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Research Paper

Senescence Signatures Predict Hospitalization Risk and Severity in COVID-19 Patients

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Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in a global pandemic associated with substantial morbidity and mortality worldwide, with a particular risk for severe disease and mortality in the elderly population. The more aged you are the higher the risk for mortality and severity due to COVID-19. Why age is the single largest risk factor for severity in COVID-19 is not known. Together virus-induced cell senesence and aging are believed to play a central role in COVID-19 severity and pathogenesis. A deeper understanding of COVID-19 pathophysiology and the involvement of senescence/aging proteins is therefore required. This can help identify patients, at an earlier stage, who are more susceptible to acquiring a severe COVID-19 infection and those who are most likely to go on to develop post-COVID-19 syndrome. This early detection remains a major challenge however largely due to limited understanding of SARS-CoV-2 pathogenesis.

In this study, we investigate whether the levels of senescence-specific plasma proteins from COVID-19 patients can be utilized to predict severity and post-COVID-19 syndrome. We performed proteomic profiling of plasma from COVID-19 patients (n = 400) using the Olink Explore 384 Inflammation Panel. Data analysis identified differences in plasma concentrations of proteins, which are linked to senescence while considering patient hospitalization status, age, and their World Health Organization (WHO) clinical progression score.

The statistically significant changes were found in the senescence-associated plasma proteome of COVID-19 patients who were hospitalized, more aged, and those with severe WHO classification (TPPI, CXCL10, HGF, VEGFA, SIRPB1, IL-6, TNFRSF11B, and B4GALT1; $p < 0.05$) and which may be linked to post-COVID-19 syndrome. Epigenetic analysis of the methylome, using the GrimAge Clock, found that biological and chronological age did not correlate in hospitalized patients. We also identified that PTX3, CXCL10, KYNU, and SIRPB1 genes had increased promoter methylation in hospitalized patients.

Machine learning analysis showed that characteristic protein changes perform with a similar accuracy to that of a whole panel biomarker signature in terms of hospitalization, age, and WHO clinical progression score. This study revealed senescence specific protein changes (sendotypes) in the plasma of COVID-19 patients, which can be used as determinants for predicting COVID-19 severity, viral signature persistence, and ultimately which may lead to post-COVID-19 syndrome. We propose that the identification of such sendotypes could be exploited for therapeutic intervention via senolytics in COVID-19.

Introduction

The impact of the coronavirus disease 2019 (COVID-19) global pandemic has been felt worldwide, with substantial morbidity and mortality evident in many countries[1–4]. As of 28 June 2023, there had been 767,518,723 people infected with the disease and 6,947,192 deaths globally[5]. COVID-19 disease, caused by severe acute respiratory syndrome coronavirus 2

(SARS-CoV-2), varies considerably, ranging from an asymptomatic to symptomatic carrier state[1,6–9], with mortality from infection more prevalent in the elderly population[10,11]. Indeed, some patients infected with COVID-19 develop mild illness/symptoms and have a good overall prognosis[7,8]. Conversely, however, some patients develop severe illness/symptoms that may result in the patient requiring hospitalization, oxygen therapy, ventilation, and/or intensive care unit (ICU) admission and even lead to organ dysfunction and death[1,6–9].

Infection with SARS-CoV-2 is driven by a pathological inflammatory response, which results in a dysregulated immune response. This involves both hyperimmune and hypoimmune responses[12–14]. Even though age is a major risk factor[4,6,15–17], we believe that the phenomenon of virally induced senescence[18–20] in conjunction with aging and pre-existing senescent cells, plays a central causative role in COVID-19 severity and pathogenesis. This may be linked to the escalating immune activation and the massive cytokine storm seen following the COVID-19 infection, which appears to drive severe infection[1,12,13,21–24]. SARS-CoV-2 induces a form of cellular senescence, a stress-inducible cellular state switch that includes terminal cell-cycle arrest, known as virus-induced senescence (VIS), through numerous mechanisms[18–20].

Studies are mounting to suggest that this VIS does have immense relevance as a cellular response to SARS-CoV-2 infection. It is reported that this VIS results in the exacerbation of the senescence-associated secretory phenotype (SASP) and is characterized by the secretion of a plethora of pro-inflammatory cytokines and chemokines, including extracellular matrix-modifying, complement-activating, and procoagulatory factors by senescent cells[18,23,25–27]. As mentioned, senescence has an important role in various process, including aging and chronic disease, which are linked with elevated levels of cellular senescence, thus resulting in the accumulation of aging-associated and chronic disease-associated senescent cells[28–31]. Therefore, in the context of SARS-CoV-2 infection in elderly patients, who already have a larger number of pre-existing senescent cells in their tissues due to the aging process, these effects of the SASP factors are much more intensified. These SASP factors influence the "cytokine storm," tissue-destructive immune cell infiltration, endothelialitis (endotheliitis), fibrosis, and micro-thrombosis, all VIS-driven features[17–20].

A deeper understanding of COVID-19 pathophysiology and the involvement of SASP is therefore required as a foundation to help identify patients, at an early stage, who are more susceptible to acquiring a more clinically severe COVID-19 infection.

To date, several studies have investigated the pathways, associated genes, and proteins that are implicated in the pathophysiology of severe COVID-19 disease, with some studies adapting multi-omic approaches[32–34]. At present, to our knowledge, there are no senescence-aging-related biomarker signatures that have been identified by extensive proteomics to risk stratify COVID-19 severity. Biomarker signatures previously defined as "sendotypes," which are defined as specific senescent endotypes, are capable of differentiating disease characteristics[25] and, for example, may have potential in identifying those patients who are at an increased risk of experiencing severe disease. Early stratification of patients via such novel biomarker signatures may improve clinical outcomes by streamlining the provision of effective clinical care and would help to alleviate the overall burden of COVID-19 on healthcare systems. This remains a major challenge, however, largely due to a limited understanding of SARS-CoV-2 pathogenesis. Further targeted research is therefore needed to explore, identify, and validate novel sendotypes which have robust clinical utility in identifying the risk of severe infection versus non-severe infection. Moreover, additional studies are warranted to fully explore and elucidate the disease mechanisms of the biological factors which are potentially driving senescence, particularly in the elderly in the context of COVID-19 infection and in patients who have post-COVID-19 syndrome.

We designed a study that adopted a novel approach to investigating senescence in COVID-19 patients, utilizing the plasma proteomic profiling approach in combination with lung cell senescence models (**Fig. 1**)[35]. Other studies have performed high-throughput proteomic profiling of COVID-19 patients[36–41]. However, this is the first study to our knowledge to take this unique approach to thoroughly investigate the relationship between SARS-CoV-2 infection, senescence, aging, and post-COVID-19 syndrome. In addition, in order to gain potential mechanistic insight, we embarked on

