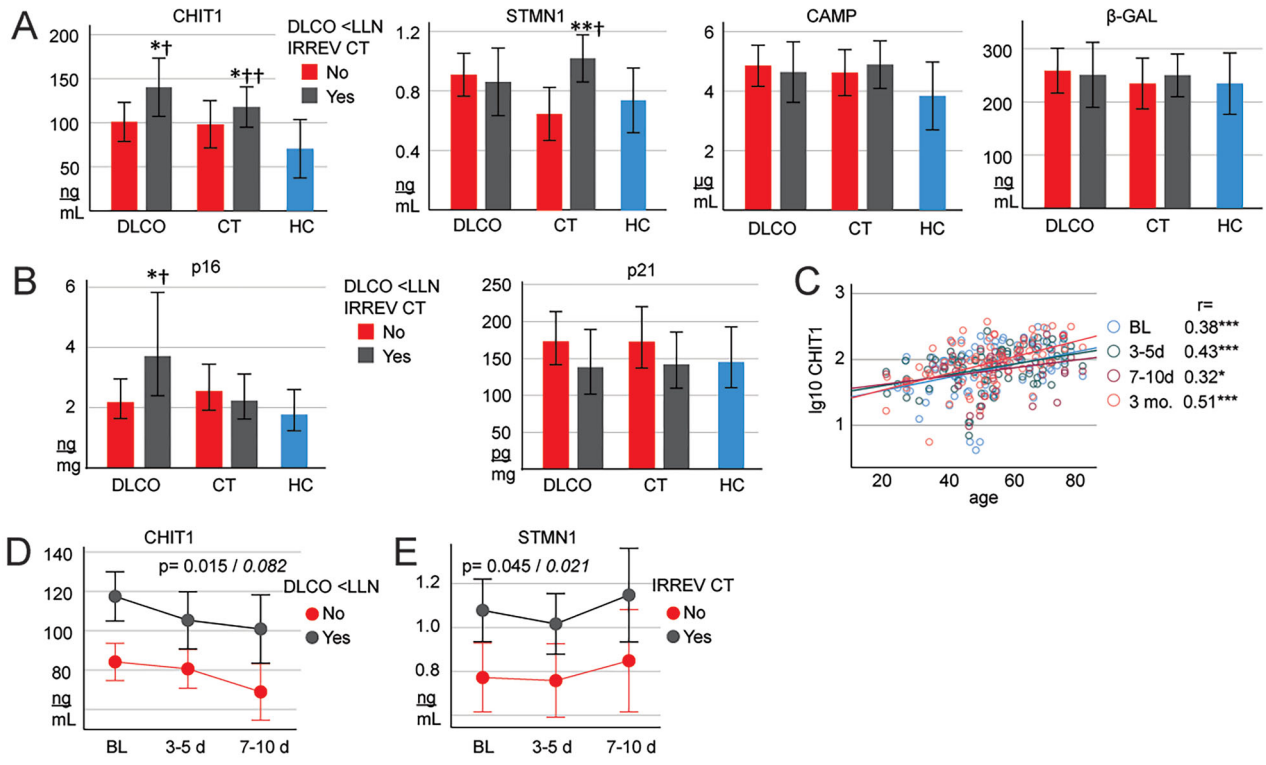

Figure 1. Telomere-associated senescence markers during hospitalization for COVID-19 disease and at three-month out-patient follow-up. (A) Chitotriosidase (CHIT1), stathmin 1 (STMN1), cathelicidin antimicrobial peptide and β-galactosidase levels in plasma and (B) p16 and p21 in peripheral blood mononuclear cell lysate of patients divided by diffusion capacity of the lungs [DL_{CO}] below lower levels of normal (DL_{CO}<LLN) and irreversible CT changes (IRREV CT) compared to healthy controls (HC) at three-months follow-up. ANCOVA with and without adjustment for age. *p<.05 vs. 'no'; †p<.05, ††p<.01 vs. HC. (C) Associations between CHIT1 (log transformed values) and age at baseline, 3–5 days, 7–10 days and three-months after hospitalization, by Spearman's correlation. Correlation coefficient, r and *p<.05, **p<.01 and ***p<.001. (D) CHIT1 and (E) STMN1 levels in plasma at baseline, 3–5 days and 7–10 days after hospitalization between patients with DL_{CO}<LLN and IRREV CT. Data are presented as back-transformed estimated marginal means with 95% confidence intervals from a mixed model analysis with patient as random and including DL_{CO} or irreversible CT, DL_{CO} or irreversible CT*time, randomized treatment. p Values represent the model without (normal p) or with age (italic p).

