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Cardiovascular signatures of COVID-19 predict mortality and identify barrier stabilizing therapies

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Summary

Background Endothelial cell (EC) activation, endotheliitis, vascular permeability, and thrombosis have been observed in patients with severe coronavirus disease 2019 (COVID-19), indicating that the vasculature is affected during the acute stages of SARS-CoV-2 infection. It remains unknown whether circulating vascular markers are sufficient to predict clinical outcomes, are unique to COVID-19, and if vascular permeability can be therapeutically targeted.

Methods Prospectively evaluating the prevalence of circulating inflammatory, cardiac, and EC activation markers as well as developing a microRNA atlas in 241 unvaccinated patients with suspected SARS-CoV-2 infection allowed for prognostic value assessment using a Random Forest model machine learning approach. Subsequent ex vivo experiments assessed EC permeability responses to patient plasma and were used to uncover modulated gene regulatory networks from which rational therapeutic design was inferred.

Findings Multiple inflammatory and EC activation biomarkers were associated with mortality in COVID-19 patients and in severity-matched SARS-CoV-2-negative patients, while dysregulation of specific microRNAs at presentation was specific for poor COVID-19-related outcomes and revealed disease-relevant pathways. Integrating the datasets using a machine learning approach further enhanced clinical risk prediction for in-hospital mortality. Exposure of ECs to COVID-19 patient plasma resulted in severity-specific gene expression responses and EC barrier dysfunction, which was ameliorated using angiopoietin-1 mimetic or recombinant Slit2-N.

Interpretation Integration of multi-omics data identified microRNA and vascular biomarkers prognostic of in-hospital mortality in COVID-19 patients and revealed that vascular stabilizing therapies should be explored as a treatment

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for endothelial dysfunction in COVID-19, and other severe diseases where endothelial dysfunction has a central role in pathogenesis.

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Research in context

Evidence before this study

While diagnostic testing has allowed for the rapid identification of COVID-19 cases, the lack of post-diagnosis risk assessment metrics, especially among the highest-risk subgroups, has hampered decisions regarding the cascade and allocation of care. To date, the integration of clinical data with broad omics technologies has opened new avenues for efficiently delineating complex patient phenotypes and their associations with clinical outcomes, with circulating profiles of plasma microRNAs in particular, having been shown to be tightly associated with cardiovascular diseases and capable of providing not only detailed prognostic information but also mechanistic insight.

Added value of this study

Markers of endothelial dysfunction at presentation, while indicative of poor outcomes in COVID-19-positive patients, likely reflect systemic vascular dysfunction in critically ill patients and are not specific to SARS-CoV-2 infection. Moreover, the generation of a plasma microRNA atlas uncovers COVID-19-specific prognostic markers and multiple disease-specific pathways of interest, including endothelial barrier dysfunction. Furthermore, synthesis of electronic health record data with clinically relevant multi-omic datasets using a machine learning approach provides substantially better metrics by which mortality can be estimated in patients with severe COVID-19. Finally, targeted stabilization of the endothelial barrier with Q-Peptide or Slit2-N are novel therapeutic avenues that should be explored in COVID-19 patients.

Implications of all the available evidence

Together, our work provides biological insight into the role of the endothelium in SARS-CoV-2 infection, the importance of micoRNA as disease- and pathway-specific biomarkers, and the exciting possibility that endothelial barrier stabilizing treatments might hold promise in COVID-19.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly contagious betacoronavirus, which results in coronavirus disease 2019 (COVID-19).[1] While the majority of infected individuals manifest mild to moderate illness, 14-31% of symptomatic unvaccinated patients eventually require hospitalization, with intensive care unit (ICU) admission rates ranging from 2-27% among those hospitalized.[2,3] Select populations, particularly older individuals and those with underlying comorbidities (including cardiovascular disease [CVD]) have even higher rates of morbidity and mortality.[4] Vaccines against SARS-CoV-2 are highly effective in limiting hospitalization, ICU admission, and death.[5,6] However, lack of vaccine access and uptake due to global and regional inequities as well as vaccine hesitancy, coupled with highly infectious variants, means there remains a significant risk of mortality across the globe.[7-11] Given the substantial pressure being placed on healthcare systems worldwide, determining post-diagnosis risk assessment metrics in high-risk patients, particularly those with underlying CVD, is necessary to assist the cascade and allocation of care.

It has been proposed that the use of existing cardiovascular and respiratory parameters could serve as a metric of risk prediction.[12,13] However, case-fatality rates of those with comorbidities remain particularly high (e.g., preexisting CVD at ~10.5%), with cardiorespiratory markers having limited utility.[14] In this regard, while standard metrics including measures of cardiac damage (e.g., troponin values above the 99th percentile), the extent of inflammatory activation (e.g., C-reactive protein expression), and cardiovascular imaging have elucidated the spectrum of COVID-19 complications, they have only modestly elucidated the risk of adverse in-hospital outcomes and often provide limited insight into disease mechanisms.[15-18] Current COVID-19 therapeutics focus on stemming aberrant immune responses and controlling viral reproduction (e.g., tocilizumab, dexamethasone, and remdesevir), which may neglect other key elements of the host response contributing to severe outcomes.[19,20] From this perspective, clinical data and autopsy studies revealing endotheliitis and

thrombosis have raised the possibility that endothelial dysfunction, particularly alterations in vascular integrity and coagulative capacity, could be a driver of clinical outcomes.[21,22] Thrombosis, fluid extravasation, and microangiopathy observed in the small vessels and capillaries of the lungs directly support the notion that an intense vascular reaction takes place in those with severe disease.[23,24] Indeed, substantial evidence has demonstrated that the endothelium is dysregulated in COVID-19 infection,[25,26] and this has led to early investigation into supportive therapeutics, and the proposal that new therapeutics should prioritize endothelial stabilization.[27-31]

Early data suggested that viral tropism towards angiotensin-converting enzyme 2 and acetylated sialic acid residues, which are highly expressed by vascular endothelial cells (EC), could instigate cardiovascular dysfunction.[32-38] However, *in vitro* evidence has alternatively suggested that SARS-CoV-2 may have limited infectious potential and replicative ability in ECs.[39] In this respect, mechanisms secondary to direct infection, such as cascading immunological activation may instead be the driving factor behind the observed endothelial dysfunction,[40] particularly among populations with coexisting conditions where EC dysfunction is already evident.[41,42] In fact, recent single cohort analysis of select endothelium-related biomarkers such as thrombomodulin,[43] von Willebrand Factor,[44] angiopoietin-2 (Ang-2),[45] and soluble triggering receptor expressed on myeloid cells-1 (sTREM-1)[46] have shown utility in prognostication, being associated with both disease severity and in-hospital mortality. While markers of endothelial function may aid in prognostication, it seems unlikely that a simple combination of markers can provide insight significant enough to adjudicate the level of care a patient will need, nor is it apparent that these markers associate specifically with COVID-19 pathology. To date, the integration of clinical data with broad omics technologies has opened up new avenues to efficiently delineate complex patient phenotypes and their associations with clinical outcomes.[47,48] Circulating profiles of plasma microRNAs (miRNA) in particular have been shown to be tightly associated with disease and capable of providing not only detailed prognostic information but also mechanistic insight.[49,50] Indeed, circulating miRNAs are emerging as a potential biomarker for SARS-CoV-2 infection,[51-53] and may even be involved in regulating disease phenotypes,[54] and may be considered in novel treatments.[55,56] Whether miRNAs can be used as a prognosticator for disease severity and mortality in vulnerable groups such as patients with CVD remains largely unexplored. It is also unknown whether miRNA biomarkers of disease severity are distinct in COVID-19 and non-COVID-19 patients in ICU settings.

In this study, we sought to broadly characterize cardiovascular signatures, including markers of cardiac, endothelial, as well as inflammatory processes and to develop for the first time a comprehensive atlas of miRNA expression across a spectrum of unvaccinated individuals diagnosed with SARS-CoV-2 infection as well as SARS-CoV-2-negative patients from the ICU. Using clinical data and plasma samples from a large patient cohort (>240 patients), we determined that endothelial dysfunction and inflammatory markers at hospital admission were associated with mortality in COVID-19 patients, but these signatures were not specific to COVID-19 infection, as they were also elevated in a non-SARS-CoV-2 ICU population. Notably, miRNA signatures were able to specifically discriminate COVID-19 disease severity and predicted in-hospital mortality, in addition to revealing pathways relevant to disease pathogenesis. A machine learning algorithm utilizing miRNA and protein biomarkers predicted COVID-19 mortality better than clinical variables alone. In *ex-vivo* studies, analysis of the molecular pathways modulated in ECs exposed to plasma from COVID-19 patients revealed disruption of the vascular barrier and motivated us to rationally test therapeutics capable of modulating endothelial dysfunction. Here we report three key findings: 1) circulating cardiovascular biomarkers that are specific to COVID-19 mortality, 2) that the endothelium is grossly dysregulated in a COVID-19 severity-specific manner, and 3) proof-of-concept evidence for the promise of vascular stabilizing therapeutics.

Methods

Detailed materials and methods can be found in the Online Supplement and the Major Resource Tables (Online Tables I and II).