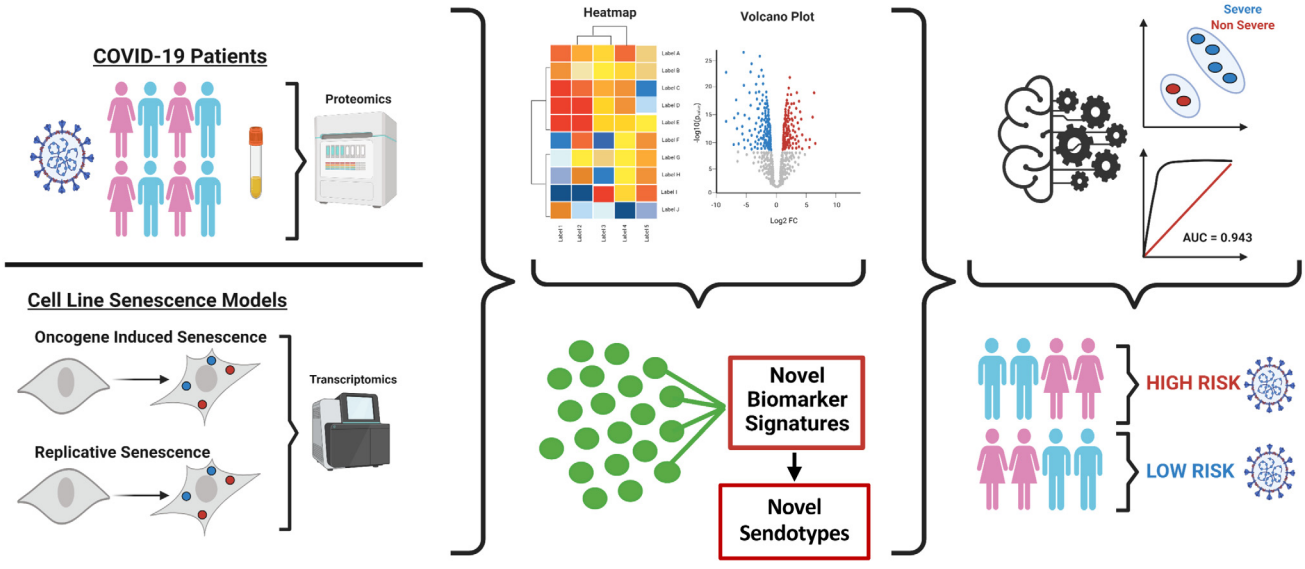

Figure 1. Study design overview. Schematic overview of the study design for this study.

investigating the role of epigenetic alterations, a hallmark of aging, in the context of COVID-19 disease. In recent years, "epigenetic clocks" have been employed in computational modeling. These epigenetic clocks are multivariate models identified by machine learning that have the ability to predict age using DNA methylation data and thus can reflect biological aging[42–45]. In this study, we performed further investigations based on methylation analysis using the GrimAge Clock, which outperforms all other DNA methylation-based biomarkers and is associated with a host of age-related conditions, lifestyle factors, and clinical biomarkers[46]. This is the first study to our knowledge that has analyzed the methylation profile of COVID-19 patients at such a large scale (n = 450) and linked this to senescence.

From this analysis, we identified predictive sendotype-specific protein changes in the plasma of COVID-19 patients, which are associated with hospitalization status, age, and severity. To gain further insights into the underlying disease mechanisms, we mapped these signatures to specific pathways and associated methylation changes in the context of the relevant sendotypes. We also investigated whether there was a potential link between epigenetics, senescence, and methylation that is associated with COVID-19. Finally, our machine learning analysis suggests that these signatures could be used as determinants for predicting COVID-19 severity and could be exploited for therapeutic intervention via senolytics or epigenetic drugs in COVID-19.

Methods

Patient cohort and clinical data collection

Patients (n = 519) were enrolled on the COVID Response Study (COVRES): A Northern Ireland population study of SARS-CoV-2 prevalence, predisposing factors, and pathology (approved by the Health and Care Research Wales Ethics Service; REC ref 20/WA/0179). All patients provided written informed consent for their samples to be used in downstream analyses. The use of patient material and information, as well as research protocols, were approved by ORECNI (Project Reference Number: IRAS 283596). The patient data and corresponding clinical data was collected and anonymized for all patients recruited as part of COVRES[47]. Clinical Trial: Trial Registration—The trial has been registered as an observational study on clinicaltrials.gov as NCT05548829.

Olink plasma proteomics assay

For this study, a randomly selected cohort of samples (n = 400) from the COVRES study was used for proteomics analysis. The demographics for the patients/samples utilized in this specific proteomics study are presented in **Table 1**. Blood samples were collected in 3 × 10 ml ethylenediamine tetraacetic acid (EDTA) tubes and immediately centrifuged at 4000 rpm (4°C) for 15 min. The plasma layer was removed and aliquoted in cryovials and stored at −80°C.

For this study, EDTA plasma samples were thawed at room temperature (20°C) and 45 µl of each sample (n = 400 [non-hospitalized n = 186; hospitalized n = 214]) were (at random) pipetted into 96-well plates with 8 × wells left empty on each plate for internal controls to be added at Olink. Once plated, the plasma samples were virus inactivated as per Olink's COVID-19 1% TritonX-100 inactivation protocol. All plates were sealed using adhesive seals. As per the COVID-19 inactivation protocol, the plates were vortexed thoroughly before centrifugation at 400 g (20°C) for 1 min followed by a 2 hour incubation at room temperature (20°C). Following this incubation, the plates were stored at −80°C until shipment. The plates with samples loaded were shipped to Olink (Olink, Uppsala, Sweden) on dry ice for proteomic analysis. Internal controls were added to each plate and protein analysis of the 400 plasma samples (using 1 µl of plasma from each patient sample) was performed using the Explore® 384 Inflammation panel (protein proximity extension assay) according to proprietary protocols. Within this 384-plex panel, overlapping assays of IL-6, IL-8 (CXCL8), and tumor necrosis factor (TNF) are included for quality control (QC) purposes. All data is presented as NPX (normalized protein expression) values, Olink Proteomics' arbitrary unit on a log2-scale, relative protein quantification calculated via normalization to the extension control (known standard), log2-transformation, and level adjustment using the plate control (plasma sample).

Table 1. Summary of patient characteristics for proteomic analysis.

Characteristics		Hospitalized n = 214	Non-hospitalized n = 186	p Value
Age	Mean (SD)	57.3 (13.1)	45.0 (14.5)	<0.001
Severity	Severe	214 (100.0)	12 (6.5)	<0.001
	Mild		174 (93.5)	
Gender	Female	90 (42.1)	109 (58.6)	0.004
	Male	120 (56.1)	75 (40.3)	
	Other	4 (1.9)	2 (1.1)	

The patient characteristics of the patients (n = 400) included in this proteomics study. Demographics described include hospitalization status, age, World Health Organization (WHO) coronavirus disease 2019 (COVID-19) classification (patients were classified according to the WHO clinical progression score established by the WHO Working Team 2020), and time since polymerase chain reaction (PCR) positive result. Patients with a WHO classification score of 1–3 were denoted as mild and those with a score of 4–9 denoted as severe.

Selection of senescence-specific proteins from Olink panel

A group of 68 proteins (**Supplementary Appendix 1**) from the 368 proteins measured using the Olink assay were selected for senescence-specific analysis. The 68 candidate proteins selected were those that had significant expression levels (RNA-sequencing data; $p < 0.05$, confidence interval [CI] 95%) measured from the human cells (IMR90; lung fibroblasts) induced to develop oncogene-induced senescence using a retrovirus expressing G12V oncogenic mutation or replicative senescence in a previous study[35]. Gene names, p values, and log2fc can be found in **Supplementary Appendix 1 (Tables 1 and 2)**.

Methylation assay

Patient saliva samples (n=450) from the COVRES study were used for downstream methylome analysis. The demographics for the patients/samples utilized in the methylome study are presented in **Supplementary Table S1**. As previously described[47], saliva samples were collected using 1x DNA Genotek Oragene DNA (OG-500) collection tubes. Saliva (2 ml) was mixed with proprietary stabilization buffer (2 ml) immediately upon collection and stored at room temperature. To extract DNA, samples were incubated for 2 hours at 56°C, followed by DNA isolation using PrepIT.L2P as per the manufacturer's instructions and eluted in 100 µl elution buffer. The quantity and purity of the extracted DNA were evaluated using the Qubit® 3.0 fluorometer.