(UK) Company Limited (Cambridge, UK) performed the stranded library preparation and sequencing on the Illumina platform (San Diego, CA). Differentially expressed genes were obtained using the DESeq2 R package [9,10]. Protein pellets were dissolved in cell lysis buffer (Cell Signaling Technology, Danvers, MA) and levels of the cyclin-dependent kinase inhibitors (CDKN) 2A (p16) and (1A) p21 were measured using enzyme immuno-assay with antibodies ab227903 (Abcam, Cambridge, UK) and DYC147 (R&D System, Minneapolis, MN), respectively, and normalized against total protein content in each sample (Bicinchoninic Acid Pierce, Thermo Fisher, Waltham, MA).

Results

Of 90 patients evaluated with DL_{CO} measurement, 28 (31%) had diffusion capacity below lower limit of normal (LLN), while 45 (50%) of 91 patients who underwent CT of the chest displayed perceived irreversible changes (consolidations, reticular opacifications, parenchymal bands, interlobular septal thickening or any bronchiectasis) [7] at three-month follow-up. Patients with DL_{CO}<LLN had elevated CHIT1 levels at three months compared to patients with DL_{CO}>LLN and healthy controls (Figure 1(A)). Similarly, patients with irreversible changes on chest CT had elevated levels of CHIT1 and STMN1 at three months compared to patients with irreversible CT changes and healthy controls. No differences in β-galactosidase or cathelicidin antimicrobial peptide levels were observed between these COVID-19 patient subsets and healthy controls.

Focussing on CHIT1 and STMN1 at three-month follow-up, we evaluated the influence of age and comorbidities (Table 1). Patients with comorbid disease (66 vs. 51 years, p<.001), DL_{CO}<LLN (63 vs. 56 years, p=.061) and irreversible CT changes (63 vs. 52 years, p<.001) were older than patients without these conditions. CHIT1, but not STMN1 (r between 0.02 and −0.17), was correlated with age both during the acute phase and at

three months (Figure 1(C)). The increased CHIT1 levels in patients with DL_{CO}<LLN and irreversible CT changes were largely mitigated by age (p=.25 and p=.56 after adjustment, respectively) but not comorbidity (p=.07 and p=.041 after adjustment, respectively). The increased STMN1 in patients with DC_{LO}<LLN and irreversible CT changes were not influenced by age or comorbidity (p=.001 and p=.006, respectively).

We also assessed the temporal course of CHIT1 and STMN1 during the acute phase according to the pulmonary sequelae at three-month follow-up. CHIT1 was increased in patients with DL_{CO}<LLN (overall p=.015), but this was attenuated by age adjustment (p=.082) (Figure 1(D)). No changes in CHIT1 in patients with irreversible CT changes were observed during the acute phase, but STMN1 was increased (p=.045) and not influenced by age (p=.021 after adjustment) (Figure 1(E)).

We next evaluated RNAseq data in patients ($n = 6$) vs. healthy controls ($n = 15$) in peripheral blood mononuclear cells during the acute phase for the classical senescence markers CDKN2A encoding p16 and CDKN1A encoding p21. CDKN2A mRNA expression was increased log2fold 1.69 p=.009 (adjusted p value) and CDKN1A was increased log2fold = 0.77, p=.138 (adjusted p value) in patients vs. controls.