Synopsis of study design, patient demographics, and clinical severity

Two hundred and forty-one patients with suspected, community-acquired SARS-CoV-2 (acute infection) were enrolled prospectively in the emergency departments or upon admission at two urban, quaternary-care hospitals in Toronto, Canada (University Health Network and St. Michael's Hospital, Table 1 and Online Figure I) between May 2020 to December 2020 (prior to vaccine availability in Canada). Infection status of admitted patients was confirmed by at least two SARS-CoV-2 polymerase-chain reaction tests. Patients with SARS-CoV-2 but noninfectious etiologies for admission were not enrolled (e.g., blunt force trauma). Medical history, physical examination, and clinical laboratory values were recorded upon admission (day 0 or 1; t_{0-1}), two to three days later (t_{2-3}), and up to five days (t_{4-5}), along with the synchronous collection of blood samples (Online Figure I). Acute illness scores (Acute Physiologic Assessment and Chronic Health Evaluation II and

Characteristics*	Disease Severity					P-value
	Mild (COVID-19 Negative) (n=30)	Mild (COVID-19 Positive) (n=27)	Moderate (COVID-19 Positive) (n=39)	Severe (COVID-19 Positive) (n=76)	Severe (COVID-19 Negative) (n=69)	
Demographics						
Age, median (IQR) — yr.	47.0 (33.0-60.5)	59.0 (41.0-70.0)	74.0 (59.0-86.0)	61.0 (52.0-71.0)	61.0 (51.0-72.0)	**<0.0001**
Distribution — no. (%)						
18-40 yr.	11 (36.7)	6 (22.2)	4 (10.3)	2 (2.6)	11 (15.9)	**<0.0001**
41-64 yr.	13 (43.3)	9 (33.3)	9 (23.1)	45 (59.2)	26 (37.7)	**0.0025**
≥65 yr.	6 (20.0)	12 (44.4)	26 (66.7)	29 (38.2)	32 (46.4)	**0.0023**
Male Sex, no./total no. (%)	17/30 (56.7)	15/27 (55.6)	25/39 (64.1)	52/76 (68.4)	46/69 (66.7)	0.6564
BMI, median (IQR)†	25.2 (21.3-32.6)	25.9 (22.5-35.0)	26.0 (21.1-30.0)	28.1 (24.2-31.8)	25.7 (21.8-32.5)	0.4798
Obesity‡, no./total no. (%)	4/19 (21.1)	6/18 (33.3)	6/27 (22.2)	38/61 (62.3)	35/69 (50.7)	**0.0006**
Smoking History — no./total no. (%)§						
Current smoker	4/12 (33.3)	3/9 (33.3)	3/5 (60.0)	2/43 (4.7)	12/69 (17.4)	**0.0027**
Previous smoker	5/12 (41.7)	0/9 (0.0)	2/5 (40.0)	5/43 (11.6)	4/69 (5.8)	**0.0032**
Never smoked	3/12 (25.0)	6/9 (66.7)	0/5 (0.0)	36/43 (83.7)	53/69 (76.8)	**<0.0001**
Length of Admission — days (IQR)	0 (0-2.3)	2 (0-13)	12 (7-24)	28 (16-65)	14 (7.5-28)	**<0.0001**
Mortality	-	3/27 (11.1)	1/39 (2.6)	29/76 (38.2)	21/69 (30.4)	**<0.0001**
Co-Existing Disorder — no./total no. (%)						
Arrythmia	3/30 (10.0)	3/27 (11.1)	9/39 (23.1)	10/69 (14.5)	12/69 (17.4)	0.6140
Asthma	1/30 (3.3)	1/27 (3.7)	2/39 (5.1)	8/70 (11.4)	3/69 (4.3)	0.4970
Cardiac procedure	2/30 (6.7)	2/27 (7.4)	10/39 (25.6)	6/69 (8.7)	7/69 (10.1)	0.9740
Chronic kidney disease	1/30 (3.3)	1/27 (3.7)	13/39 (33.3)	10/70 (14.3)	6/69 (87.0)	**0.0204**
Congestive heart failure	3/30 (10.0)	3/27 (11.1)	5/39 (12.8)	5/69 (7.2)	10/69 (14.5)	0.7389
COPD	3/30 (10.0)	5/27 (18.5)	5/39 (12.8)	6/69 (8.7)	14/69 (20.3)	0.3134
Coronary artery disease	6/30 (20.0)	5/27 (18.5)	8/39 (20.5)	6/69 (8.7)	8/69 (11.6)	0.2802
Diabetes mellitus	2/30 (6.7)	11/27 (40.7)	16/39 (41.0)	35/69 (50.7)	19/69 (27.5)	**<0.0001**
Dyslipidemia	5/30 (16.7)	6/27 (22.2)	19/39 (48.7)	22/69 (31.9)	16/69 (23.2)	**0.0266**
Gastroesophageal reflux disease	0/30 (0.0)	1/27 (3.7)	0/39 (0.0)	10/69 (14.5)	5/69 (7.2)	**0.0019**
Gout	0/30 (0.0)	1/27 (3.7)	0/39 (0.0)	2/70 (2.9)	0/69 (0.0)	0.2165
Hypertension	6/30 (20.0)	12/27 (44.4)	20/39 (51.3)	40/69 (58.0)	30/69 (43.5)	**0.0105**
Immunocompromised	2/30 (6.7)	1/27 (3.7)	1/39 (2.6)	1/70 (1.4)	2/69 (2.9)	0.6173
Malignancy						
None	29/30 (96.7)	22/27 (81.5)	33/39 (84.6)	65/70 (92.9)	63/69 (91.3)	0.2279
Active	0/30 (0.0)	3/27 (11.1)	2/39 (5.1)	2/70 (2.9)	2/69 (2.9)	0.2605
Previous	1/30 (3.3)	2/27 (7.4)	4/39 (10.3)	3/70 (4.3)	4/69 (5.8)	0.7546
Myocardial infarction	3/30 (10.0)	2/27 (7.4)	3/39 (7.7)	5/69 (7.2)	4/69 (5.8)	0.9505
Obstructive sleep apnea	2/30 (6.7)	3/27 (11.1)	4/39 (10.3)	8/70 (11.4)	5/69 (7.2)	0.9038
Non-COVID-19 Pneumonia	0/30 (0.0)	0/27 (0.0)	0/39 (0.0)	2/70 (2.9)	2/69 (2.9)	0.8958
Peripheral vascular disease	1/30 (3.3)	2/27 (7.4)	1/39 (2.6)	3/70 (4.3)	3/69 (4.3)	0.9263
Stroke	0/30 (0.0)	0/27 (0.0)	0/39 (0.0)	3/70 (4.3)	0/69 (0.0)	0.3294
Concurrent Therapeutics — no./total no. (%)						
Angiotensin II receptor blockers	1/30 (3.3)	4/26 (15.4)	2/39 (5.1)	3/64 (4.7)	1/68 (1.5)	0.1054
ACE inhibitor	1/30 (3.3)	2/26 (7.7)	2/39 (5.1)	14/64 (21.9)	7/68 (10.3)	0.1007
Anticoagulant	7/30 (23.3)	5/26 (19.2)	4/39 (10.3)	6/64 (9.4)	7/68 (10.3)	0.2749
Beta blocker	4/30 (13.3)	4/26 (15.4)	15/39 (38.5)	16/64 (25.0)	12/68 (17.6)	0.0781
Calcium channel blocker	3/30 (10.0)	4/26 (15.4)	6/39 (15.4)	16/64 (25.0)	4/68 (5.9)	**0.0338**
Diuretics	1/30 (3.3)	1/26 (3.8)	5/39 (12.8)	14/64 (21.9)	18/68 (26.5)	**0.0088**
Statin	3/30 (10.0)	7/26 (26.9)	18/39 (46.2)	21/64 (32.8)	20/68 (29.4)	0.2992

***Table 1*: Patient Demographics and Clinical Characteristics of Patients at T_{0-1}.**

Abbreviations: ACE = Angiotensin-converting-enzyme inhibitors; COPD = Chronic obstructive pulmonary disease; BMI = Body Mass Index; IQR = Interquartile range. Percentages may not add up to 100% due to rounding.

* Summary statistics are based on the full population indicated in the column heading.

† Body mass index is the weight in kilograms divided by the square of the height in meters.

‡ Obesity is classified according to World Health Organization guidelines (i.e., >25).

§ Smoker is defined as an aggregate of self-reported current and previous smokers.

Sequential Organ Failure Assessment) were adjudicated upon admission. As a result of sampling logistics, t_0 or t_1 (days) were grouped as the earliest timepoint available for admitted patients. Similarly, collapsed sampling timepoints at t_{2-3} and t_{4-5} allowed evaluation of patient trajectories. Since the analysis was conducted retrospectively, clinical care was dictated by individual care providers with the primary outcome being mortality.

The cohort of 241 patients was categorized into three groups that reflected conventional concepts of COVID-19 severity (National Institutes of Health, 'Clinical Spectrum of SARS-CoV-2 Infection'), as well as two analogous symptom/severity matched control groups.[57] The resulting five groups were: SARS-CoV-2 negative patients presenting to outpatient clinics with symptoms consistent with a respiratory tract illness (n=30, "mild negative"); SARS-CoV-2 positive patients presenting to outpatient clinics with symptoms consistent with a respiratory tract illness (n=27, "mild COVID-19"); admitted SARS-CoV-2 positive patients requiring supplemental oxygenation (n=39, "moderate COVID-19"); SARS-CoV-2 positive patients requiring high-level inpatient ICU care (n=76, "severe COVID-19"); and SARS-CoV-2 negative patients who exhibited symptoms of a severe respiratory disease requiring high-level inpatient ICU care (n=69, "severe negative"). Patients testing negative by nasopharyngeal SARS-CoV-2 polymerase chain reaction had immunological history (i.e., antibody reactivity) ascertained through spike antigen cross-reactivity using a Federal Drug Administration-approved enzyme-linked immunosorbent sandwich assay (Online Figure II).

Data visualization and statistical analysis

All data generated and analyzed that support the findings of this study are included in this article. Associated supplementary information files are available on a publicly accessible archive (see below).

Descriptive analysis. Clinical variables were characterized using summary statistics. Continuous variables were described using median and inter-quartile range (IQR), and dichotomous or polytomous variables were described using frequencies. Between-group differences were evaluated using Wilcoxon rank-sum tests for continuous variables and Fisher's exact tests for dichotomous/polytomous variables. Correlation between continuous variables were quantified using Spearman rank correlation.

Descriptive outcome analysis. The Kaplan-Meier survival method was applied to assess in-hospital death, and between-group differences in freedom from death were evaluated using Gray's test. The length of hospital admission was characterized using competing risk models in terms of cumulative incidence rate function. Univariable Cox proportional hazard regression was applied to assess and quantify the association of the baseline clinical characteristics with in-hospital death. The associations of continuous variables were modeled using natural cubic splines. *Biomarker Analysis* - Comparisons between two independent groups were made using *t*-tests for normally distributed continuous variables or Wilcoxon rank-sum tests for non-normally distributed continuous variables. When more than two groups were compared, either a one-way ANOVA with a Tukey or Bonferroni post-hoc test (where appropriate) for multiple testing correction, or Kruskal-Wallis one-way analysis of variance with Dunn's multiple comparison correction was used. Two-way ANOVA was used to estimate how the mean quantitative variable changes according to time and for group differences in EC barrier disruption experiments. Where appropriate, Benjamini-Hochberg false discovery rate (FDR) was utilized with adjusted P values (or Q value where stated) of <0.05 being considered statistically significant and indicated in the graphs as reported by the analysis software with significance thresholds of $P<0.05$, $P<0.01$, $P<0.001$, and $P<0.0001$ indicated as *, **, ***, **** respectively. MiRNA pathway analysis was conducted using BioCarta/KEGG/Reactome databases and tested for enrichment by a hypergeometric test with adjustment for multiple comparisons using the Benjamini-Hochberg FDR, with $P \leq 0.05$ considered to be statistically enriched in a gene set of interest.[58-60] Although many hypotheses were tested throughout the manuscript, no experiment-wide multiple test correction was applied.