The samples were stored for downstream analysis at −20°C. As previously described[48], the Illumina Infinium MethylationEPIC (EPIC) array was used for the methylation analysis of the DNA samples collected from saliva (n = 450). In brief, 500 ng of DNA underwent sodium bisulfite conversion to convert unmethylated cytosines to uracil. The DNA was denatured and neutralized followed by isothermal whole genome amplification overnight. Following amplification, DNA was fragmented using enzymes and precipitated with isopropanol. It was then resuspended before being hybridized to the EPIC BeadChip arrays. Chips were washed before single base extension and labeling of the oligos. Imaging was performed on the iScan System. A single Rscript analysis pipeline utilizing the approach by ref.[49] with modification was used to initially process array image data files (.IDAT) to aid sample and process quality control.

Statistical analysis

For the differential analysis, the Welch's two-tailed t-test has been applied to each assay individually with fold change, p value, degrees of freedom, and t-stat recorded. The adjusted p value was generated using Benjamini and Hochberg post hoc adjustment. For the heatmap generation, significantly differentiated proteins found above were selected. Heatmaps are generated using normalized data with the raw log2/NPX values. Patients are clustered according to their proteomic values and are labeled according to their demographics. Columns were labeled using relevant patient data. For volcano plots, the −Log10 of the adjusted p value is plotted against the Log2FoldChange (centered at 0). Points are colored based on two criteria; points with a p.adjust value < 0.05 and a log2FoldChange value > 0.1 are colored red (upregulated) and points with a p.adjust value < 0.05 and a log2FoldChange value < -0.1 are colored blue (downregulated), all other points are colored gray. The principal component analysis (PCA) is plotted using all the combinations of the top five principal components. For each of these plots, the points represent the two-dimensional location of a multidimensional sample with the percentage of variance captured by each component displayed in the x/y labels. Each point is then colored based on patient data (each plot is plotted several times using different coloring). The boxplots with violin follow the methods found on ggplot2-based plots with statistical details at ggstatsplot (indrajeetpatil.github.io) via the function ggbetweenstats() function. All analyses were performed via R version 4.1.2 (2021-11-01). Detailed statistics, p-values, degrees of freedom, means, and log2fc of each identified protein in each group can be found in **Supplementary Appendix 1 (Tables 3–8)**.

Enriched pathway analysis

Proteins with differences in their plasma concentrations had their associated gene symbol, log2FC, and adjusted p-values taken and ran with pathfindR, an integrative omics package used to perform pathway analysis. pathfindR returned pathway enrichment maps based on pre-existing differential expression/methylation data from various omic studies and pathway/gene set annotations from reputable sources such as the Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, BioCarta, and Gene Ontology (GO). The results displayed the top 10 most significant enriched signaling pathways based on the lowest adjusted p-value. Terms were labeled on the y axis, enrichment labeled on the x axis, and the size of the data point is correlated with the number of genes found in that specific network that are significant.

Machine learning

Machine learning analysis was undertaken using the KNIME Analytics Platform[50] running on a 64 core Windows 10 server. Patient data were bootstrapped 1000 times to generate an ensemble of data sets. On each dataset, the forward feature selection of protein biomarkers was undertaken to identify a panel with a maximum of five proteins and the panels were selected based on their accuracy in fivefold stratified cross validation. Logistic regression and random forest classifiers were developed with the former running for 1000 epochs using a stochastic average gradient solver and the latter running with between 50 and 500 decision trees optimized by hill climbing. In all cases, the best-performing classifier was selected.

From the ensemble of receiver operator characteristic (ROC) curves, linear interpolation was used to enable regular sampling along the specificity axis. For each value of specificity, the corresponding sensitivities were ranked with the 5%, 50%, and 95% values taken as the lower CI, median and upper CI ROC curves were generated for the median and CI.

Methylation analysis: RnBeads pipeline

Data import of n = 450 was performed with all samples retained. Sample groups were divided into hospitalized versus non-hospitalized. Quality control checks were performed (including removal of single nucleotide polymorphisms (SNPs) and "bad" probes, large probe drop out and retained 699,922 probes, sex chromosome also removed). Preprocessing and bmiq normalization performed. Covariate analysis was performed (sva module = be, eight surrogate variables, and covariates controlled for: sample sex). Inference module was also used (sample age, LUMP immune cell compositions estimate, and sex prediction). Exploratory analysis (PCA, methylation density plots) and differential methylation analysis were also performed. The DNA methylation clock used in the analysis was the GrimAge Clock.

Results

Characteristics of COVID-19 patients for proteomic analysis

The characteristics of the participants (n = 400) in the current proteomic analysis are shown in **Table 1**. Patients were grouped according to various demographics including hospitalization, (patients were classified as hospitalized if they attended/admitted to the hospital within 14 days of positive polymerase chain reaction (PCR) result), age, WHO clinical progression score (WHO scores are based on the overall highest WHO score during their infection regardless of hospitalization or not)[51], and the time since the patient's PCR positive result.

The study cohort consisted of non-hospitalized (n = 186) and hospitalized (n = 214) patients with the age breakdown as ≤50 y old (n = 183) or >50 y old (n = 217). The rationale for splitting for age group was based on the median of the entire COVRES cohort which was 51 y old. In addition, according to Centre for Disease Control and Prevention, older adults (especially those aged 50 y and older) are more likely to get very sick from COVID-19 than younger people. The WHO clinical progression score was categorized into two categories: 1. Patients denoted as mild (n = 174) had a WHO classification of 1–3 and 2. Patients denoted as severe (n = 226) had a WHO classification of 4–9. Finally, patients were also categorized according to the length of time between positive PCR result and sample collection

Table 2. Summary of patient characteristics into ≤8 weeks or >8 weeks since positive PCR result.

Characteristics		≤8 Weeks PCR n = 121	>8 Weeks PCR n = 279	p Value
Age	Mean (SD)	51.2 (15.2)	51.7 (15.1)	0.749
Hospitalization	Hospitalized	64 (52.9)	150 (53.8)	0.959
	Non-hospitalized	57 (47.1)	129 (46.2)	
Severity	Mild 1–3	55 (45.5)	119 (42.7)	0.682
	Severe 4–9	66 (54.5)	160 (57.3)	
Gender	Female	62 (51.2)	137 (49.1)	0.262
	Male	59 (48.8)	136 (48.7)	
	Other		6 (2.2)	

The patient characteristics of the patients (n = 400) included in this proteomics study. Demographics described include hospitalization status, age, WHO COVID-19 classification (patients were classified according to the WHO clinical progression score established by the WHO Working Team 2020), and time since PCR positive result. Patients with a WHO classification score of 1–3 were denoted as mild and those with a score of 4–9 denoted as severe.

with patients divided into ≤8 weeks or >8 weeks since positive PCR result (Table 2).

Significant differences in plasma concentrations of senescence proteins in hospitalized, aged and severe WHO-classified patients

High-throughput proteomic analysis was performed using the Explore® 384 Inflammation panel with a total of 368 unique proteins analyzed. As explained in the Methods section, the analysis was specifically focused on proteins linked to senescence. Transcripts which had significant expression levels in previous RNA-sequencing data ($p < 0.05$, CI 95%) measured from cells (IMR90; primary lung fibroblasts) induced to develop oncogene-induced senescence or replicative senescence in a previous study[35] were identified. These identified transcripts were then overlapped with proteins measured using the Olink panel to identify proteins linked to senescence based on the cell line senescence models. In total, 68 proteins from the total of 368 proteins measured using the Olink assay were selected for senescence-specific proteomic analysis (**Supplementary Appendix 1, Tables 1 and 2**).

