OBSERVATIONAL STUDY

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Angiopoietin-Like4 Is a Novel Marker of COVID-19 Severity

IMPORTANCE: Vascular dysfunction and capillary leak are common in critically ill COVID-19 patients, but identification of endothelial pathways involved in COVID-19 pathogenesis has been limited. Angiopoietin-like 4 (ANGPTL4) is a protein secreted in response to hypoxic and nutrient-poor conditions that has a variety of biological effects including vascular injury and capillary leak.

OBJECTIVES: To assess the role of ANGPTL4 in COVID-19–related outcomes.

DESIGN, SETTING, AND PARTICIPANTS: Two hundred twenty-five COVID-19 ICU patients were enrolled from April 2020 to May 2021 in a prospective, multicenter cohort study from three different medical centers, University of Washington, University of Southern California and New York University.

MAIN OUTCOMES AND MEASURES: Plasma ANGPTL4 was measured on days 1, 7, and 14 after ICU admission. We used previously published tissue proteomic data and lung single nucleus RNA (snRNA) sequencing data from specimens collected from COVID-19 patients to determine the tissues and cells that produce ANGPTL4.

RESULTS: Higher plasma ANGPTL4 concentrations were significantly associated with worse hospital mortality (adjusted odds ratio per $\log_2$ increase, 1.53; 95% CI, 1.17–2.00; $p = 0.002$). Higher ANGPTL4 concentrations were also associated with higher proportions of venous thromboembolism and acute respiratory distress syndrome. Longitudinal ANGPTL4 concentrations were significantly different during the first 2 weeks of hospitalization in patients who subsequently died compared with survivors (p for interaction $= 8.1 \times 10^{-5}$). Proteomics analysis demonstrated abundance of ANGPTL4 in lung tissue compared with other organs in COVID-19. *ANGPTL4* single-nuclear RNA gene expression was significantly increased in pulmonary alveolar type 2 epithelial cells and fibroblasts in COVID-19 lung tissue compared with controls.

CONCLUSIONS AND RELEVANCE: ANGPTL4 is expressed in pulmonary epithelial cells and fibroblasts and is associated with clinical prognosis in critically ill COVID-19 patients.

KEY WORDS: angiopoietin-like 4; COVID-19; endothelial dysfunction; intensive care unit; sepsis

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Several studies have demonstrated that dysregulation of the vascular endothelium in COVID-19 patients is associated with capillary leak leading to pulmonary edema, higher rates of venous and arterial thrombi, and bleeding diatheses (1–3). However, traditional markers of endothelial dysfunction, such as circulating angiopoietin-2 (Ang-2) concentrations, may, in fact, be lower in COVID-19 than non-COVID-19 patients and may be less prognostic for clinical outcomes early after ICU admission (4, 5). These results suggest that there may be additional pathways involved in endothelial dysfunction that are associated with COVID-19 severity.

KEY POINTS

Question: The host endothelial response in COVID-19 is understudied. What is the association of angiopoietin-like 4 (ANGPTL4) in COVID-19 critical illness?

Findings: We used a combination of plasma ANGPTL4 measured on ICU admission and longitudinally in a prospectively enrolled clinical cohort and previously published proteomics and single nucleus RNA sequencing from COVID-19 lung tissue to demonstrate that ANGPTL4 is expressed in pulmonary epithelial cells and fibroblasts and plasma **concentrations** are associated with clinical prognosis in critically ill COVID-19 patients.

Meaning: This study demonstrates an association between ANGPTL4 signaling and COVID-19 pathogenesis. ANGPTL4 may be a therapeutic target to prevent poor outcomes in COVID-19.

Angiopoietin-like 4 (ANGPTL4) is a protein secreted in response to hypoxic and nutrient-poor conditions through a hypoxia inducible factor-1 subunit alpha dependent pathway (6). ANGPTL4 has a variety of biological effects including vascular injury, wound healing, and lipid metabolism (7). Full-length ANGPTL4 can be cleaved by a proprotein convertase into an n-terminal fragment (n-ANGPTL4) that confers effects on lipid metabolism and a c-terminal fragment (c-ANGPTL4) that has effects on vascular function (8). In murine models of influenza and pneumococcal pneumonia, upregulation of ANGPTL4 expression resulted in vascular leak and pulmonary edema that was partially reversed by pretreatment with an antibody against ANGPTL4 (9, 10). One study in patients with acute respiratory distress syndrome (ARDS) with paired bronchoalveolar lavage and serum samples found that ANGPTL4 concentrations were higher in ARDS than controls and levels were associated with hospital mortality (11). These findings led us to hypothesize that ANGPTL4 may contribute to severe capillary leak in respiratory infection that characterizes many patients with severe COVID-19. There is little published information about ANGPTL4 in COVID-19. One published study in postmortem lung tissue investigators found elevated *ANGPTL4* gene expression from patients with COVID-19 or influenza A (H1N1)pdm09 relative to controls. Another study demonstrated that elevated levels of circulating ANGPTL4 were associated with encephalitis in patients with COVID-19 (1, 12).