Risk assessment using machine learning. We performed 250 experiments using repeated randomized stratified sub-sampling cross-validation into 80% training and 20% testing using Python (v3.8.8) and scikit-learn[61] (v.0.24.1). Categorical features were hot encoded, with missing variables recorded as additional categorical variables, having -1.0 for numerical features. For each experiment, a Random Decision Forest model was fit to the training dataset and evaluated on the independent testing set.[62] Model performance was assessed by the area under the receiver operating characteristic (AUROC) calculated on the testing set. Average AUROC and 95% confidence intervals were calculated across the 250 runs using the percentile method. Feature importance was estimated using permutation feature importance and aggregated across the 250 runs. Clinical data models included age, diabetes at baseline, hypertension at baseline, dyslipidemia at baseline, coronary artery disease at baseline, myocardial infarction at baseline, heart failure at baseline, valvular heart disease at baseline, arrhythmia at baseline, family history of cardiac disease, relationship between patient and family member with

history of cardiac disease, diagnosis of family member with history of cardiac disease, history of chronic obstructive pulmonary disease at baseline, smoking history, and COVID-19 diagnosis as covariates within the model. Protein models included all available protein biomarker data while the miRNA model had a multiphase selection, whereby all miRNA were inputted into the first phase (feature selection via collinearity reduction), with the remaining 102 miRNA that experienced limited collinearity being used to train the model. Subsequent combinatorial iterations (e.g., Clinical data and Protein Expression) combined the features of these datasets.

Visualization. Unless indicated otherwise, graphs depict averaged values of independent data points with technical replicates and have error bars displayed as mean +/- standard deviation (±S.D.). Data were analyzed with GraphPad Prism 9.0.0 for MacOS (GraphPad Software, Inc., La Jolla, CA, USA; Biomarker Multiple Comparisons), R[63] (v4.0.3; Spearman Correlation Plots), and FIJI[64] (v2.1.0/1.53c; Quantifying Image Intensities). Final figures were assembled for publication purposes using Adobe Illustrator (v25.4.1).

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Results

The baseline characteristics for the cohort are indicated in Table 1. The median age (interquartile range [IQR]) of the entire cohort (n=241; 155 male patients [64.3%]) was 61 [51-72] years, and of those, 201 were admitted, having a median hospital stay of 18 (IQR, 8-40) days during which 54 (26.9%) died (Table 1). Regardless of admission or SARS-CoV-2 status, co-existing medical conditions were common amongst the population, including 83 (out of a total of 234; 35.5%) with diabetes mellitus, 80 (34.4%) with underlying CVD, 31 (12.9%) having chronic kidney disease, and 159 (66.0%) patients having more than one co-existing condition. In this respect, there were significant differences in age (P<0.0001), and the frequency of obesity (P=0.0006), smoking history (P<0.0001), chronic kidney disease (P=0.0204), diabetes mellitus (P<0.0001), dyslipidemia (P=0.0266), gastroesophageal reflux disease (P=0.0019), hypertension (P=0.0105), calcium channel blocker treatment (P=0.0338), and use of diuretics (P=0.0088) between groups (Table 1). Although a small proportion of patients had documented smoking status, there were 40 (total 138; 29.0%) current or former smokers, with 33 (total 234; 14.1%) patients having chronic obstructive pulmonary disease.

On admission to hospital, clinical lab data highlighted disparities between the groups with leukocytosis and lymphopenia seen amongst more severe COVID-19-positive and -negative groups (e.g., white blood cell count [P<0.0001]; lymphocytes [P<0.0023, Online table III]). Amongst cardiovascular markers, there were significant differences in the frequency of elevated creatine kinase (P<0.0001) and D-dimers (P<0.0001). Although the etiology of admissions was predominantly extracardiac in nature, high-sensitivity troponin I (hs-CnTI) values at t_{0-1} were detectable (>3pg/mL) in 157 patients (65.1%), with values significantly elevated (>15pg/mL in males; >10pg/mL in females) in 90 patients (37.3%, Online Table III), primarily in the severe COVID-19-positive and -negative groups (P<0.0001).

COVID-19 outcomes during hospital admission

Amongst the 132 patients admitted with COVID-19, 33 died, with Kaplan-Meier survival curves indicating that patients with severe disease had higher mortality risk than did patients with less severe phenotypes (Gray's test, P<0.001, Figure 1A). Furthermore, the only baseline clinical metrics associated with mortality within admitted patients were the history of coronary artery disease (Log-rank, P=0.011) and age at hospital admission (Log-rank, P=0.046, Online Table IV and Online Figure III). While gout was also significant (P=0.003), there were only two patients with this condition in the severe COVID-19 group and none in the severe COVID-19-negative group. During hospitalization, 76 COVID-19 patients (44.2%) were transferred to the ICU (severe COVID-19) immediately upon admission. Of these, 23 (30.3%) were treated with non-invasive ventilation, 53 (69.7%) with invasive ventilation, and 21 (27.6%) underwent extracorporeal membrane oxygenation. When compared to severity-matched (i.e., SOFA and APACHE II assessments completed at time of admission before COVID-19 status was determined) SARS-CoV-2-negative controls, patients with severe COVID-19 had on average longer ICU stays (mean 13 [IQR, 7-35] days versus mean 8 [IQR, 3-15] days, P<0.0001), were more likely to display acute respiratory distress syndrome (ARDS, P=0.0195), and had overall worse oxygenation, requiring higher FiO2 (P<0.0001) as well as having lower PaO_2/FiO_2 ratios (P=0.0003, Online Table V). Interestingly, although many cardiovascular metrics were unchanged, admitted COVID-19 patients had an increase in the number of noted arrhythmic events (P=0.007) and a higher number of secondary cardiovascular events (P=0.001, Online Table V, Defined in Online Methods). However, when compared to severity

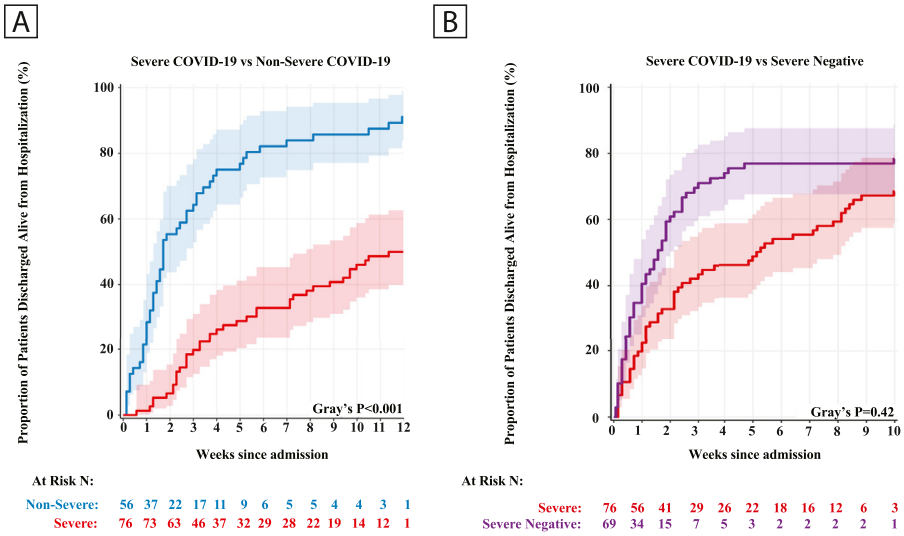

Figure 1. Unadjusted Kaplan-Meier Estimates of Survival. (a) Gray's test comparing curves of all admitted patients with severe COVID-19 to all admitted non-severe COVID-19 patients (i.e., mild and moderate vs severe, P<0.001). (b) Gray's test comparing curves of those with severe COVID-19 to severity matched SARS-CoV-2 negative patients (P=0.42). Refer to Online Figure III as well as Online Tables IV-VI for more information.

matched SARS-CoV-2 negative patients, there were no significant differences in overall survival (P=0.42; Figure 1B).

Associations of cardiovascular and inflammatory biomarkers with outcomes

The vascular endothelium acts as the crucial interface between blood components and tissues, displaying a series of properties that maintain homeostasis (i.e., maintenance of vascular barrier, coagulative capacity, and modulation of immunological responses).[42,65,66] While these functions participate in the dynamic regulation of cardiovascular homeostasis and coordinate many host defense mechanisms, proinflammatory cytokines are known to elicit a change in endothelial phenotype, promoting thrombosis, local tissue injury, propagating inflammation and potentially contributing to mortality.[67] In order to test the hypothesis that metrics of cardiac damage and inflammatory endothelial dysfunction/activation may better reflect COVID-19 severity and subsequent mortality than standard clinical metrics, a custom Simple Plex assay for markers robustly associated with inflammation and endothelial dysfunction was performed (i.e., Ang-2, endothelin-1 [ET-1], soluble intercellular adhesion molecule [sICAM], soluble vascular cell adhesion molecule [sVCAM], soluble E-Selectin [sE-selectin], sTREM-1, interleukin-6 [IL], and IL-8), along with standalone assays measuring myeloperoxidase (MPO) and high-sensitivity cardiac troponin (hs-cTnI). Correlation analysis revealed that Ang-2, sE-Selectin, and sICAM had moderate correlations with numerous other inflammatory markers, with correlation coefficients ranging from -0.89 (IL-6:MPO) to 0.89 (sICAM-1:Ang-2; Online Figures IV-IX). Myeloperoxidase was the only biomarker without a significant correlation. Among these markers, two were significantly different between mild SARS-CoV-2 positive patients and SARS-CoV-2 negative patients with mild illness at t_{0-1} (elevated Ang-2, P=0.0200; elevated MPO, P=0.0439, Figure 2A, B, Online Figure X). In contrast, nine of the markers (Ang-2, ET-1, sICAM-1, sVCAM-1, sE-Selectin, sTREM-1, IL-6, IL-8, and MPO, Figure 2A, B, Online Figure X) reflected differences among severity within the SARS-CoV-2 positive cohort but failed to demonstrate significance between the critically ill patient groups that did or did not have COVID-19. Of note, hs-cTnI was only elevated in the severe groups, and no differences were observed between those that did or did not have COVID-19-related critical illness.