We also investigated p16 and p21 in peripheral blood mononuclear cell lysate isolated from patients ($n = 54$) and controls ($n = 18$) at three-month follow-up. As shown in Figure 1(B), p16 levels, but not p21 levels, were increased in patients with DL_{CO}<LLN compared to patients with DL_{CO}>LLN and healthy controls. These markers were not related to CT changes (data not shown).

The study population was derived from a randomized controlled trial where the patients receiving local standard of care alone or standard of care plus either remdesivir or hydroxychloroquine (Table 1) [6]. We have previously reported no differences between the treatment arms with respect to clinical outcomes or pulmonary functional test/chest CT findings at three months [6,7]. In agreement with these findings, levels of CHIT1 (p=.83) and STMN1 (p=.44) similarly did not differ between the treatment arms.

Discussion

The mechanisms promoting long-term COVID-19 pulmonary complications remain unclear. Herein, we found that persistently elevated plasma levels of the senescence markers CHIT1 and STMN1 and cellular levels of p16 at three months after hospitalization of COVID-19 patients were associated with pulmonary pathology, as assessed by DC_{LO}<LLN and/or the presence of chest CT findings consistent with irreversible lung injury. Notably, the elevated levels of these plasma markers seem to reflect both age-dependent (CHIT1) and age-independent (STMN1) processes. We suggest that accelerated ageing or senescence could be important for long-term pulmonary complications of COVID-19.

The circulating markers selected were based on their roles in response to telomere-induced senescence. Analysis of these proteins has revealed increased levels of expression in human ageing, age-related disease, and in chronic diseases. STMN1 is a cytosolic phosphoprotein that mediates many cellular functions. Of particular relevance, Tian et al. [11] suggest that STMN1 could be involved in endothelial cell permeability and endothelial cell barrier disruption within the lung, and knockdown of STMN1 was found to reduce pulmonary endothelial cell permeability and acute lung injury. CHIT1 has also been shown to play important roles in lung diseases [12], potentially involving pro-fibrotic mechanisms. Whether such mechanisms are operating in long-COVID-19 pulmonary pathology remains unknown.

Senescence is triggered by a numerous of stressors (including telomere damage) that promote a DNA damage response leading to p53-dependent upregulation of p21 and/or expression of p16. In a recent correspondence [13], the increased expression of the senescence markers p16, p21 and the DNA damage marker γ-H2AX and p53-binding protein 1 in relation to COVID-19 lung complications was discussed. Early massive senescent lung cell accumulation was observed in areas of severe COVID-19 related lung damage and persisted in the lungs over time, and many of them appeared alongside with the development of long-term lung alterations. Our finding of increased p16 in peripheral blood mononuclear cell lysates from patients with impaired pulmonary function supports persistent cellular senescence in subgroups of patients with pulmonary complications following severe COVID-19. The distinct increase in p16 vs. p21 could be related to their different regulation during senescence. In a previous study, p21 was shown to decrease after senescence is achieved, while upregulation of p16 may be essential for maintenance of the senescent-cell-cycle arrest [14].

Previous studies have reported that the SARS-CoV-2 surface antigen Spike-1 protein, which signals through angiotensin-converting enzyme 2 receptors, the putative receptor for SARS-CoV-2 in humans, can cause