This approach was adapted for investigating the regulation of senescence-associated proteins in COVID-19 patients based on the hospitalization status, the WHO clinical progression score, and age. First, the analysis by hospitalization status revealed a substantial number of significant differences in plasma concentrations of senescence proteins (n = 43) in hospitalized patients compared with non-hospitalized patients who had a sample collection of ≤8 weeks since a positive PCR result, as illustrated in the cluster analysis heatmap (Fig. 2A). Indeed, many of these senescence proteins appeared to exhibit an increased levels; however, there was also a few senescence proteins which appeared to have a decreased levels in the hospitalized patients compared with the non-hospitalized patients (Fig. 2B). The top 10 significant senescence proteins ($p < 0.01$) identified includes TPP1, PTX3, ITGA11, B4GALT1, CXCL10, HGF, VEGFA, PCDH1, SIRPB1, and TIMP3 (top three proteins shown in Fig. 2C), all of which have significantly increased levels in patients who were hospitalized, compared with non-hospitalized patients except ITGA11 which was downregulated in hospitalized patients ($p < 0.05$; **Supplementary Appendix 1, Tables 3–8**).

Parallel analysis also revealed a similar number of significant differences in plasma concentrations of senescence proteins (n = 48) in patients who were categorized to have a severe WHO clinical progression score compared to patients with a mild WHO clinical progression score and who had sample collection ≤8 weeks since a positive PCR result, as illustrated in the cluster analysis heatmap (**Supplementary Fig. S1A**). Similar to the regulation pattern observed in the hospitalized patients, it was evident that many of these senescence proteins appeared to exhibit increased levels; however, there was a few senescence proteins which appeared to have decreased levels in the severe WHO-categorized patients compared with the mild WHO-categorized patients (**Supplementary Fig. S1B**). The top 10 significant senescence proteins ($p < 0.01$) includes PTX3, TPP1, B4GALT1, ITGA11, HGF, CXCL10, SIRPB1, VEGFA, KYNU, and PCDH1 (top eight proteins shown in **Supplementary Fig. S1C**), all of which have increased levels in severe WHO-classified patients with the exception of ITGA11 which was downregulated in severe WHO-classified patients. This again adds confidence to the analysis to see the strong overlap between the top 10 senescence proteins and associated regulation levels (upregulated/downregulated) for both hospitalized and severe WHO clinical progression score.

The samples were then evaluated for differences in senescence-specific protein levels for patients who had sample collection >8 weeks since a positive PCR result. Analysis revealed several significant differences in plasma concentrations of senescence proteins (n = 47) in hospitalized patients compared with non-hospitalized patients, as illustrated in the cluster analysis heatmap (Fig. 2D). Indeed, most of these senescence proteins also appeared to demonstrate increased levels; however, there were a few senescence proteins which appeared to have decreased levels in the hospitalized patients compared with the non-hospitalized patients (Fig. 2E). The top 10 significant senescence proteins ($p < 0.01$) identified includes HGF, BTN2A1, LTBR, ANGPTL2, CXCL10, TPP1, CKAP4, TNFRSF11B, IL-6, and B4GALT1 (top three proteins shown in Fig. 2F), all of which have increased levels in patients who were hospitalized compared with non-hospitalized patients.

On the other hand, analysis for patients who were categorized to have a severe WHO clinical progression score compared to patients with a mild WHO clinical progression score and who had sample collection >8 weeks since a positive PCR result, identified a slightly lower number of significant differences in plasma concentrations of senescence proteins (n = 30) in patients, as illustrated in the cluster analysis heatmap (**Supplementary Fig. S1D**). In line with the regulation pattern observed in hospitalized patients, it was evident that most of these senescence proteins appeared to exhibit increased levels; however there was also some, although few, senescence proteins which appeared to have decreased levels in the severe WHO-categorized patients compared with the mild WHO-categorized patients (**Supplementary Fig. S1E**). The top 10 significant proteins ($p < 0.01$) identified includes HGF, ANGPTL2, LTBR, BTN2A1, CXCL10, TPP1, B4GALT1, IL-6, TNFRSF11B, and KYNU (top eight proteins shown in **Supplementary Fig. S1F**), all of which have increased levels in patients who had a severe WHO clinical progression score compared to patients with a mild WHO clinical progression score. Interestingly again, nine of these top proteins evident in the severe WHO-classified patients also are the same