Amongst all patients admitted with COVID-19, univariable analysis revealed that only Ang-2 was associated with mortality (P=0.015; Online Table IV), suggesting higher concentrations are associated with higher mortality, while both Ang-2 (P=0.020) and sVCAM-1 (P=0.012) were associated with mortality when looking specifically at the severe COVID-19 patients (Figure 2C, D, Online Table VI). Sub-stratifying these markers by severity, there were significantly higher t_{0-1} concentrations of Ang-2, IL-6, and MPO in non-survivors with COVID-19 when compared to the severe SARS-CoV-2 negative patients (Online Figure XI). Over time, only IL-6 and MPO remained significantly different between severe COVID-19 and severe negative patients (Online Figure XII and XIII). Taken together, while markers of inflammation/endothelial dysfunction were observed at early

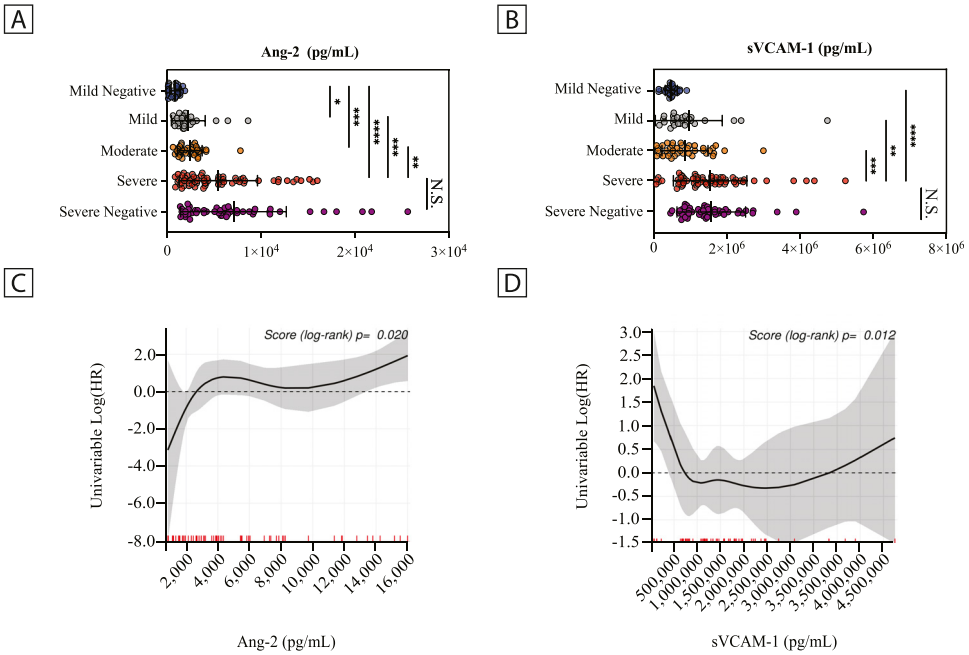

Figure 2. Plasma Concentration of Endothelial Dysfunction and Inflammatory Markers at $T_{0\text{-}1}$. (a) Levels of Ang-2 and (b) sVCAM-1 stratified among disease severity. Data shown are for all patients with an available $t_{0\text{-}1}$ sample (n=210), with values representing the mean and error bars are (±S.D.). (c) Unadjusted log hazard ratio of Ang-2 and (d) sVCAM-1 in severe disease patients (n=76) with univariable p-value in association with mortality. P values for multiple group comparisons were determined by Kruskal-Wallis test with Dunn's multiple comparisons test. Severe negative statistical comparisons are only shown in reference to the concordant severe group. Red hash marks indicate individual samples. Abbreviations: Ang-2, Angiopoietin-2; VCAM-1, Vascular cell adhesion molecule 1; N.S., non-significant. Refer to Online Figure IV-XIII for more information and additional endothelial and inflammatory markers.

time points and associated with severity, the majority were not specific to either COVID-19 status or mortality.

Plasma miRNA atlas of SARS-CoV-2 infection reveals markers specific to COVID-19 and mortality

While select inflammatory/EC activation markers were informative for ICU mortality, they lacked the ability to distinguish between COVID-19 and non-COVID-19 pathology. We postulated that assessment of the circulating miRNA transcriptome may provide further precision with respect to patient subgroups, since miRNA profiling (in contrast to circulating protein markers) has been shown to effectively differentiate complex disease etiologies.[68,69] Using whole transcriptome miRNA sequencing (2,083 mature miRNAs) we screened plasma obtained from the differing groups of disease severity to identify meaningful differences in miRNA composition (n=30, negative mild; n=14, mild COVID-19; n=15, moderate COVID-19; n=36, severe COVID-19; n=33, negative severe). Comparative analyses indicated that there were substantially higher numbers of differentially expressed miRNA as disease severity progressed (Figure 3A; Fold ≥±1.5 and Q<0.05). Comparing severe COVID-19 to severe SARS-CoV-2 negative patients revealed 765 differentially expressed miRNAs that could subsequently be used for group differentiation (Figure 3B). DIANA-mirPath pathway analysis on the differentially expressed miRNAs suggested broad enrichment of pathways, including those related to cardiomyocyte function (i.e., ErbB2 signaling and arrhythmogenic right ventricular cardiomyopathy) as well as adherens junctions (Figure 3C). Sub-analysis of the severe COVID-19 patients for mortality revealed 207 differentially expressed miRNA between survivors and non-survivors (Figure 3D), including pathway enrichment for platelet activation, extracellular matrix-receptor interactions, Ras, and ErbB2 (Figure 3E). A full list of differentially expressed miRNA and predicted KEGG pathways is available in Online Data Files IV and V as well as Online Figures XIV and XV.

Predictive power of clinical, protein, and miRNA data on mortality using machine learning

Given the high dimensionality of the datasets generated, we sought to examine the utility of models developed using machine learning to predict in-hospital mortality of COVID-19 patients at admission, based on common clinical data, protein expression data, and miRNA expression data. We performed 250 experiments using repeated randomized stratified sub-

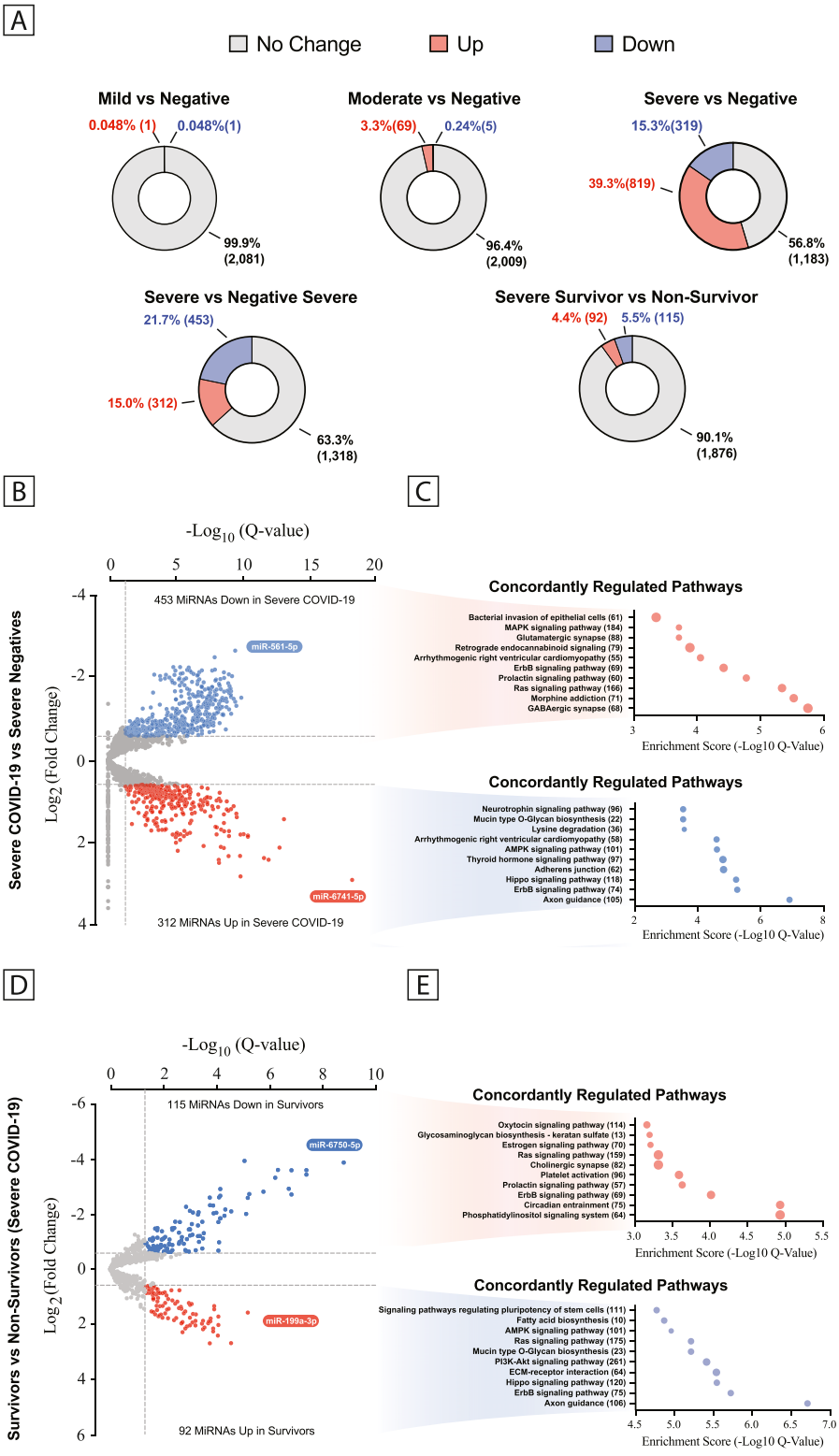

Figure 3. Plasma MiRNA Transcriptome Across the COVID-19 Severity. (a) Pie chart percent modulation of the transcriptome for all subgroups studied. Volcano plots of differentially expressed miRNA between patient groups (b, d) with predicted KEGG terms (with enrichment score below and number of genes to the right) for pathways of deregulated miRNAs shown beside each corresponding region of the volcano plot (c, e). Data are displayed as false discovery rate (FDR) adjusted P values (Q values) vs the $\log_2$ fold change, with dashed lines drawn to define restriction boundaries. Refer to Online Figure XIV-XV for more information.