To assess the role of ANGPTL4 in critically ill COVID-19 patients, we measured plasma ANGPTL4 concentrations in a multicenter, prospective, observational ICU cohort of COVID-19 patients that afforded an opportunity to relate biomarker concentrations to clinical outcomes. We tested for associations between ANGPTL4 concentrations measured on ICU admission with the primary outcome of hospital mortality and secondary outcomes of ARDS, venous thromboembolism (VTE), and days receiving invasive mechanical ventilation. We also tested the relationship between longitudinal ANGPTL4 concentrations with hospital mortality in COVID-19. Finally, we used previously published tissue proteomics and single nucleus RNA (snRNA) sequencing data from lung specimens collected from COVID-19 patients to determine whether lung pathology contributes to circulating ANGPTL4 concentrations. Our conjoined clinical, proteomics and snRNA in lung tissue suggest that ANGPTL4 reflects a pathophysiologic pathway in COVID-19 linked to worsened clinical outcomes; and, that targeting ANGPTL4 blockade may provide a therapeutic avenue to prevent endothelial dysfunction.

METHODS

Patient Cohort and Study Design

Patients were prospectively enrolled in the Severe Acute Respiratory Infection Preparedness (SARI-PREP) cohort from March 2020 to May 2021. SARI-PREP is a multicenter, prospective, observational cohort study enrolling hospitalized patients with SARI from a viral etiology (NCT04786301). SARI-PREP inclusion criteria included age greater than or equal to 18 years, presence of fever, respiratory symptoms, and evidence of lower respiratory involvement (13). All subjects had nasopharyngeal swabs collected for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) testing and a case of COVID-19 was confirmed by a positive reverse transcription-polymerase chain reaction assay for SARS-CoV-2. Full inclusion and exclusion criteria

can be found in the **Online Supplement** (http://links.lww.com/CCX/B117). We included a convenience sample of 225 COVID-19 patients enrolled in SARI-PREP from three sites (14). Blood samples collected within 24 hours of ICU admission were used for the primary analysis and samples collected on days 7 and 14 were used for longitudinal analyses. The research was approved at participating sites by the University of Nebraska Medical Center Institutional Review Board (544-20-FB) in March 27, 2020, for the study titled, Severe Acute Respiratory Infection Preparedness (SARI-PREP). Procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975. Informed consent was collected on all patients or was waived by the local regulatory board early during the pandemic. The clinical trials registration number is NCT04786301. All procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975.

Clinical Data Abstraction and Outcome Definitions

Clinical data were abstracted from the electronic medical record into standardized case report forms by trained research coordinators. Clinicians and trained research coordinators reviewed the charts daily to adjudicate clinical events. Quality checks on all abstracted data were completed by two blinded reviewers on a subset of all patient charts. Cases of deep venous thrombosis or pulmonary embolism were identified through ultrasound and chest CT during hospitalization and grouped as VTE. Diagnosis of ARDS was based on a clinical diagnosis by the treating team during hospitalization. Patients diagnosed with ARDS could be receiving invasive or noninvasive mechanical ventilation or high-flow nasal cannula (15, 16).

ANGPTL4 Measurement

We used an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) to measure plasma ANGPTL4. ANGPTL4 was measured in one batch on ICU admission plasma samples (run 1) and in a second batch on days 7 and 14 plasma samples (run 2). To determine which version of ANGPTL4 was measured using the ELISA plate, we purchased human recombinant full-length, c-terminal, and n-terminal ANGPTL4 from R&D systems. We then diluted all recombinant versions to a concentration of 80 ng/mL and then six additional two-fold dilutions (i.e., 40, 20, 10, 5, 2.5, and 1.25 ng/mL). We tested each concentration of ANGPTL4 on the R&D ELISA assay to determine which product of ANGPTL4 the assay was detecting. Additional information about the performance of the ELISA assay can be found in the Online Supplement (http://links.lww.com/CCX/B117).

Statistical Analysis

ANGPTL4 protein concentrations were $\log_2$ transformed and stratified into tertiles. We used logistic regression with generalized linear models and the binomial family to test for the association between ANGPTL4 concentrations and the primary outcome of hospital mortality (R v4.2.1, Vienna, Austria). Primary results are reported as the odds ratio (OR) with CIs and *p* values. We also used generalized additive models with the binomial family to model logistic regression for hospital mortality across splines of the untransformed ANGPTL4 concentrations (R package "gam" [v1.20.2]). All regression analyses were controlled for age, enrollment site, sex, body mass index (BMI), diabetes mellitus (DM), hypertension, and chronic kidney disease (CKD). Additional secondary outcomes of ARDS, VTE and days of mechanical ventilation were tested. Methods for secondary outcomes can be found in the Online Supplement (http://links.lww.com/CCX/B117).