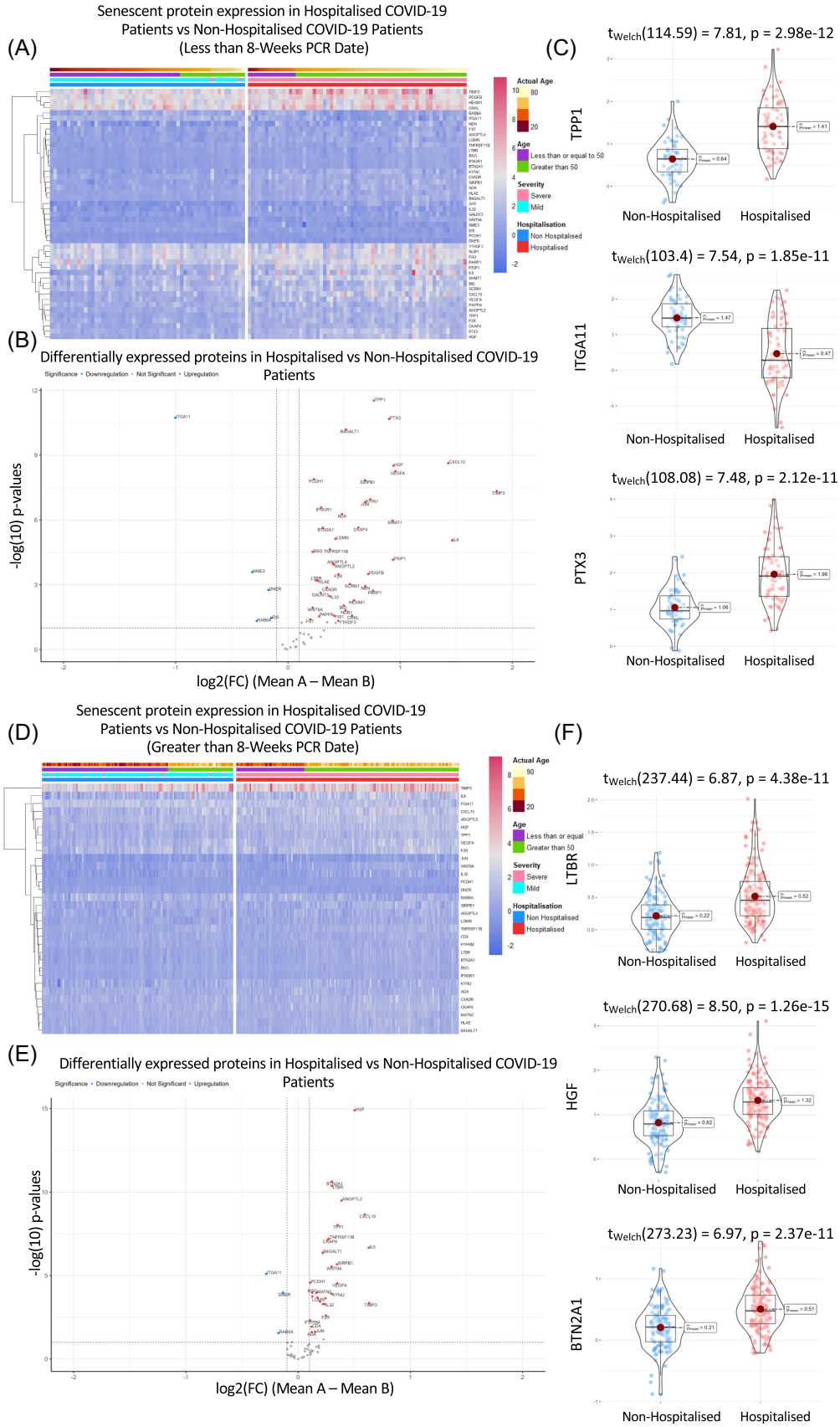

Figure 2. Senescence proteins with significant differences in their plasma concentrations in hospitalized patients. (A) Heatmap and **(B)** volcano plot showing all differentially regulated senescence-specific proteins in hospitalized versus non-hospitalized patients ≤8 weeks since their positive

(legend continued on next page)

polymerase chain reaction (PCR) test. **(C)** Boxplots for the top three differentially regulated senescence-specific proteins in hospitalized versus non-hospitalized patients ≤8 weeks since their positive PCR test. **(D)** Heatmap and **(E)** volcano plot showing all differentially regulated senescence-specific proteins in hospitalized versus non-hospitalized patients >8 weeks since their positive PCR test. **(F)** Boxplots for the top three differentially regulated senescence-specific proteins in hospitalized versus non-hospitalized patients >8 weeks since their positive PCR test.

top proteins evident in the hospitalized patients thus highlighting the robustness of the statistical analysis performed.

As senescence is closely linked with aging, we investigated the impact of age on senescence proteins in COVID-19-infected patients. As hypothesized, the data revealed that the age of patients had a significant impact on the regulation levels of several senescence proteins. This analysis revealed a substantial number of significant differences in plasma concentrations of senescence proteins ($n = 31$) in aged patients, >50 y old compared with patients ≤50 y old, who had sample collection ≤8 weeks since a positive PCR result, this is illustrated in the cluster analysis heatmap (**Fig. 3A**). Indeed, as observed previously for hospitalization status and the WHO clinical progression score, several of these senescence proteins appeared to exhibit increased levels with only a few senescence proteins appearing to have decreased levels in aged patients, >50 y old compared with patients ≤50 y old, (**Fig. 3B**). The top 10 significant proteins ($p < 0.01$) identified includes WNT9A, PTX3, CXCL10, HGF, PCDH1, IFNGR1, TNFRSF11B, JUN, ITGA11, and B4GALT1 (top three proteins shown in **Fig. 3C**), all of which have increased levels in patients who were >50 y compared with patients ≤50 y old apart from ITGA11 which was downregulated in more aged patients. Of note, ITGA11 is also downregulated in both hospitalized and severe WHO-classified patients who had sample collection ≤8 weeks since a positive PCR result.

Likewise, a comparable observation was apparent when looking at aged patients, >50 y old compared with patients ≤50 y old, who had sample collection >8 weeks since a positive PCR result. This analysis revealed a substantial number of significant differences in plasma concentrations of senescence proteins ($n = 33$) in aged patients, >50 y old compared with patients ≤50 y old, who had sample collection >8 weeks since a positive PCR result, this is illustrated in the cluster analysis heatmap (**Fig. 3D**). As observed in the previous analyses, most of these senescence proteins appear to exhibit increased levels with very few senescence proteins having decreased levels in aged patients, >50 y old compared with patients ≤50 y old (**Fig. 3E**). The top 10 significant proteins ($p < 0.01$) identified includes WNT9A, TNFRSF11B, PCDH1, HGF, LTBR, BTN2A1, IL32, CXCL10, SIRPB1, and CKAP4 (top three proteins shown in **Fig. 3F**), all of which have increased levels in patients who were >50 y compared with patients ≤50 y old.

PCA was performed to identify strong patterns or trends linked to senescence protein regulation and the respective hospitalization, age, and WHO clinical progression scores within COVID-19 patients either ≤8 weeks or >8 weeks since positive PCR result. This analysis permitted the visualization of the large data set in which the data segregated into two groups for each of the variables and excitingly which is associated and driven by senescence protein regulation. This is illustrated in the PCA plots for hospitalized/non-hospitalized patients ≤8 weeks (**Supplementary Fig. S2A**) or >8 weeks (**Supplementary Fig. S2B**) since positive PCR result, for patients with a mild/severe WHO clinical progression score ≤8 weeks (**Supplementary Fig. S2C**) or >8 weeks (**Supplementary Fig. S2D**) since positive PCR result, and also for patients according to their age (≤50 y old or >50 y old) ≤8 weeks (**Supplementary Fig. S2E**) or >8 weeks (**Supplementary Fig. S2F**) since positive PCR result.

Pathway and network analysis of senescence proteins with significant differences in their plasma concentrations in hospitalized, aged and severe WHO-classified patients

Further investigation into the implication of senescence protein regulation in hospitalized, aged, and severe WHO-classified patients was performed via pathway and network analysis to identify the most commonly implicated pathways and interactions.

This analysis was performed using all senescence proteins with significant differences in their plasma concentrations within hospitalized patients ≤8 weeks (**Fig. 4A**) or >8 weeks (**Fig. 4B**) since positive PCR result, for patients with a mild/severe WHO clinical progression score ≤8 weeks (**Supplementary Fig. S3A**) or >8 weeks (**Supplementary Fig. S3B**) since positive PCR result, and also for patients according to their age (≤50 y old or >50 y old) ≤8 weeks (**Fig. 4C**) or >8 weeks (**Fig. 4D**) since positive PCR result. The observation of the pathway enrichment analysis revealed various molecular interactions mapped to several aspects including cytokine–cytokine receptor interactions, MAPK signaling pathways, JAK–STAT signaling pathways, and cell differentiation.

Persistent signatures of COVID-19

As a major focus of this study was linked to senescence, we therefore also wanted to identify a senescence-specific signature, otherwise known as a sendotype, which could also be considered as a persistent signature of COVID-19 syndrome. In order to assess the potential and associated performance with utilizing a smaller senescence-specific biomarker signature panel versus a full Olink panel biomarker signature (separate manuscript in preparation), machine learning was employed, and ROC curves were generated with area under the curve (AUC) values. Forward feature selection of protein biomarkers (senescence and whole panel) was undertaken to a identify panels with a maximum of five proteins. From this analysis, it was identified that a biomarker signature comprising senescence only proteins was able to perform with a similar accuracy to that of a whole panel biomarker signature in terms of hospitalization (AUC 0.85 vs. 0.92 ≤8 weeks, 0.70 vs. 0.78 >8 weeks), age (AUC 0.90 vs. 0.93 ≤8 weeks, 0.85 vs. 0.90 >8 weeks), and WHO clinical progression score (AUC 0.87 vs. 0.93 ≤8 weeks, 0.70 vs. 0.79 >8 weeks) (**Fig. 5A–D** and **Supplementary Fig. S4A,B**).