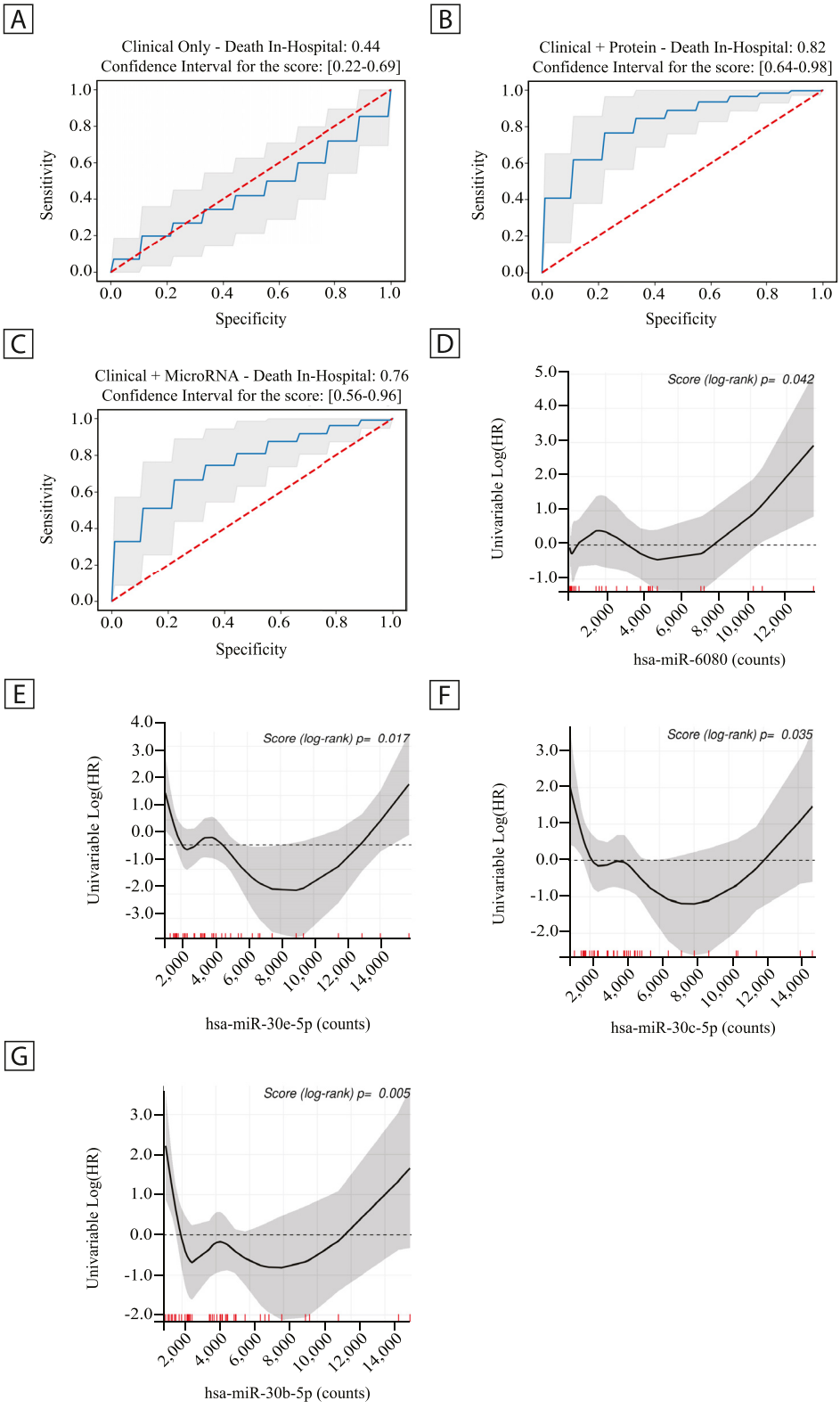

Figure 4. Machine Learning Approach to Risk Assessment and Association of Biomarkers with In-Hospital Mortality for Severe COVID-19 Patients. Assessment of datasets using repeated randomized stratified sub-sampling cross-validation (Random Forest machine learning) for (a) clinical data, (b) clinical data and protein expression metrics, and (c) clinical data and miRNA atlas

sampling cross-validation into sets of 80% training and 20% testing, to train a set of Random Forest models.[62] We assessed model performance by the area under the receiver operating characteristic (AUROC) calculated on the testing sets. Aggregate statistics on AUROC were calculated across the 250 experiments. We observed a low AUROC using only clinical features available at the time of admission (AUROC 0.44, 95% CI 0.22-0.69, Figure 4A).[62] However, incorporation of either the protein expression data or miRNA data improved the performance over the models that used conventional clinical predictors alone, having AUROCs of 0.82 (95% CI 0.64-0.98) and 0.76 (95% CI 0.56-0.96) respectively (Figure 4B, C). In the clinical data only models, ranking input variables that contributed to model predictivity revealed age and body mass index were amongst the most important (Online Figure XVI). Furthermore, in contrast to the traditional univariate statistical analyses, MPO, sTREM-1, and Ang-2 were listed as the top contributing features in the synthesized clinical and protein multivariate machine learning model, coming before any other traditional clinical factors (Online Figure XVII). Owing to the exceedingly high dimensionality (2,083 features), machine learning did not rank specific variables within the synthesized clinical and miRNA model with statistical reliability.

Identification of miRNAs associated with severe COVID-19 mortality

Since miRNAs provided superior specificity for COVID-19 mortality compared to protein biomarkers (Figure 2, 3), we next sought to identify candidate miRNA markers that significantly contribute to mortality risk. We considered miRNAs within the top 50% of abundance and cross-examined the differential expression between both survivors and non-survivors of COVID-19 as well as SARS-CoV-2 severe negatives. Additionally, we took into consideration existing biological relevance, thereby specifically analyzing miR-1, which is associated with myocardial injury,[70,71] miR-199a-3p which has been shown to be cardioprotective,[72] miR-181a-5p which has been shown to restrict vascular inflammation,[73] along with members of the miR-30 family which are enriched in ECs and capable of modulating inflammation,[74–76] as well miR-339-3p and miR-6080 which were among the highest differentially expressed. Univariable hazard ratios and log-rank P-values were generated to determine the relationship of the miRNA expression measured in plasma with mortality. When ranking by the significance of independent association with mortality, miRNAs were among the highest-ranking factors compared to other clinical metrics (Online Table IV and VI) highlighting that miR-30b/c/e, -6080, -181a-5p, -199a-3p, and -339 (Figure 4D-G, Online Figures XVIII) were specific for COVID-19 severity and mortality. In contrast, miR-1 failed to show a significant association with mortality (Online Table VI).

Endothelial barrier disruption is rapidly induced during co-incubation with plasma from patients with moderate to severe COVID-19

With studies showing conflicting evidence that SARS-CoV-2 can directly infect the endothelium in a physiologically meaningful fashion,[21,77,78] we reasoned that modulation of the extracellular milieu (i.e., changes to circulating plasma components such as sTREM-1, Ang-2, and MPO) as a result of the systemic immune response may be a driving factor behind the observed endothelial dysfunction. In this context, data from previous studies conducted in systemic inflammatory response syndrome and sepsis have underscored the impact of endothelial barrier disruption on disease outcomes.[79] The data presented so far suggest that dysfunction of the endothelium may also accompany COVID-19 and may be associated with poor outcomes. To further examine this hypothesis, we utilized two validated permeability platforms using an endothelial monolayer model: continuous monitoring of transendothelial electrical resistance (Figure 5A) using the xCelligence platform and a transwell system consisting of a confluent monolayer of pooled human umbilical vein ECs on a semi-permeable membrane. In a timeframe that would exclude viral replication,[39,80] cells were treated with 20% (v/v) plasma from the $t_{0\text{-}1}$ samples from across the disease severity spectrum of COVID positive and negative patients. Moderate and severe COVID-19 patient plasma induced significant endothelial barrier dysfunction, while the mild and mild negative patient plasma did not induce significant EC leak in the xCelligence assay (Figure 5B).

Similarly, using a validation cohort, the integrity of the monolayer was gauged through leakage of a large dextran tracer across the EC barrier in both acute (i.e., one-hour) and longer-term (i.e., six-hour) treatment. This revealed barrier disruption in response to moderate and severe COVID-19 patient plasma, but not in response to plasma from severe SARS-CoV-2 negative patients (Online Figure XIX). Of the mediators tested, EC permeability correlated with levels of Ang-2, hs-cTnI, ET-1, IL-6, IL-8, sTREM-1, and MPO (Online Figure XX). These experiments provided *in vitro* evidence that barrier dysfunction can be independent of

expression metrics. Univariable log hazard ratios of candidate miRNAs (d) hsa-miR-6080, (e) hsa-miR-30e-5p, (f) hsa-miR-30c-5p, and (g) hsa-miR-30b-5p in relation to mortality. Red hash marks indicate individual samples. Abbreviations: HR, Hazard Ratio; miRNA, MicroRNA. Refer to Online Figure XVI-XVIII for more information.