Plasma samples were collected longitudinally on days 1, 7, and 14 after ICU admission in a subset of patients that were enrolled from University of Washington (n = 180). Using longitudinal data, we completed two different analyses. First, we used the generalized least squares fits by restricted maximum likelihood with four types of correlation matrices to model repeated measures in longitudinal data (nlme R package, v3.1.158, Vienna, Austria). Analysis of variance (ANOVA) was used to select the longitudinal models with the lowest Bayesian information criterion. We tested whether longitudinal ANGPTL4 concentrations were associated with hospital mortality with an interaction term between hospital day and mortality, adjusting for age, sex, and BMI. Second, among patients still hospitalized and with ANGPTL4 measured, we tested the association of ANGPTL4 concentrations on days 7 and 14 using logistic regression adjusting for age, sex, BMI, CKD, hypertension, and DM

for hospital mortality to demonstrate the change in risk during the first 2 weeks of hospitalization. In a sensitivity analysis, we tested the association of ANGPTL4 with in-hospital mortality in patients who survived at least 14 days after ICU admission and had ANGPTL4 measured at three timepoints (admission, 7 and 14 d). We report the bootstrapped 95% CI of values of the mean difference as it does not assume the data are normally distributed.

Tissue Proteomics

We downloaded proteomics data from postmortem tissue of 19 COVID-19 patients that quantified abundance of 11,394 proteins (17). We graphed the protein abundance of ANGPTL4 in seven different tissues (testis, kidney, liver, lung, spleen, heart, and thyroid). Pairwise *t* tests were used to compare ANGPTL4 concentrations between lung and other tissues.

Single Nucleus RNA sequencing Analysis

We downloaded snRNA sequencing data from postmortem lungs of 19 COVID-19 and 7 control individuals from Melms et al (17) (Gene Expression Omnibus, GSE171524) that comprised 116,314 cells (18). In the article by Melms et al (17), ANGPTL4 gene expression was not provided; thus, we completed a secondary analysis of the primary data. Additional data on RNA sequencing analyses can be found in the **eMethods** (http://links.lww.com/CCX/B117).

SARS-CoV-2 Infection of Human Distal Airway and Alveolar Epithelial Cells

Small airway epithelial cells immortalized via expression of telomerase reverse transcriptase (HSAEC-KT CRL-4050, American Type Culture Collection, Manassas, VA) were expanded in culture, using small airway epithelial cell growth medium (PromoCell, Heidelberg, Germany) and infected with SARS-CoV-2 or mock. These distal airway epithelial cells demonstrated gene expression consistent with distal respiratory/alveolar epithelial cells. Additional data on SARS-CoV-2 infection of human epithelial cells can be found in the eMethods (http://links.lww.com/CCX/B117).

Data Availability

Data and codes used for the analyses presented in this article are available upon request.

RESULTS

Characteristics of COVID-19 Population

Among 225 enrolled patients with COVID-19, the mean age was 56 ± 16 years, 66% were male, 53% had a prior history of hypertension, and 34% had DM. On study enrollment, 75% of patients received invasive mechanical ventilation or high-flow nasal cannula. Use of COVID-19 therapeutics during hospitalization were common, 74% of patients received corticosteroids, 43% remdesivir, and 8% tocilizumab (Table 1). A subset of COVID-19 patients had SARS-CoV-2 sequencing performed (n = 38) that demonstrated variants predominantly in the A (original strain) and B clades (**Table S1**, http://links.lww.com/CCX/B117).

Plasma ANGPTL4 Concentrations Are Associated With COVID-19 Clinical Outcomes

The primary outcome of hospital mortality occurred in 75 patients (33%). Complications likely to involve endothelial dysfunction were common, 44 patients (20%) were diagnosed with a VTE during hospitalization and 130 (58%) with ARDS. The median plasma ANGPTL4 concentration on study enrollment was 642 ng/mL (interquartile range, 237–696 ng/mL). In cross-sectional analyses, we tested the association of ANGPTL4 concentrations with the National Institutes of Health COVID Ordinal Scale. We found that ANGPTL4 concentrations were not associated with severity of respiratory failure on ICU admission (**Fig. S1**, http://links.lww.com/CCX/B117). In contrast, higher plasma ANGPTL4 concentrations were independently associated with a greater risk of hospital mortality (adjusted OR [aOR], 1.53; 95% CI, 1.17–2.00 per doubling of ANGPTL4; p = 0.002) adjusted for demographics and comorbidities (Table 2 and **Fig. 1)**. Patients in the highest tertile of ANGPTL4 had a three times greater risk for hospital mortality (aOR, 3.07; 95% CI, 1.31–7.46; p = 0.011) compared with patients in the lowest tertile (Table 2). Higher ANGPTL4 concentrations were also associated with a greater risk for VTE (aOR, 1.48; 95% CI, 1.11–1.98; p = 0.008) and a greater risk for ARDS during hospitalization (aOR, 1.30; 95% CI, 1.02–1.65; p = 0.036) (Table 3). ARDS may be present on ICU admission and so we completed a sensitivity analysis in patients not receiving IMV on ICU admission to identify whether ANGPTL4 was associated