Methylation-associated analysis

As mentioned, methylation and epigenetic changes are implicated in senescence. As part of this study, saliva samples ($n = 450$) from the COVRES study were used for downstream methylome analysis which was performed using the Illumina Infinium MethylationEPIC (EPIC) array. The demographics for the patients/samples utilized in the methylome study are presented in **Supplementary Table S1**. Chronological age was correlated with biological age using the GrimAge clock. Data were

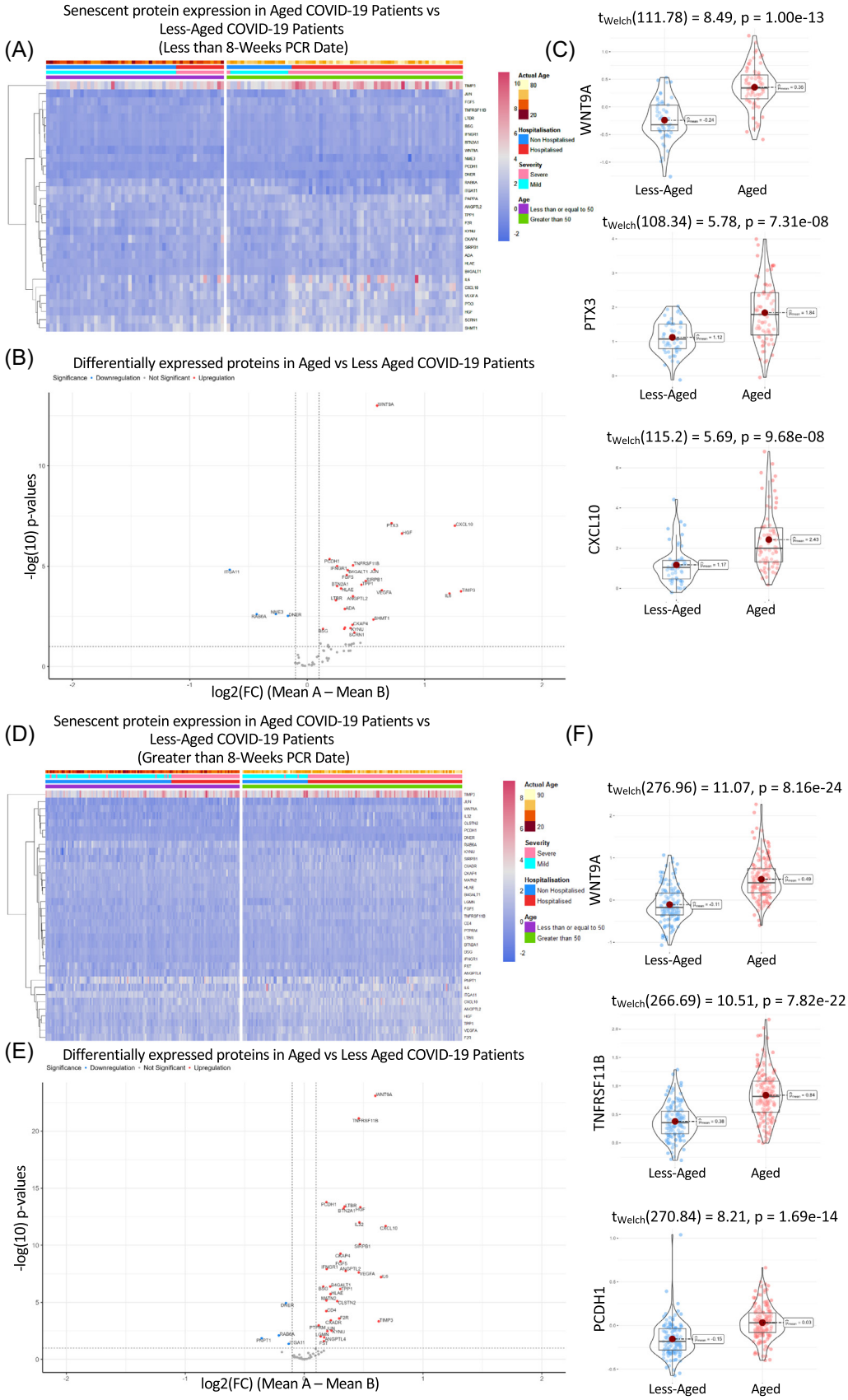

Figure 3. Senescence proteins with significant differences in their plasma concentrations in aged patients. (A) Heatmap and **(B)** volcano plot showing all differentially regulated senescence-specific proteins in ≤50 y versus >50 y old patients ≤8 weeks since their positive PCR test.

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(C) Boxplots for the top three differentially regulated senescence-specific proteins in ≤50 y versus >50 y old patients ≤8 weeks since their positive PCR test. **(D)** Heatmap and **(E)** volcano plot showing all differentially regulated senescence-specific proteins in ≤50 y versus >50 y old patients >8 weeks since their positive PCR test. **(F)** Box plots for the top three differentially regulated senescence-specific proteins in ≤50 y versus >50 years old patients >8 weeks since their positive PCR test.

normalized, as shown in the QQ plot (**Supplementary Fig. S5A**). From this analysis, it was evident that hospitalized patients have a higher epigenetic age than chronological age (Pearson's correlation coefficient = 0.9525337) with the r scale correlation values indicating a very good correlation between chronological and epigenetic age. They also appear to be aging at a faster rate (**Fig. 5E**, red vs. blue line at left, $p < 2.2e\text{-}16$). Hospitalized patients have accelerated biological aging as per the GrimAge clock with the difference in age acceleration (AgeAcc) between the two groups appearing as significant ($p < 0.001$) (**Fig. 5E**, right). Furthermore, analysis also reveals that ≤50 y old cluster more tightly together, while patients >50 y old have a greater variance (**Supplementary Fig. S5B**) thus indicating that age has an important role.

In addition, we evaluated the methylation levels of senescence-specific proteins that were profiled on the Olink proteomics panel. Upon analysis, we identified a subset of genes (n = 27) which had differences in their methylation levels at the promoter region in hospitalized patients compared with non-hospitalized patients (**Supplementary Fig. S5C**). Out of this subset, the genes (n = 9) which had the largest changes in their methylation levels in the promotor region of hospitalized patients compared with non-hospitalized patients were PCDH1, TIMP3, LGMN, IL-32, LTBR, PTX3, KYNU, CXCL10, and SIRPB1 (**Fig. 5F**). Moreover, 25.93% of significant senescence proteins overlap with FDR-significant promoters between hospitalized and non-hospitalized patients (**Fig. 5G**).

Finally, we also identified a subset of genes (n = 26) which had differences in their methylation levels at the promoter region in patients who had sample collection ≤8 weeks compared with patients who had sample collection >8 weeks positive PCR result (**Supplementary Fig. S5D**). Out of this subset, the genes (n = 3) which had the largest changes in their methylation levels in the promotor region of patients who had sample collection ≤8 weeks compared with patients who had sample collection >8 weeks positive PCR result were KYNU, LGMN, and PCDH1 (**Fig. 5H**).

Discussion

In this study, we have measured and identified novel biomarker signatures and specific sendotypes using plasma samples obtained from COVID-19-infected patients. We have identified senescence-associated proteomic changes in the plasma of hospitalized patients, more aged, and those with severe WHO classification. Machine learning showed sendotypes perform as accurately with high sensitivity and specificity as compared to a larger proteomic panel. Specifically, our study has identified several senescence-associated proteins in both hospitalized and aged patients with a predominant elevation in levels evident for most proteins. Indeed, PTX3 and CXCL10 were identified to have elevated levels in hospitalized and aged patients, this correlates with other findings that have shown that an increased levels of PTX3 and CXCL10 predict COVID-19 outcome and are strongly associated with COVID severity and mortality[34,52–55].