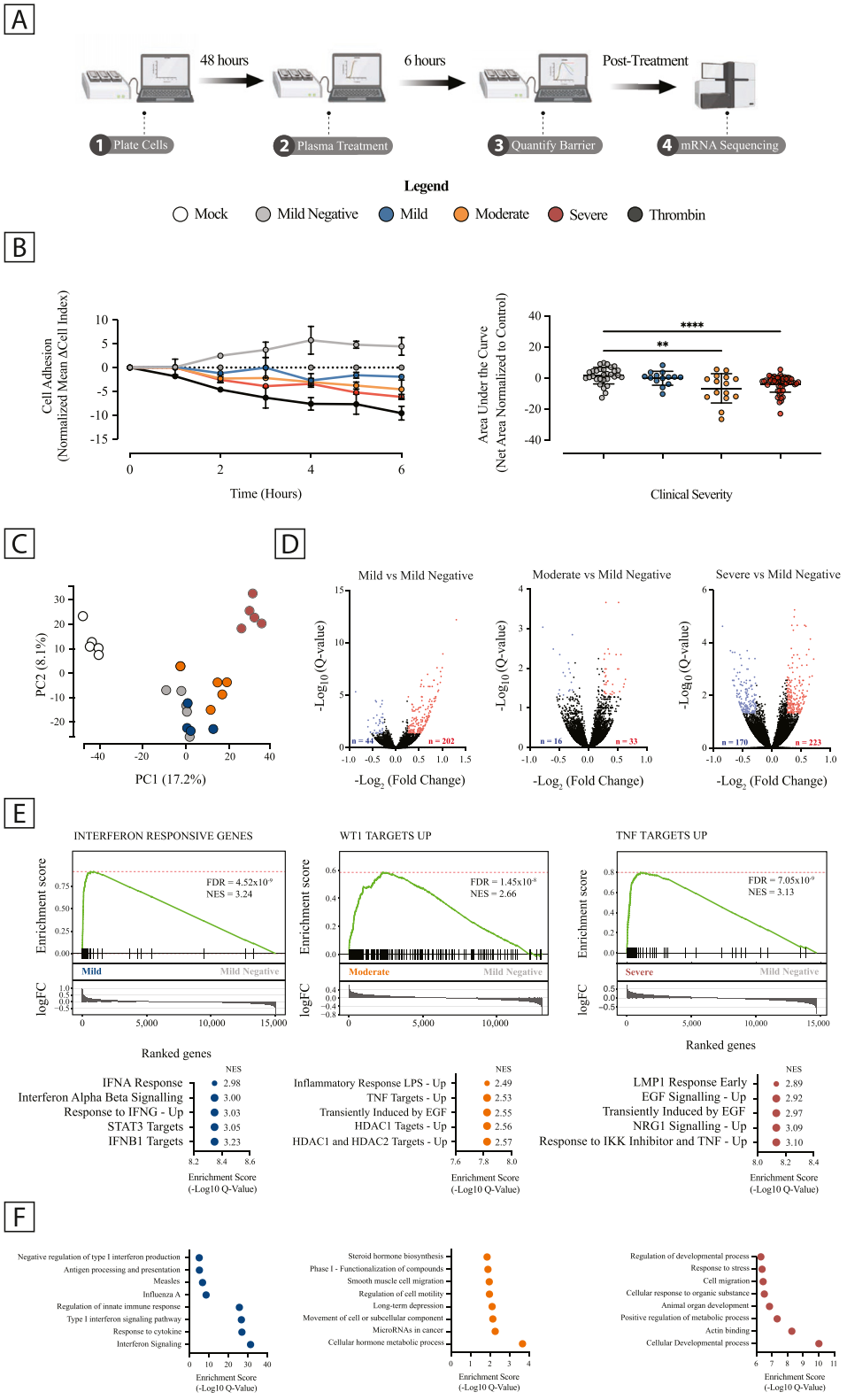

Figure 5. Endothelial Barrier Disruption Driven by Modulation of Inflammatory and Cytoskeletal Pathways is Disease Severity Dependent. (a) Overview of parallel endothelial phenotype monitoring. The overall scheme is shown, representing the real-time monitoring of EC barrier function (xCelligence) followed by both mRNA sequencing and multiplexed immunohistochemistry. (b) Time-

direct SARS-CoV-2 infection. We reasoned that elucidating the unique pathways through which the more severe COVID-19 phenotypes exert their barrier disruptive effects may reveal potential therapeutic approaches to maintain EC barrier.

Modulation of inflammatory and cytoskeletal processes are coincident with barrier dysfunction

To gain a better understanding of how the endothelium is modulated by plasma components, we investigated the gene-level changes in ECs after six hours of co-incubation with patient-derived plasma using RNA-sequencing. On the transcriptome level (16,285 total quantified genes, having ≥10 reads in at least five samples), biological replicates within groups were tightly correlated (Pearson r=0.975-0.988, n=4-5), suggesting robust intra-group clustering even among a heterogeneous patient group, similar to previous studies.[47] Principal component analysis of the normalized transcriptome showed segregation between experimental groups, with the severe group being clearly distinct from the mild and moderate groups (Figure 5C). Pair-wise differential expression analysis with the mild SARS-CoV-2 negative cohort as the control revealed 393, 49, and 246 genes are differentially expressed (FDR < 0.05, $\log_2$ fold change >±0.58) in the mild, moderate, and severe COVID-19 cohorts, respectively (Figure 5D). Gene set enrichment analysis revealed that co-incubation with either severe or moderate COVID-19 patient plasma altered the expression of endothelial genes related to acute inflammatory response, angiogenic programs, or histone deacetylase activity, whereas administration of plasma from mild COVID-19 patients altered the expression of genes involved in priming of antiviral responses (Figure 5E and Online Data Files VI, VII, and VIII). Similarly, pathway enrichment analysis using gProfiler with significant differentially expressed genes highlighted a predominance of KEGG pathways relating to interferon in the mild group, while in contrast, pathways in ECs exposed to moderate and severe COVID-19 plasma related more broadly to cell motility, developmental processes, cell stress responses, cell structure reorganization, and actin mobilization (Figure 5F). Collectively, these results suggested endothelial structural changes could be occurring that might be amenable to treatment with barrier stabilizing agents.

Vascular barrier stabilizing drugs prevent EC permeability induced by COVID-19 patient plasma exposure *in vitro*

We next examined two principal structural contributors to the EC barrier, vascular endothelial-cadherin (VE-Cadherin) and Claudin-5. VE-cadherin is an essential adherens junction protein that regulates cell-cell junctional stability, and Claudin-5 is a tight junction protein that regulates size-dependent paracellular permeability pathways.[81] Immunostaining confirmed significant disruptions to both VE-cadherin and Claudin-5 expression as well as junctional localization, particularly in ECs exposed to the moderate and severe COVID-19 plasma (Figure 6A). To gauge the potential clinical importance of these changes in relation to vascular leak, we next investigated whether targeted therapeutics that are known to stabilize the vascular barrier or suppress EC activation can prevent COVID-19 plasma-induced permeability *in vitro*. The following drugs that have been

course monitoring of pHUVEC barrier function during coincubation with 20% (v/v) plasma (left) sampled from $t_{0\text{-}1}$ (negative, n=30; mild, n=14; moderate disease, n=15; severe disease, n=52; thrombin, n=7) compared to 'mock' treatment (i.e., PBS control) and quantification of area under the curve across the six-hour coincubation period (right). Thrombin treatment was included as a barrier disrupting positive control. Barrier data displayed depicts $t_{0\text{-}1}$ adjusted values (media only) that were subsequently normalized to mock-treated cells (PBS, dashed line) and displayed as the mean of the change in cell index from experiment initiation. Note: some error bars are too small to be visible on the figure. Quantified values are relative to 'mock' treatment and represent mean and error bars (±S.D.). P values determined by a Kruskal-Wallis one-way analysis of variance with Dunn's multiple comparison correction. moderate vs negative, $p=3.1 \times 10^{-3}$; severe vs negative, $p=4.7 \times 10^{-5}$. (c) Principal component analysis plot, with the 2D coordinates of each profile based on the scores of the first two principal components. (d) Volcano plot displaying the $-\log_{10}$ of the adjusted P values vs the $\log_2$ fold change of respective disease severities compared with COVID-19-negative control transcript expression. Red and blue markers indicate adjusted FDR-adjusted P values <0.05 for up- and down-regulation, respectively, based on a log fold-change of >±0.58. (e) Gene set enrichment plot of the top-ranked gene set, TNF Targets Up (FDR = 7.05×10^{-9}, NES = 3.13), WT1 Targets Up (FDR = 1.45×10^{-8}, NES = 2.66), and interferon responsive genes (FDR = 4.52×10^{-9}, NES = 3.24) using all genes ranked by their magnitude of association with each respective disease severity group (the enrichment P value shown was computed from the GSEA test) along with top ranked gene sets (below). The tick marks denote the location of the genes in each respective module. Fold change of all genes between the compared conditions are shown as bar plots in the bottom panels (x axis: genes ranked by -$\log_2$ fold change; y axis: -$\log_{10}$ fold change). (f) Top ten significantly enriched pathways based on all genes ranked by fold change identified by gProfiler are shown for each comparison. Abbreviations: COVID-19, Coronavirus Disease 2019; EC, Endothelial cell; F.C., Fold change; FDR, False discovery rate; PBS, Phosphate buffered saline; P.C., Principal component; pHUVEC, Pool human umbilical vein endothelial cells; mRNA, Messenger RNA; NES, Normalized enrichment score; S.D., Standard deviation; TNF, Tumor necrosis factor; WT1, Wilms' tumor-suppressor. See Online Data Files IV, V, and VI for a full list of differentially expressed genes, GSEAs, and pathways. Refer to Online Figures XIX-XX for more information.