TABLE 1.
Baseline Characteristics

Patient Characteristics	(*n* = 225)
Demographics and comorbidities	
Site of enrollment, *n* (%)	
University of Washington	180 (80)
New York University	37 (16)
University of Southern California	8 (4)
Age, mean ± SD	56 ± 16
Male, *n* (%)	149 (66)
Body mass index, kg/m^2, mean ± SD	31 ± 9
Race, *n* (%)	
American Indian	8 (4)
Asian	28 (12)
Black/African American	27 (12)
Pacific Islander	4 (2)
White	130 (58)
Other	6 (3)
Unknown	22 (10)
Ethnicity, *n* (%)	
Hispanic/Latinx	73 (32)
Coexisting disease, *n* (%)	
Asthma	71 (32)
Chronic kidney disease	40 (18)
Diabetes mellitus	76 (34)
Hypertension	120 (53)
Characteristics upon ICU admission	
National Institutes of Health 8-point COVID Ordinal Score, *n* (%)	
4 (hospitalization)	26 (12)
5 (any supplemental oxygen)	31 (14)
6 (high-flow nasal cannula or noninvasive positive pressure ventilation)	53 (24)
7 (invasive mechanical ventilation or extracorporeal membrane oxygenation)	115 (51)
Acute Physiology and Chronic Health Evaluation II, mean ± SD	24 ± 10
Treatments during hospitalization, *n* (%)	
Corticosteroids	167 (74)
Remdesivir	96 (43)
Tocilizumab	18 (8)
Outcomes during hospitalization, *n* (%)	
Acute respiratory distress syndrome	130 (58)
Venous thromboembolism	44 (20)
In-hospital mortality	75 (33)

with subsequent development of ARDS. Among this subset of 120 patients, ANGPTL4 concentrations on ICU admission continued to be associated with ARDS risk (aOR, 1.61; 95% CI, 1.05–2.48 per doubling of ANGPTL4; $p = 0.03$). Finally, among patients who received IMV, lower ANGPTL4 concentrations on ICU admission were associated with higher likelihood of extubation from mechanical ventilation (**Fig. S2**, http://links.lww.com/CCX/B117).

Longitudinal Analyses Demonstrate That ANGPTL4 Trajectories Are Different in Survivors Compared With Patients Who Died During Hospitalization

COVID-19 is characterized by prolonged hospitalization with late mortality in ICU patients. Thus, we compared plasma ANGPTL4 concentrations in ICU patients with longitudinal blood measurements and clinical outcomes. Among 180 patients with at least two ANGPTL4 values, 67 patients died (37%) with the majority of deaths occurring after 14 days of ICU admission (n = 43 [64%]). In longitudinal repeated measures time series regression analyses, the interaction between hospital day and mortality was significant ($p = 8.2 \times 10^{-5}$), showing that the association of ANGPTL4 and mortality significantly increases with time (**Fig. 2**).

In patients who remained in the hospital 7 and 14 days after ICU admission, we tested whether ANGPTL4 concentrations continue to discriminate patients at higher risk for subsequent hospital mortality. Multivariable adjusted models demonstrated that increasing ANGPTL4 concentrations remained associated with increasing hospital mortality during the two weeks of hospitalization; ANGPTL4 day 1 (OR, 1.56; 95% CI, 1.16–2.09), day 7 (OR, 2.42; 95% CI, 1.53–3.84), and day 14 (OR, 2.80; 95% CI, 1.61–4.86). To account for limitations of differential dropout of patients for longitudinal analyses, we completed a separate analysis that evaluated the association of ANGPTL4 concentrations restricted to patients who have blood samples available at all three timepoints (ICU admission, 7 and 14 d). In this population of 76 patients, 35 died (46%). In longitudinal repeated measures time series regression analyses, the interaction between hospital day and mortality was significant (p = 0.002), showing that the association of ANGPTL4 and

TABLE 2.
Association of Plasma Angiopoietin-Like 4 Concentration With Hospital Mortality

			Baseline Demographic Adjusted[a]		Multivariable Adjusted[b]	
Plasma Angiopoietin-Like 4	*n* at Risk	*n* Events	OR (95% CI)	*p*	OR (95% CI)	*p*
Tertile 1	75	15	1.0 (Reference)	–	1.0 (Reference)	–
Tertile 2	75	25	1.51 (0.67–3.45)	0.320	1.59 (0.70–3.69)	0.270
Tertile 3	75	35	2.75 (1.25–6.24)	0.013	3.07 (1.31–7.46)	0.011
Per doubling	225	75	1.48 (1.15–1.91)	0.003	1.53 (1.17–2.0)	0.002

OR = odds ratio.