The role of the senescence-aging-COVID-19 axis and the phenomena of immunosenescence, aging, and novel sendotypes (senescent endotypes specifically) in COVID-19 infection and post-COVID-19 syndrome has not been fully explored. SARS-CoV-2 induces cellular senescence, specifically known as VIS, through numerous mechanisms[18,26,27]. Studies are mounting to suggest that this VIS does have immense relevance as a cellular response to SARS-CoV-2 infection[17–20]. This VIS results in the exacerbation of SASP and is characterized by the secretion of a plethora of pro-inflammatory cytokines and chemokines including extracellular matrix-modifying, complement-activating, and pro-coagulatory factors by senescent cells[25]. As mentioned, senescence has an important role in various processes including aging and chronic disease which are linked with elevated levels of cellular senescence thus resulting in the accumulation of aging-associated and chronic disease-associated senescent cells[28–31].

Cellular senescence in general is known to be challenging within the field because no single marker exists that defines senescence and especially senescence specific to SARS-CoV-2 infection. In addition to this, cryopreserved clinical samples from COVID-19-infected patients need to undergo virus deactivation fixation steps thus preventing their use for gold standard assays used for detecting senescence such as SA-β-gal staining. Therefore, it remains challenging to fully understand the role of senescence in COVID-19. More recent studies are beginning to address this challenge. A panel of senescence markers could robustly discriminate between nasopharyngeal mucosa samples obtained from COVID-19-infected patients and prepandemic biopsy samples from patients without a respiratory tract infection. A panel of senescence markers, which includes p16INK4a, p21CIP1, H3K9me3, lipofuscin, and IL-8, had significantly higher reactivity in the mucosa samples from the COVID-19-infected patients compared with the prepandemic biopsy samples from patients without a respiratory tract infection[18,26,56,57]. In addition, much stronger signs of senescence were evident in the lung specimens of COVID-19-infected patients compared to negative controls[18].

Single cell and high-throughput transcriptomic analyses further support the indication that VIS is indeed linked to the severity of SARS-CoV-2 infection. It has been reported that the patients infected with SARS-CoV-2 had elevated markers of senescence, including elevated levels of transcription of p16INK4a, present in cells of the upper and lower airway mucosa and also had an increased levels of SASP factors, including IL-1α, IL-6, CCL2, CXCL10, MMP9, PAI1, and TIMP1, within their serum compared to healthy controls[18,41,57–62]. Furthermore, another study reported that the removal of senescent cells from COVID-19 infection reduced the viral load in aged mice[17] thus adding to our hypothesis that age-associated pre-existing senescent cells have a causative role and clear clinical relevance in determining COVID-19 severity.

In order to gain a potential mechanistic insight, we embarked on investigating the role of epigenetic alterations in COVID-19. These epigenetic clocks are multivariate machine learning models that can predict age using DNA methylation data and thus can reflect biological aging[42–46]. In this study, we performed

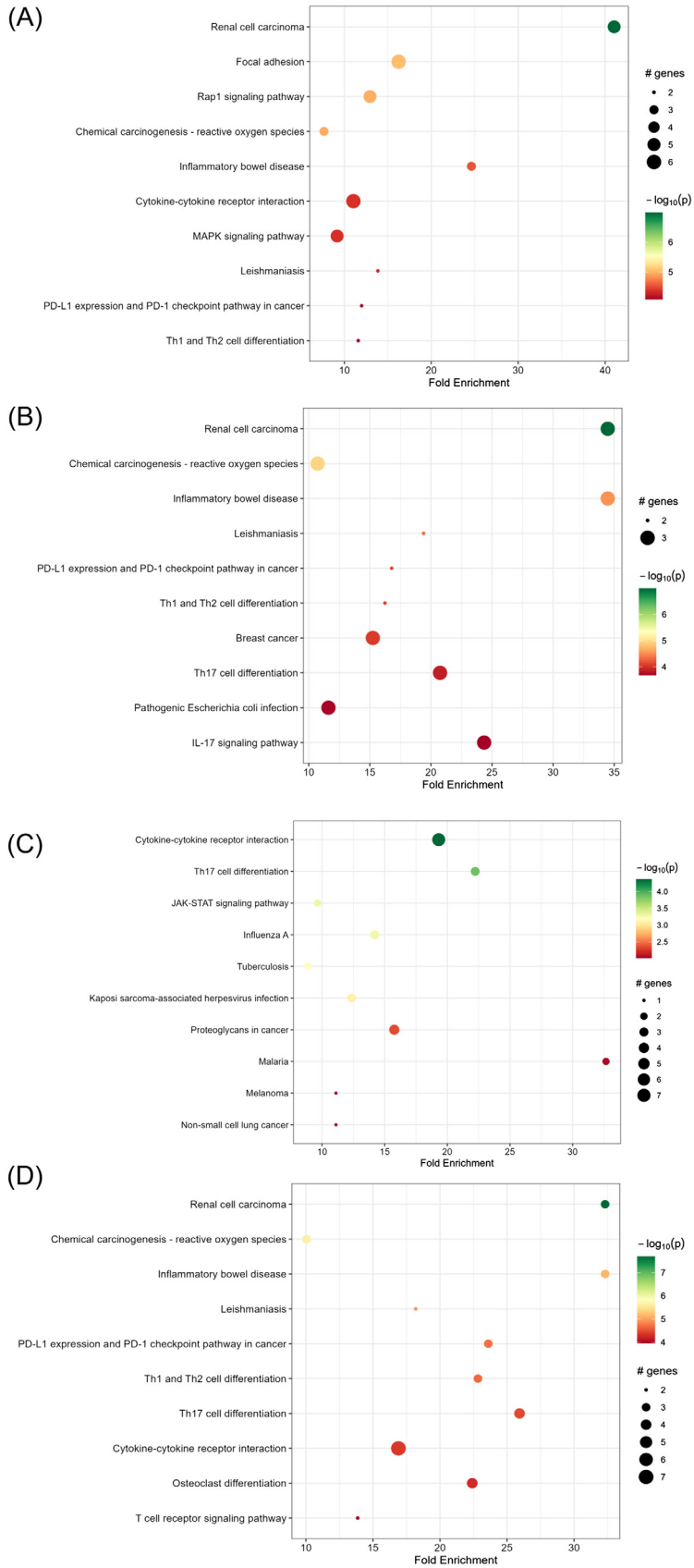

Figure 4. Pathway analysis of senescence proteins with significant differences in their plasma concentrations. Pathway analysis of all the significantly differentially regulated senescence proteins in hospitalized patients **(A)** ≤8 weeks since their positive PCR test and **(B)** >8 weeks since their positive PCR test. Pathway analysis of all the significantly differentially regulated senescence proteins in aged (>50 y old) patients **(C)** ≤8 weeks since their positive PCR test and **(D)** >8 weeks since their positive PCR test.