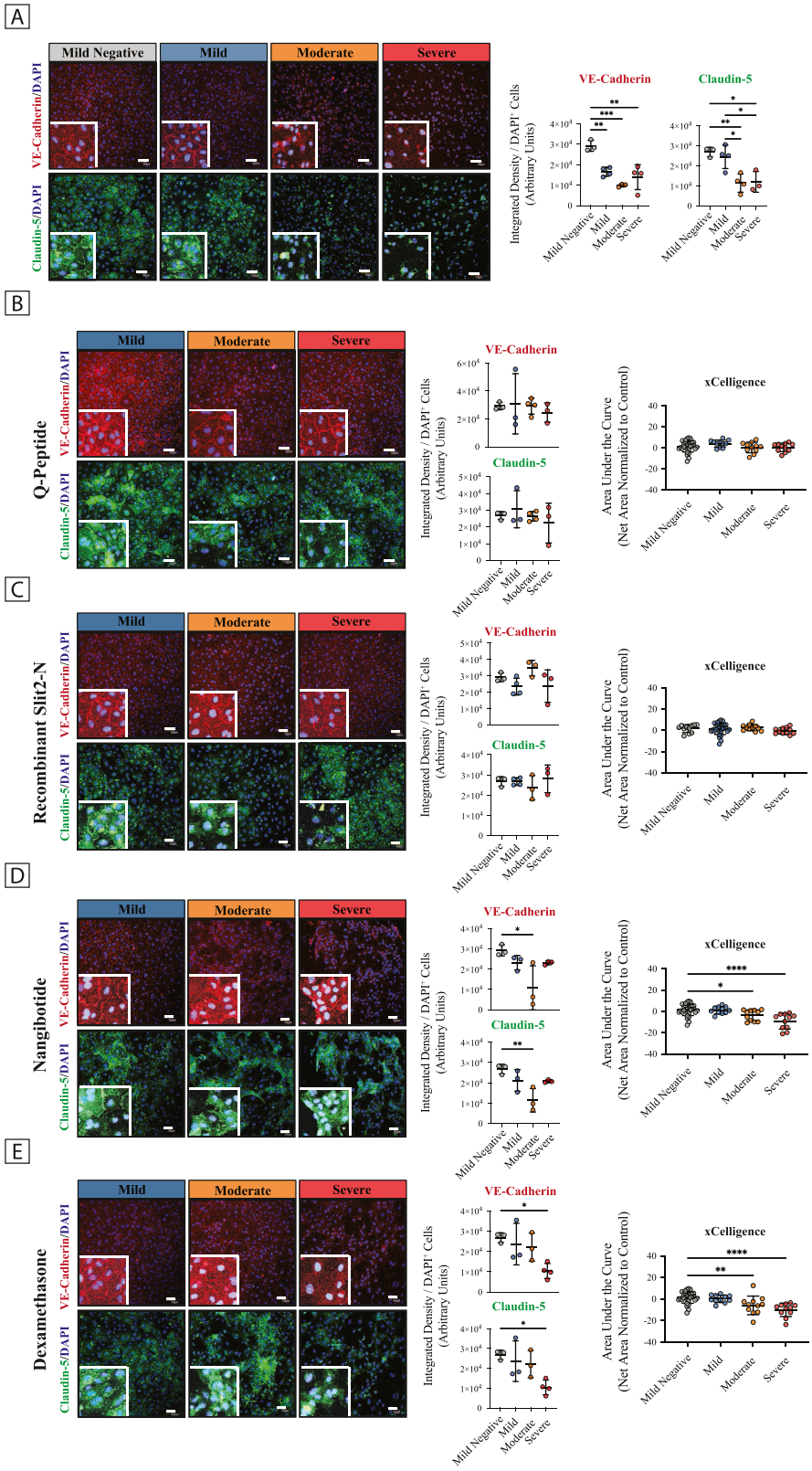

Figure 6. Targeted Modulators of Endothelial Barrier Protect Against COVID-19 Plasma-Induced Endothelial Barrier Dysfunction. (a) Left; Representative confocal microscopy images and brightness enhanced zooms depicting VE-cadherin (red) and Claudin-5 (green)

reported to reduce endothelial dysfunction were utilized: Q-peptide, a synthetic integrin-binding motif of angiopoietin-1[82]; Slit2-N, a recombinant member of the Slit family of secreted extracellular matrix glycoproteins that stabilizes adherens junctions[83]; Nangibotide, a TREM-1 inhibitor[84]; and dexamethasone, a potent synthetic adrenal corticosteroid currently used in COVID-19 treatment.[85] Co-treatment with either Q-peptide or Slit2-N prevented the disruption of the EC barrier, as examined through junctional protein expression (Figure 6B, C, left) and permeability as examined by xCelligence readout (Figure 6B, C, right). While other agents could maintain junctional protein expression (e.g., Nangibotide, dexamethasone, Figure 6D, E) in specific settings, no other agents were universally able to maintain barrier protein expression in the face of moderate or severe disease plasma exposure and prevent the physiologic barrier disruption measured by electrical resistance.

Discussion

Recent literature on COVID-19 pathogenesis has suggested that the induction of substantial acute respiratory distress phenotypes is driven by a mismatched inflammatory response together with broad vascular dysfunction.[86,87] While several detailed reports implementing multi-omic approaches have provided insight into the immune cell phenotypes involved in these processes,[88–90] risk stratifying immune markers specific to COVID-19 have not been fully elucidated. Herein, we provide a comprehensive, multi-omics-based description of the molecular antecedents to COVID-19 mortality, yielding new insights pertaining to the vasculature. Our study further delineates the gradient of vascular dysfunction observed in patients across the spectrum of COVID-19 severity, particularly among those with severe illness. While our findings are consistent with smaller cohort studies examining single markers of cardiovascular dysfunction,[46,91] our report is the first to use a complete set of disease-negative controls, which allowed us to ask etiological questions. Our analysis revealed that although markers of cardiovascular dysfunction (such as Ang-2 and sVCAM-1, Figure 2A, B) tracked well with COVID-19 severity and outcomes, these markers were similarly elevated in ICU patients without COVID-19, which indicates they are more appropriate as general markers of disease severity and are not COVID-19 specific.

In recent years, circulating miRNAs have emerged as exquisite biomarkers for complex pathological conditions, including influenza and sepsis,[92,93] and studies are beginning to emerge assessing select circulating miRNAs in COVID-19 cohorts.[94,95] The inherent stability of plasma miRNAs under harsh conditions and reproducible quantification makes them attractive candidates for use as noninvasive biomarkers.[50] Incorporating whole transcriptome miRNA sequencing as an added metric allowed for the effective differentiation between severities of COVID-19 and severe SARS-CoV-2 negative controls using samples gathered on admission. Through this study, we provide further empirical evidence for the value of data from novel biomarkers over metrics traditionally utilized in healthcare systems (i.e., clinical demographics and laboratory data). Notably, we show that the data from electronic medical records failed to adequately capture the risk of in-hospital mortality. Using a Random Decision Forest machine learning model on our multi-omic biologically relevant datasets we were able to develop an exploratory prognostic risk prediction model that incorporates markers that are COVID-19- and vascular-specific. Importantly, there are several platforms that allow the interrogation of candidate miRNAs within clinically relevant timeframes.[96,97] While still investigational, given the

junctional staining after six hours of treatment of pHUVECs with t_{0-1} COVID-19 plasma and matched controls; n=3-4 per group. Scale bars: 50 µm. Right; Quantification of VE-cadherin and Claudin-5 pixel intensity for each respective group. Each dot represents mean protein expression across at least four representative fields of view of a biological replicate. Center bars represent mean and error bars are (±S.D.). P values were determined by one-way ANOVA with Tukey's multiple comparisons test. Mild vs negative: VE-cadherin, $P=6.0 \times 10^{-3}$. Moderate vs. negative: VE-cadherin, $P=4.0 \times 10^{-4}$; Claudin-5, $p=1.6 \times 10^{-2}$. Negative vs severe: VE-cadherin, $P=1.6 \times 10^{-3}$; Claudin-5, $P=8.0 \times 10^{-3}$. Mild vs moderate: Claudin-5, $P=2.9 \times 10^{-2}$. Mild vs severe: Claudin-5, $P=1.4 \times 10^{-2}$. (b-e) Left; Representative confocal microscopy images depicting a combinatorial screen of COVID-19 plasma and matched controls treated with either (b) Q-peptide, (c) Slit2-N, (d) Nangibotide, and (e) Dexamethasone on pHUVECs reveals modulated maintenance of VE-cadherin (red) and Claudin-5 (green) expression; n=3-4 per group. Scale bars: 50 µm. Middle; Quantification of VE-cadherin and Claudin-5 for each respective group. Imaging P values were determined by one-way ANOVA with Dunnett's multiple comparisons test (all compared to control). Nangibotide; Moderate vs negative: VE-cadherin $*P=1.3 \times 10^{-2}$, Claudin-5 $**P=8.2 \times 10^{-3}$. Dexamethasone; Severe vs negative: VE-cadherin $*P=2.4 \times 10^{-2}$, Claudin-5 $*P=3.3 \times 10^{-3}$. Right; Quantification of VE-cadherin and Claudin-5 for each respective group. Each dot represents mean protein expression across at least four representative fields of view of a biological replicate. Center bars represent mean and error bars are (±S.D.). Right; TEER quantification of corresponding treatment groups. P values were determined by Kruskal-Wallis test with Dunn's multiple comparisons test (all compared to control). Nangibotide; Negative vs moderate (P): $*P=3.9 \times 10^{-2}$; Severe vs negative $****P=4.3 \times 10^{-5}$. Dexamethasone; Moderate vs negative (P): $**P=6.2 \times 10^{-3}$; Severe vs negative$****P=2.2 \times 10^{-5}$. Abbreviations: COVID-19, Coronavirus Disease 2019; pHUVEC, Pool human umbilical vein endothelial cells; TEER, transendothelial electrical resistance; S.D., Standard deviation. Refer to Online Figure XXI for more information.

extent of pre-clinical and clinical research on miRNAs,[68] it is reasonable to expect miRNA datasets will soon be amenable for clinical implementation.[94,95] As an example of the importance of a multi-omic approach that includes clinically relevant disease negative controls, miR-1 (which has been intensively studied as a cardiac enriched miRNA and associated with numerous cardiac etiologies[69-71,98]) failed to show an association with in-hospital mortality in our study. If the promising miRNAs, miR-30b/c/e, -6080, -181a-5p, -199a-3p, and -339 identified here are validated in larger populations, they would represent new biomarkers that could be utilized for rapid in-hospital risk assessment. As miRNAs represent functional biomarkers, having active roles in gene regulation, they also present an important opportunity to understand the pathophysiological relevance of endothelial-based processes affected by SARS-CoV-2 infection. For example, it has been shown that miR-98 may regulate TMPRSS2, an established primer protein for cellular SARS-COV-2 entry.[99,100]