[a]Baseline demographics adjustment: age, sex, body mass index, and site.

[b]Multivariable adjusted: Baseline demographic covariates and diabetes mellitus, hypertension, and chronic kidney disease.

Multivariable logistic regression and a series of nested adjustment models were used to test for an association between angiopoietin-like 4 (ANGPTL4) tertiles or ANGPTL4 continuous (doubling) with the primary outcome of hospital mortality.

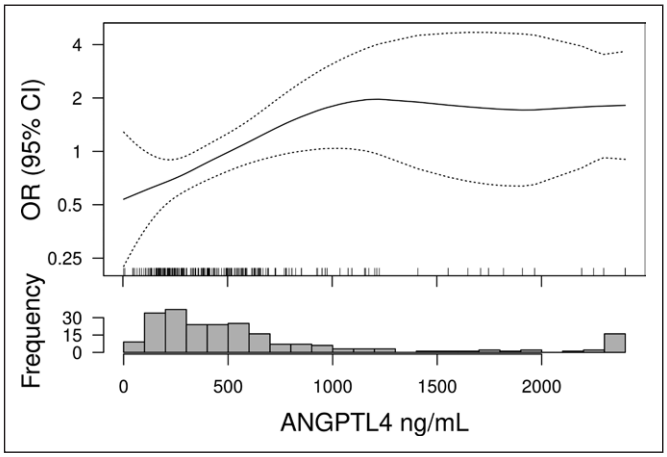

Figure 1. Association of plasma angiopoietin-like 4 (ANGPTL4) concentrations with hospital mortality. The smooth spline estimates the odds ratio (OR) of hospital mortality according to ICU admission plasma ANGPTL4 concentrations. Analyses are adjusted for age, sex, and body mass index. *Dotted lines* represent 95% CIs. Below the spline is the histogram of the distribution of ANGPTL4 to indicate the range of the data.

mortality significantly increases with time (**Fig. S3**, http://links.lww.com/CCX/B117).

Targeted ELISA Immunoassay Measures c-ANGPTL4

ANGPTL4 is a multifunctional circulating protein that undergoes proteolytic processing by membrane proprotein convertases upon secretion (8). The n-ANGPTL4 domain inhibits lipoprotein lipase (LPL), while the c-ANGPTL4 is involved in vascular hyperpermeability and angiogenesis. To determine which product of ANGPTL4 that the ELISA measures, we tested human recombinant full-length ANGPTL4, c-ANGPTL4, and n-ANGPTL4. We found that the ELISA was detecting with highest avidity c-ANGPTL4 and then full-length ANGPTL4 (**Fig. S4**, http://links.lww.com/CCX/B117). The n-ANGPTL4 product had little affinity for the ANGPTL4 ELISA assay even at very high concentrations. These findings highlight that the ELISA assay is measuring with high-affinity c-ANGPTL4 and minimally measuring n-ANGPTL4.

Tissue Proteomics Demonstrates Abundance of ANGPTL4 in Lung Compared With Other Organs

Having demonstrated that plasma ANGPTL4 concentrations are associated with clinical outcomes in COVID-19, we examined tissues that potentially contribute to circulating ANGPTL4 in COVID-19. We used a published database of proteomic measurements in 19 autopsy samples from seven different organs from patients with COVID-19. We found that ANGPTL4 protein abundance was high in multiple tissues, including kidney, liver, testis, and others, with lung having the highest abundance of ANGPTL4 (**Fig. S5**, http://links.lww.com/CCX/B117). In this database, ANGPTL4 abundance in the lung was not different between COVID and non-COVID-19 but was different in kidney and liver tissue. Due to the large abundance of ANGPTL4 in lung tissue in COVID-19, we sought to determine whether different lung cell types may

TABLE 3.
Association of Plasma Angiopoietin-Like 4 Concentration With Acute Respiratory Distress Syndrome and Venous Thromboembolism

Plasma Angiopoietin-Like 4 per Doubling	*n* at Risk	*n* Events	Baseline Demographic Adjusted[a]		Multivariable Adjusted[b]	
			OR (95% CI)	*p*	OR (95% CI)	*p*
Acute respiratory distress syndrome	225	130	1.23 (0.98–1.55)	0.073	1.30 (1.02–1.65)	0.036
Venous thromboembolism	225	44	1.32 (1.02–1.71)	0.035	1.48 (1.11–1.98)	0.008

OR = odds ratio.
[a]Baseline demographics adjustment: age, sex, body mass index, and site.
[b]Multivariable adjusted: Baseline demographic covariates and diabetes mellitus, hypertension, and chronic kidney disease.