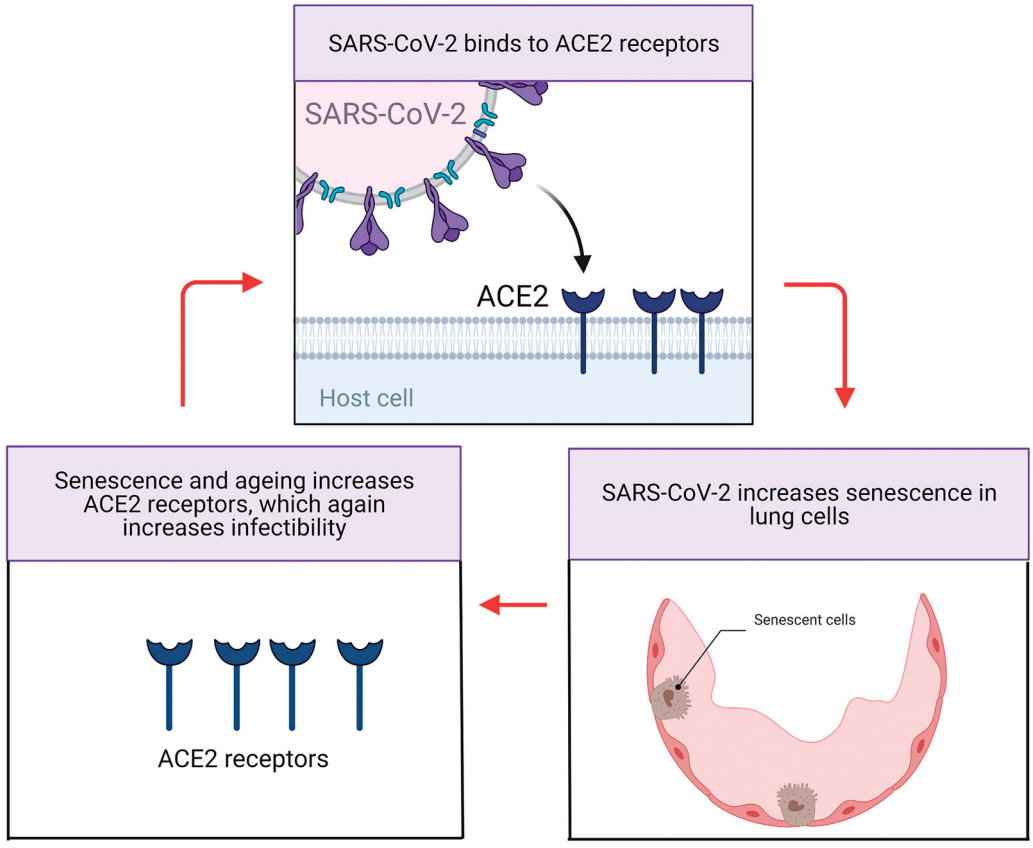

Figure 2. Overview over the interaction between senescence and SARS-CoV-2 where SARS-CoV-2 promotes senescence and senescence enhances SARS-CoV-2 infectivity. Created with BioRender.com.

amplification of the tissue-destructive, pro-inflammatory, senescence-associated secretory phenotype of already senescent human cells and spike pseudotyped vesicular stomatitis virus can cause non-senescent lung epithelial cells become senescent [15]. Angiotensin-converting enzyme 2 increased upon telomere shortening ($Terc^{-/-}$ mice) and ageing in the lungs [16]. It was proposed that telomere dysfunction due to telomeric shortening or damage triggers DNA damage response activation during ageing, which causes the upregulation of angiotensin-converting enzyme 2, accordingly contributing to making the elderly more susceptible to the COVID-19 infection since angiotensin-converting enzyme 2 favours infectability. In summary, there are indications of a bilateral interaction between senescence and SARS-CoV-2, in which the virus promotes senescence, and senescence enhances SARS-CoV-2 infectivity (Figure 2).

The present study has some limitations, including lack of RNAseq data from three months follow-up, lack of data from additional senescence markers and lack of more established methods for measurements of the markers, such as western blot, immunofluorescence and immunohistochemistry. In addition, the number of patients was relatively low, in particular for the RNAseq analyses.

Based on the present data, we suggest that increased cellular senescence could be involved in the pathogenesis of pulmonary pathology following hospitalization for COVID-19. This includes both age-dependent and age-independent mechanisms, which could have potential direct impact on the development of pulmonary fibrosis. These findings may be of relevance for future research exploring the pathophysiology and management of post-COVID-19 lung disease.

Disclosure statement

The authors report no conflict of interest.

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ORCID

Tove Lekva http://orcid.org/0000-0002-7962-4726
Bente Halvorsen http://orcid.org/0000-0002-6529-6485

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