Given that the endothelium is the gatekeeper of vascular permeability, which is a main pathophysiological process that is dysregulated in systemic inflammatory conditions, there is intense research on its role in ARDS, systemic inflammatory response syndrome, and sepsis (among others).[101-103] Several lines of evidence support the hypothesis that endothelial function is a major determinant of COVID-19 outcome.[91,104] Studies have shown endothelial derangements (i.e., pathological sprouting angiogenesis), increased endothelial apoptosis, modified metabolism, and a strong correlation between underlying CVD and mortality as COVID-19 severity increases.[43,45] Often forgotten, endothelial dysfunction−associated molecular patterns are broad and strong activators of innate immune responses, leading to innate immunity-mediated organ injury. To this end, we observed that plasma from patients with moderate and severe COVID-19 induced profound barrier disruption, as assessed through the modulation of VE-Cadherin and Claudin-5 at EC-EC junctions. Transcriptomic analysis revealed that COVID-19 plasma from moderate and severe patients appears to preferentially induce pro-inflammatory immune gene processes, while plasma from mild patients induces an interferon (IFN) response. This is in line with several studies on the immune response of mild vs severe COVID-19 patients and is confirmed by the over-representation of type I IFN genes in ECs exposed to mild COVID-19 plasma.[91,105,106] Type I IFNs are critical mediators of the antiviral immune response, and subverting the early type I IFN response has been shown repeatedly to be a contributor to coronavirus infection.[105] Looking at the moderate and severe phenotypes where endothelial pro-inflammatory responses (e.g., TNF) and histone deacetylase dysregulation occurred, both epigenetic modifications as well as TNF responses have been documented in COVID-19.[91,106]

Increased pro-inflammatory cytokine expression, specifically TNF and IL-6, has been shown to upregulate trypsin, resulting in the loss of endothelial tight junctions, subsequently inducing vascular hyperpermeability.[107,108] We are encouraged by our findings that both Q-peptide and recombinant Slit2-N, could effectively prevent the induction of endothelial permeability *in vitro*. With the known roles of Ang-1 and Ang-2 as ligands of the endothelial cell-specific receptor Tie2 and their antagonistic relationship as modulators of endothelial survival, the barrier maintenance seen through Q-peptide could be due to antagonism of higher Ang-2 levels in more severe COVID-19.[109,110] Additionally, measurement of the levels of endogenous soluble Slit2 in SARS-CoV-2 patient plasma revealed a significant upregulation only in patients with severe disease suggesting a possible compensatory mechanism and furthering the biological relevance of Slit2-N treatment (Online Figure XXI). Therefore, as a proof-of-concept, these data support the hypothesis that disruption of endothelial barrier function, particularly as a result of loss of adherens and tight junctions, is induced during COVID-19. These findings support a model in which systemic induction of pro-inflammatory processes, rather than direct infection, may be the primary driver of systemic endothelial perturbations observed in COVID-19 patients, and that in addition to current treatments aimed at dampening immune responses, preserving endothelial barrier may be a viable adjuvant strategy to reduce the mortality of SARS-CoV-2.

Furthermore, Q-peptide and Slit2N act via different pharmacological pathways, suggesting that preservation of endothelial barrier itself is the key therapeutic mechanism. It will nonetheless be important to determine whether pre-existing and potentially chronic endothelial permeability − layered on top of a permeabilizing stimulus such as infection − can be reversed through biologics that interact directly at the endothelial interface. If the significant induction of endothelial permeability can be overridden, even in patients with an ongoing inflammatory insult, then pharmacological intervention at the level of the vasculature may be an effective adjuvant therapy for patients at a higher risk of adverse events. Indeed, previous studies using animal models of sepsis, influenza, and malaria have reaffirmed that pharmacological treatments that enhance vascular resiliency in the face of an aberrant host immune response may be a therapeutically feasible approach for managing vascular dysfunction without compromising immunity.[111-113] Although stabilizing the vasculature may be more practical than attenuating individual cytokines, it remains to be determined whether severe vascular damage can be reversed in a clinically meaningful way. Anecdotally, recent reports of adrenomedullin, a peptide hormone capable of endothelial stabilization, have suggested high *in vivo* efficacy and positive results from both Phase I and II trials in patients with sepsis.[114,115] A

prospective study comparing endothelial function in critical illness of non-viral etiology and viral etiology may provide clearer insights into the translatability of these findings. Lastly, it is worth noting that dexamethasone, a widely used glucocorticoid in the treatment of COVID-19, failed to stabilize barrier function in our acute EC model. Within the context of COVID-19, it may be that synthetic glucocorticoids provide efficacy against myeloid cell-driven inflammation and the resultant endothelial dysfunction.[116] While evidence suggests glucocorticoids – including dexamethasone – can directly modulate the endothelium, their effects appear dependent on pro-inflammatory contexts. In particular, glucocorticoids appear to lead to impaired endothelial dysfunction within settings of limited inflammatory insult,[117,118] while paradoxically benefiting the vasculature under pro-inflammatory conditions.[119] The mechanisms, timing, and context-dependent responses seen through glucocorticoid treatment remain to be clearly delineated.

Taken together, we have provided the first highly salient comparison of a diverse group of cardiovascular markers between COVID-19 positive and negative patients, highlighting that well-known markers of inflammation, cardiac damage, and endothelial dysfunction are not specific to COVID-19 pathology. Notably, with a targeted miRNA transcriptomic approach, we were able to discern specific markers that showed better discrimination between these two groups. Incorporating protein and miRNA markers using machine learning approaches improved the prediction of in-hospital mortality over baseline clinical variables. While exploratory, this is a clinically feasible approach that has the advantage of using pathophysiologically relevant SARS-CoV-2 markers, as opposed to the surrogate markers used in most other published risk stratification models.[17,46] Finally, our data provide several lines of evidence supporting the notion that endothelial barrier function is affected in a SARS-CoV-2 specific manner that is distinct from the pathways involved in critically ill patients with non-COVID-19 severe respiratory illnesses. Our data reinforce the idea that barrier dysfunction is likely independent of direct viral infection and instead secondary to yet undiscovered mediators in the plasma. We further pursued our observations using assays of EC function using an *in vitro* model, which served as a platform for rational therapeutic selection. Here, we show that EC barrier is compromised by the addition of COVID-19 patient plasma in a disease severity-dependent manner and that this can be prevented by stabilization with synthetic Ang-1 (Q-peptide) or Slit2-N. Together, our work provides biological insight into the role of the endothelium in SARS-CoV-2 infection, the importance of miRNA as disease- and pathway-specific biomarkers, and the exciting possibility that endothelial barrier stabilizing treatments might hold promise in COVID-19. Moreover, we provide insights into the use of this approach to find therapeutic options that might prove useful in other critical illnesses and emerging infectious diseases where endothelial permeability is central to disease pathophysiology.

Limitations

The results of this study should be viewed in the context of its design, sample size, and socio-geographical context (i.e., access to advanced care life support). Variation between studies may represent temporal differences in circulating viral strains or vaccination status (notably, this study was done in a timeframe prior to B.1.1.7 [Alpha], B.1.351 [Beta], B.1.617.2 [Delta], P.1 [Gamma], and B.1.1.529 [Omicron] lineage circulation and prior to public vaccination programs), which can impact severity and mortality. In this respect, the sample size of patients hospitalized with COVID-19 and those who experienced clinical outcomes in our cohorts may be considered modest compared to studies utilizing larger hospital registries or based out of geographical areas with a substantially higher case prevalence; this may have made the study susceptible to sampling bias. *A priori* sample size calculations were not conducted for the matching of control cohorts as effect size calculations were not possible given the emergence of this new pathogen with little information on expected rates of outcomes. Therefore, biomarkers identified and their associations with clinical outcomes should be interpreted with caution given the caveats relating to missing data points, long-term outcomes, and the infeasibility of adjusted multivariable analysis. Findings from the miRNA atlas and machine learning algorithms should be validated in additional cohorts before implementation in clinical practice. The *in vitro* system used in this manuscript represents a reductionist approach to disease modelling, and as such, vascular cell co-culture or examination of specific endothelial beds (i.e., coronary or pulmonary) may better inform us about microenvironment changes in COVID-19. This approach, along with treatment concentrations and timeframes, may explain the failure to see an effect with either TREM-1 inhibitor, nangibotide, or dexamethasone. Putative therapies could be further assessed in co-culture models (e.g., organ on a chip[120]) that better mimic tissue complexity and will require testing in animal models. Nevertheless, our findings provide important knowledge relating to the pathophysiology and risk stratification of betacoronavirus infection and provide avenues for future research in infectious disease.

Declaration of interests

The authors would like to disclose the following potential conflicts of interest: S.M reports personal fees from Quthero Inc.; M.R reports ownership in Quthero Inc;

Quthero is working to develop QHREDGS peptides for wound healing applications and had no influence on the design, interpretation, or conclusions associated with this study. E.F. reports personal fees from ALung Technologies, Baxter, Aerogen, GE Healthcare, Inspira, and Vasomune outside the scope of the submitted work. K.H. reports personal fees from Sanofi Genzyme, Amicus, and Medscape outside the scope of the submitted work. D.G., M.R., K.K., P.T., K.H., J.E.F. and K.L.H. have submitted patents on the findings disclosed in this manuscript. All other authors declare an absence of any commercial, financial, or intellectual relationships that could be construed as a potential conflict of interest.

Contributors

D.G., M.N., R.W., A.S., C.E., S.M., F.B., M.W., M.R., E. F., K.K., C.D., K.H., P.T., J.E.F, and K.L.H. contributed to the study design. D.G., A.S., P.B., S.F., U.T., A.B., C. D., P.T., and K.H. contributed to patient recruitment, data collection, and clinical analysis. D.G., M.N., R.W., C.E., and S.M. contributed to wet-lab data generation and *in vitro* analysis. S.F. and C.M. contributed to clinical data analysis, machine learning model construction, model testing, and model comparisons. H.H. and M.W. contributed to the mRNA sequencing analysis. D.G., M. N., R.W., C.E., J.E.F, and K.L.H. wrote and revised the manuscript. All authors discussed and commented on the manuscript. K.H., P.T., J.E.F., and K.L.H. provided funding to support this study.

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Contact for reagent, resource, and data sharing

The authors declare that all supporting data are available within the article and its supplementary data files. Transcriptomic data (i.e., messenger RNA and miRNA sequencing) have been deposited at the Gene Expression Omnibus and the RStudio analysis pipeline is contained within the Online Data Supplement. The data generated in this study have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through the GEO Series accession number (GSE; GSE178331, mRNA) and (GSE178246, miRNA). Further information and requests for resources, reagents, or patient-level datasets from qualified researchers trained in human subject confidentiality protocols may be sent to the lead contact, Dr. Kathryn Howe (kathryn.howe@uhn.ca).

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.103982.

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