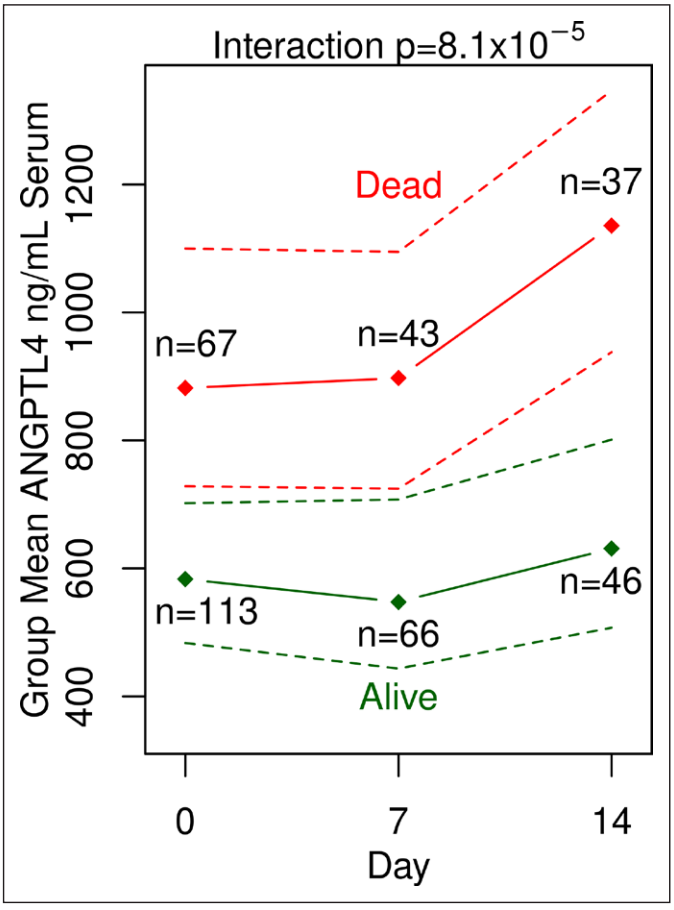

Figure 2. Longitudinal plasma angiopoietin-like 4 (ANGPTL4) measurements are associated with hospital mortality. Plasma ANGPTL4 markers measured on days 1, 7, and 14 after ICU admission stratified by hospital mortality. The *dots* represent the mean ANGPTL4 concentrations and the *n* provides the total number of patients with ANGPTL4 concentrations measured on a given day. The *dashed lines* provide the bootstrap 95% CI of the mean.

differentially express ANGPTL4 between COVID and non-COVID-19.

snRNA Sequencing Data Demonstrates Upregulation of ANGPTL4 in Pulmonary Alveolar Epithelial Cells and Fibroblasts After SARS-CoV-2 Infection

We examined whether ANGPTL4 gene expression was upregulated in postmortem lung specimens from patients with COVID-19 using snRNA sequencing. For these analyses, we used a published snRNA sequencing dataset of lung tissue from 19 individuals who died from COVID-19 and seven individuals without COVID-19 who underwent lung resection or biopsy (18). ANGPTL4 gene expression was significantly upregulated in pulmonary alveolar type 2 epithelial cells (false discovery rate = 4.38×10^{-48}) and fibroblasts (false discovery rate = 1.48×10^{-22}) in COVID-19 decedents compared with controls (**Fig. 3*A***). In addition, gene expression of neuropilin-1 and neuropilin-2, the proposed receptors for ANGPTL4 binding (19, 20), were upregulated in a number of cell types, including endothelial cells (**Figs. S6** and **S7**, and Online Supplement File 1, http://links.lww.com/CCX/B117). Next, we cultured immortalized distal small airway epithelial cells (includes distal bronchiolar and alveolar epithelial cells) and infected with SARS-CoV-2. We then collected RNA over serial timepoints during the first 72 hours. Consistent with our single-cell RNA sequencing data, a two-way ANOVA revealed there was a significant interaction between the effects