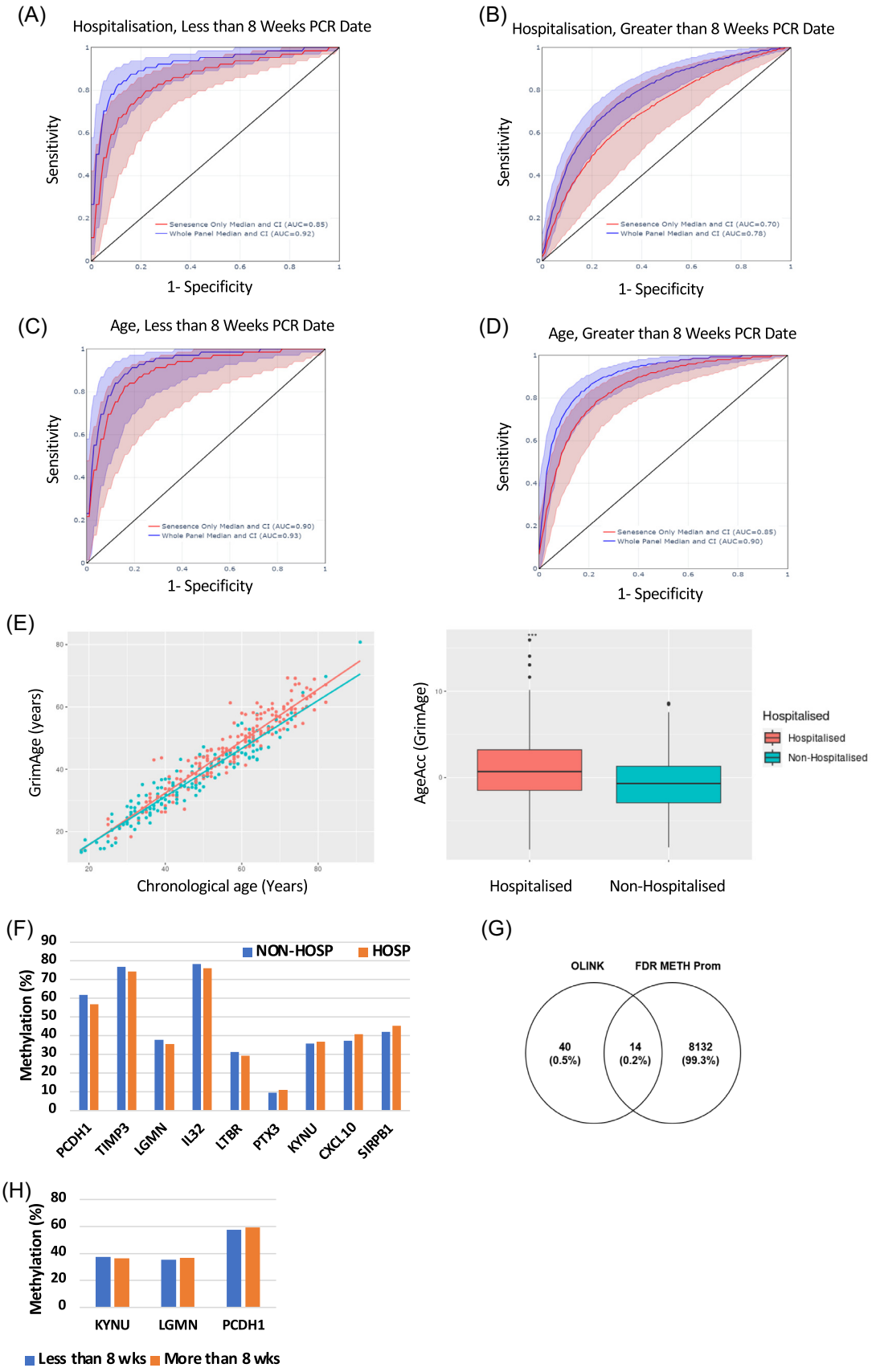

Figure 5. Persistent signatures and methylation-associated analysis. (A–D) Receiver operating characteristic (ROC) curve of levels of the whole panel signature (n = 5 proteins) versus a senescence-specific signature (n = 5 proteins) based on their hospitalization status and age ≤8 weeks or

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>8 weeks since their positive PCR test. **(E)** Correlation plot between the chronological age and biological age according to the GrimAge clock in hospitalized and non-hospitalized patients. **(F)** Top genes (n = 9) which had the largest change in methylation levels in the promoter region of senescence-specific proteins in hospitalized patients compared with non-hospitalized patients. **(G)** Venn diagram showing 25.93% of significant senescence proteins overlap with FDR-significant promoters between hospitalized and non-hospitalized patients. **(H)** Top genes (n = 3) which had the largest change in methylation levels in the promoter region of senescence-specific proteins in patients ≤8 weeks since their positive PCR test compared with patients >8 weeks since their positive PCR test.

methylation analysis using the GrimAge clock[46]. Studies to date are very limited[63–66] and this is the first study to our knowledge that has analyzed the methylation profile and specifically linked this to senescence of COVID-19 patients at such a large scale. We found that hospitalized patients have a higher epigenetic age than chronological age and appear to be aging at a faster rate. This may explain the higher risk of severe disease in these older patients. Interestingly, we found elevated protein levels of PTX3 and CXCL10 were counterbalanced by hypermethylation of promotor regions of both genes (**Fig. 5F**). We propose that the hypermethylation evident is not enough to dampen the cytokine storm in hospitalized and aged patients. Furthermore, we also identified genes which exhibited differences in their methylation profiles and maintained these for a longer-timeframe post-COVID-19 infection. These methylation changes may indeed be associated and indicative of post-COVID-19 syndrome.

Taken together, the findings from these studies suggest that the paradigm of senescence, an aging-related switch, underpins and is a critical regulator of SARS-CoV-2-triggered senescence which drives pathology in COVID-19. We believe that a predisposition to this senescence-evoked immune cascade, which is linked to aging, can be triggered by SARS-CoV-2 infection and lead to severe disease. However, this area of research is very much in infancy and substantial studies are needed to understand this SARS-CoV-2-evoked COVID-19 pathobiology. The clinical promise of senolytics as a novel treatment against COVID-19 is a promising area and previous studies have demonstrated that treatment with senolytics was able to eradicate VIS cells, alleviated COVID-19 lung disease, and reduced inflammation in two COVID-19-driven animal models[18–20] and also reduced viral load in aged hamsters[17]. This may allow new insights into the dynamics of COVID-19 disease, and we propose that utilizing sendotypes and the removal of such senescent cells or VIS cells could help prevent or mitigate severe COVID-19 and poor clinical outcomes.

Limitations

We acknowledge limitations in our study such as comorbidities, body mass index (BMI), age, gender, and antiviral use, which may also serve as important confounder/effect modifiers. We also acknowledge that the study design should compare concentrations of circulating proteins between hospitalized and non-hospitalized older adults that are matched for at least chronological age; however, this is not possible in this study design, as the sample size would become too small for machine learning to be performed.

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Author Contributions

All the authors have seen and approved the final version of the scientific article being submitted. All the authors warrant that this article is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere. S.M.L. wrote majority of the scientific article with intellectual inputs and write-up from T.S.R., S.M.L., A.J.B., and T.S.R. conceived the idea, designed the research, and critically revised it. Initial COVRES study design, ethics and governance approvals and recruitment drive was managed by D.S.G., E.K.M., C.W., A.J.B., and T.S.R., SM.M.L., E.C., D.M., G.G., S.W., T.M., A.E., M.B., and J.M. helped with data collection and formal analysis with intellectual inputs from T.S.R, S.W., D.S.G., E.K.M., V.E.M., S.D.Z., C.P.W., and P.S. L.F. and R.E.I. performed methylation analysis with intellectual inputs from C.P.W. M.K., and M.B. performed detailed clinical characterization of patients. A.P., M.O.K., D.S.G., A.J.B., and T.S.R. secured funding. All authors have read and agreed to the published version of the article.

Supplementary Materials

Supplemental information can be found here: Supplementary.

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