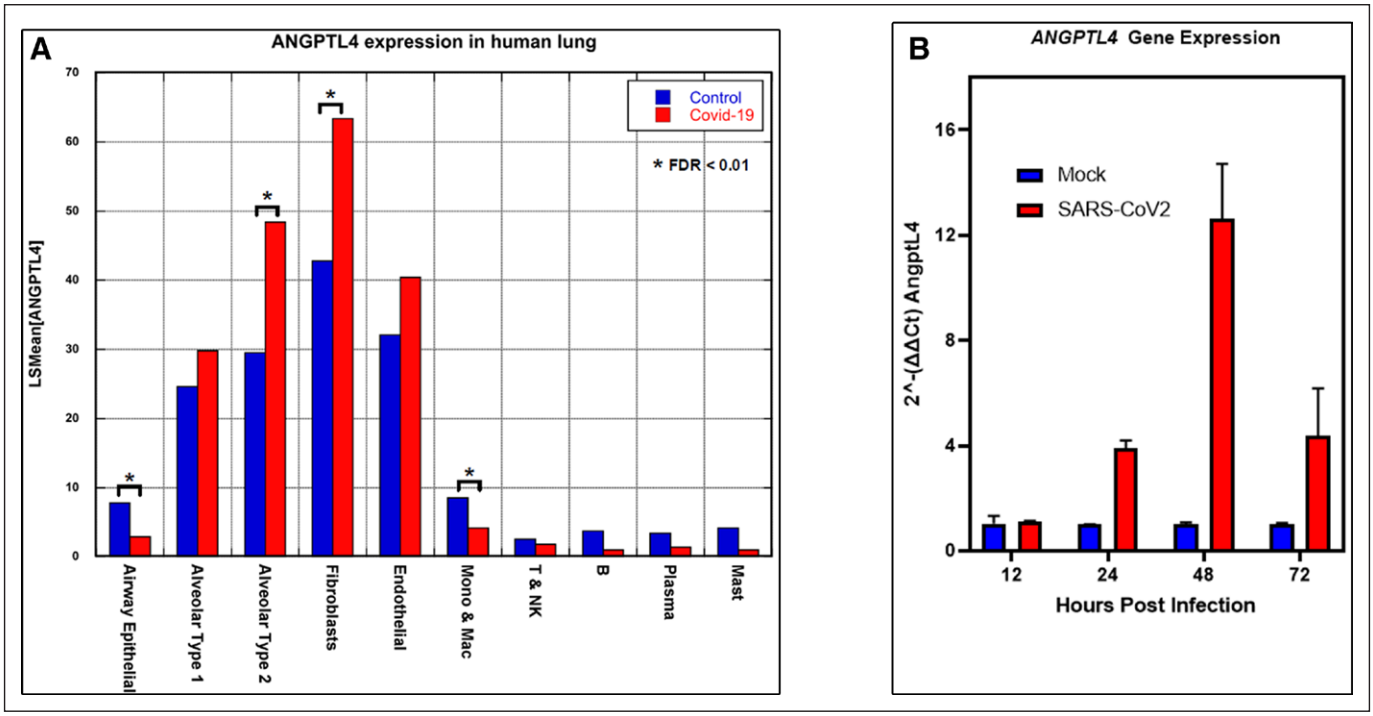

Figure 3. Upregulation of angiopoietin-like 4 (*ANGPTL4*) gene expression in human lung alveolar type 2 epithelial cells and fibroblasts. **A**, Single-cell RNA sequencing data comparing *ANGPTL4* gene expression in 20 COVID-19 patients to seven without COVID-19. Transcript counts are cell specific. Analysis demonstrates significant upregulation of *ANGPTL4* in alveolar type 2 epithelial cells and fibroblasts and downregulation in monocytes (Mono) and macrophages (Mac). Cells classified as airway epithelial cells demonstrated high expression of dynein axonemal heavy chain 9 gene expression (*DNAH9*), whose product is essential for ciliary function. **B**, *ANGPTL4* gene expression in immortalized human small airway epithelial cells infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). These cells gene expression pattern was consistent with distal respiratory/alveolar epithelium (no DNAH9 expression and high podoplanin, a gene expressed by type 1 alveolar epithelial cells. A two-way analysis of variance revealed there was a significant interaction between the effects of SARS-CoV-2 and time on ANGPTL4 expression ($p = 0.0027$). Simple main effects analysis showed that SARS-CoV-2 did have a statistically significant effect on ANGPTL4 expression ($p = 0.04$). FDR = false discovery rate; LSMean = Least squares mean; B = B cells; T and NK = T and Natural Killer Cells; 2^-(ΔΔCt) =Cycle thresholds.

of SARS-CoV-2 and time on ANGPTL4 expression ($p = 0.0027$). Simple main effects analysis showed that SARS-CoV-2 did have a statistically significant effect on ANGPTL4 expression ($p = 0.04$) (**Fig. 3*B***).

DISCUSSION

Vascular permeability leading to pulmonary edema, respiratory failure, and acute lung injury are hallmarks of SARS-CoV-2 infection in critically ill COVID-19 patients; however, the identification of specific endothelial pathways leading to this pathology remains limited. We used a targeted immunoassay to measure circulating ANGPTL4 levels and test associations with outcomes in a multicenter, ICU cohort of COVID-19 adult patients. Longitudinal plasma measurements demonstrated the dynamics of ANGPTL4 plasma concentrations and the association of ANGPTL4 with clinical outcomes, such as ARDS, VTE, days of mechanical ventilation, and in-hospital mortality. Leveraging a publicly available proteomic and lung snRNA sequencing data, we demonstrated that ANGPTL4 is highly abundant in lung tissue and that its expression is upregulated in a number of lung cell types in COVID-19 compared with controls. Finally, we identify that the ELISA assay used to measure ANGPTL4 has high affinity for c-ANGPTL4. Taken together, our findings suggest plasma ANGPTL4 concentrations are associated with clinical prognosis in critically ill COVID-19 patients.

The role of ANGPTL4 in the promotion of vessel permeability has been controversial (7). Preclinical data supporting a role for ANGPTL4 in preventing vessel permeability and preserving vessel integrity have been reported, mostly in cancer (21). Alternately, studies have demonstrated that ANGPTL4 may potentiate vascular permeability through binding to neuropilin-1

and neuropilin-2 surface receptors on endothelial cells leading to vascular leak (6, 22). These seemingly conflicting roles for ANGPTL4 may be due to different products of ANGPTL4 being measured. We demonstrated that the ELISA assay used in our studies was predominantly measuring c-ANGPTL4 rather than n-ANGPTL4. ANGPTL4's role in disease may also be context dependent. For example, Ang-2's biological role is context dependent with regulating angiogenesis in cancer but promoting endothelial dysfunction and capillary leak in critical illness (23, 24). Our findings demonstrate the close link between plasma c-ANGPTL4 concentrations and clinical signs of endothelial disruption, in particular, development of VTE, ARDS, days receiving IMV, and in-hospital mortality.

Currently, therapeutics that target endothelial pathways in critical illness, infection or COVID-19 are lacking. Previous studies have shown mice that are genetically deficient in *ANGPTL4* are more resistant to pulmonary tissue leak and inflammation-induced lung damage from influenza virus infection (10). The same investigators also demonstrated that treatment of mice with influenza followed by use of a neutralizing anti-ANGPTL4 antibody significantly accelerated lung recovery and improved tissue integrity (10). In a *Streptococcus pneumoniae*-infected mouse model, the investigators demonstrated that anti-ANGPTL4 treatment augmented the benefits of antibiotics with prolonged median survival of mice and less inflammation (9). Clinical studies have shown that ANGPTL4 concentrations are elevated in bronchoalveolar lavage fluid and serum samples in ARDS compared with controls and ANGPTL4 concentrations are associated with hospital mortality in ARDS (11). These results suggest that ANGPTL4 signaling may be important in viral and nonviral causes of pneumonia. Despite this strong experimental rationale, no therapy targeting the ANGPTL4 pathway has been tested in human pneumonia. Our findings highlight the importance of further elucidation of the role of ANGPTL4 in COVID-19 and potentially other severe respiratory infections. Furthermore, effective intervention on this cascade might yield observable benefit within two weeks of intervention from admission.

This study has a number of strengths. First, all blood samples were collected within a standardized time from ICU admission and handled in a rigorous and uniform fashion. Second, patients were recruited from three different medical centers across a broad time-calendar period from April 2020 to May 2021. Inclusion of patients from different time periods of the pandemic suggest that the associations of ANGPTL4 with clinical outcomes are relevant for patients experiencing different SARS-CoV-2 variants, COVID-19 treatments, or medical systems' strain. Third, multivariable models adjusted for demographics and comorbidities suggest that ANGPTL4 is independently associated with clinical outcomes in the ICU. Fourth, repeated measures of longitudinal ANGPTL4 concentrations demonstrate that this biomarker is dynamic during critical illness and associations with COVID-19–related clinical outcomes increased over the course of hospitalization. This study also has limitations. Diagnoses of ARDS and VTE were ascertained clinically by the treating team. Our use of a clinical diagnosis to define the outcomes of ARDS and VTE may have led to misclassification. However, previous studies, such as LUNG-SAFE (Lung Study to Understand the Global Impact of Severe Acute Respiratory Failure), have shown that the direction of misclassification in clinical settings is generally toward underdiagnosis, suggesting misclassification in our analyses would have likely biased our findings to the null (25). There may have been misclassification of ARDS diagnosis or lack of diagnostic workup for VTE. While we approached the relationship of ANGPTL4 and clinical outcomes using multiple lines of research (plasma ANGPTL4, ex vivo transfection of cell lines, in vivo ANGPTL4 expression), these data alone demonstrate associations and future studies will be required to define mechanisms by which ANGPTL4 is acting and establish causality. In addition, lung is not the only tissue source that produces ANGPTL4 and other organs, such as liver and kidney, likely contributed to circulating ANGPTL4 concentrations. However, in tissue proteomic analyses, we found that abundance of ANGPTL4 is high in the lung.

In conclusion, in a prospective, multicenter ICU cohort of adult COVID-19 patients, we demonstrate that ANGPTL4 plasma concentrations are associated with COVID-19 clinical outcomes. Identification of these ANGPTL4 associations in COVID-19 has several important implications. This may prove useful as a prognostic biomarker to inform clinical decisions. Its biochemical pathway may lead to therapeutic opportunities. Finally, prognostication based upon ANGPTL4 concentrations may identify patients with COVID-19 who would have

the highest potential to benefit from therapies that modulate ANGPTL4 signaling or other interventions.

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