OBSERVATIONAL STUDY

OPEN

Validating a Proteomic Signature of Severe COVID-19

Christopher V. Cosgriff, MD, MPH[1]
Todd A. Miano, PhD, PharmD[2]
Divij Mathew, PhD[3,4]
Alexander C. Huang, MD, PhD[3,5,6,7]
Heather M. Giannini, MD, MS[8]
Leticia Kuri-Cervantes, PhD[3,9]
M. Betina Pampena, PhD[3,9]
Caroline A. G. Ittner, PhD[8,10]
Ariel R. Weisman, MS[8,10]
Roseline S. Agyekum, BS[8,10]
Thomas G. Dunn, BS[8,10]
Oluwatosin Oniyide, BS[8,10]
Alexandra P. Turner, BS[8,10]
Kurt D'Andrea, BS[3]
Sharon Adamski, BS[11]
Allison R. Greenplate, PhD[3,4,11]
Brian J. Anderson, MD, MSCE[8,10]
Michael O. Harhay, PhD[2]
Tiffanie K. Jones, MD, MPH, MSCE[2,8,10]
John P. Reilly, MD, MSCE[8,10]
Nilam S. Mangalmurti, MD[8,10,3,12]
Michael G. S. Shashaty, MD, MSCE[8,10]
Michael R. Betts, PhD[3,9]
E. John Wherry, PhD[3,4,6]
Nuala J. Meyer, MD, MS[8,10,3,12]

OBJECTIVES: COVID-19 is a heterogenous disease. Biomarker-based approaches may identify patients at risk for severe disease, who may be more likely to benefit from specific therapies. Our objective was to identify and validate a plasma protein signature for severe COVID-19.

DESIGN: Prospective observational cohort study.

SETTING: Two hospitals in the United States.

PATIENTS: One hundred sixty-seven hospitalized adults with COVID-19.

INTERVENTION: None.

MEASUREMENTS AND MAIN RESULTS: We measured 713 plasma proteins in 167 hospitalized patients with COVID-19 using a high-throughput platform. We classified patients as nonsevere versus severe COVID-19, defined as the need for high-flow nasal cannula, mechanical ventilation, extracorporeal membrane oxygenation, or death, at study entry and in 7-day intervals thereafter. We compared proteins measured at baseline between these two groups by logistic regression adjusting for age, sex, symptom duration, and comorbidities. We used lead proteins from dysregulated pathways as inputs for elastic net logistic regression to identify a parsimonious signature of severe disease and validated this signature in an external COVID-19 dataset. We tested whether the association between corticosteroid use and mortality varied by protein signature. One hundred ninety-four proteins were associated with severe COVID-19 at the time of hospital admission. Pathway analysis identified multiple pathways associated with inflammatory response and tissue repair programs. Elastic net logistic regression yielded a 14-protein signature that discriminated 90-day mortality in an external cohort with an area under the receiver-operator characteristic curve of 0.92 (95% CI, 0.88–0.95). Classifying patients based on the predicted risk from the signature identified a heterogeneous response to treatment with corticosteroids ($p = 0.006$).

CONCLUSIONS: Inpatients with COVID-19 express heterogeneous patterns of plasma proteins. We propose a 14-protein signature of disease severity that may have value in developing precision medicine approaches for COVID-19 pneumonia.

KEY WORDS: acute hypoxic respiratory failure; adult; corticosteroids; COVID-19; plasma proteomics

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) results in COVID-19, the manifestations of which range from asymptotic carriage to the development of acute respiratory distress syndrome (ARDS) (1, 2). This clinical heterogeneity is mirrored by significant heterogeneity in the host immune response (3–6). Notably, whereas treatments targeting viral replication have been effective in early disease (7, 8), the treatments that have demonstrated a mortality benefit once severe COVID-19 is established have been anti-inflammatory (9–11).

DOI: 10.1097/CCE.0000000000000800

KEY POINTS

Question: We sought to identify and validate a plasma protein signature of severe COVID-19 using a high-throughput protein assay and to test whether proteomic high risk subjects exhibited a differential response to corticosteroids.

Findings: Over 190 proteins were differentially expressed between severe and nonsevere hospitalized subjects with COVID-19. We validated a 14-protein signature with strong discrimination for 90-day mortality in two populations, and the risk score demonstrated significant interaction with the mortality effect of corticosteroids.

Meaning: Our data suggest significant heterogeneity in the COVID-19 plasma proteome that could be tested as an enrichment factor for anti-inflammatory treatments.

Although immunomodulation has demonstrated a mortality benefit, it is not without risks, and the benefits in these trials appear limited to those with more severe disease. In the Randomized Evaluation of COVID-19 Therapy (RECOVERY) trial, administration of dexamethasone was beneficial in patients with hypoxemia, and there was a suggestion of harm in patients not receiving oxygen therapy (9). Disease severity may increase the number of patients who will have the outcome of interest (prognostic enrichment) and thus improve the power to detect a difference between groups. Alternatively, the presence of hypoxemia may serve as a proxy to identify patients with a more dysregulated immune response and thus identify patients more likely to respond to therapy (predictive enrichment) (12).

Working toward predictive enrichment, critical illness syndrome subphenotypes have been proposed based on unsupervised and supervised machine learning approaches using both clinical features and biomarkers (13–15). In ARDS, subphenotypes identified by latent class analysis (LCA) applied to clinical and biomarker features have been reproduced in multiple cohorts and have been applied retrospectively to identify heterogeneous treatment effects (15–18). Applying LCA to a COVID-19 cohort again identified two subphenotypes with a heterogeneous treatment effect for corticosteroid administration (19). Such work suggests that the use of biomarkers in feature sets may be used to identify groups of patients most likely to benefit from therapies and least likely to experience harm with a precision that exceeds clinical features alone.

Many of these prior works have used established candidate biomarkers for inflammation such as interleukin (IL)–6 and IL-8 (20). However, the secreted proteome in COVID-19 may differ from other forms of sepsis, and higher throughput protein arrays may identify more specific candidates. We sought to develop a proteomic signature of severe COVID-19 that broadly surveyed both familiar and novel proteins. As was recently applied in the development of a proteomic signature for progression of interstitial lung disease, we performed a high-dimensional sampling of the plasma proteome and used a combination of biological insight and machine learning to develop a parsimonious protein signature (21). We then examined whether the effect of corticosteroid administration on mortality varied with respect to subgroups derived from our protein signature. We propose this feature set for future work in endotype identification, as well as investigations into the differential molecular pathophysiology that may explain ARDS subphenotypes.

MATERIALS AND METHODS

Study Design and Participants

Our discovery cohort were patients admitted to one of two hospitals in our health system with a positive SARS-CoV-2 plolymerase chain reaction (PCR) test who provided informed consent within 3 days of admission between March 23, 2020, and July 27, 2020. We selected late July 2020 as our termination date because at that time COVID-19 admissions had significantly decreased in Philadelphia and samples were batched for analysis. As such, these subjects represent the first wave of what we now recognize as the Alpha variant of SARS-CoV-2. At enrollment, blood was collected and processed for plasma. Clinical data were abstracted from the electronic health record. Each patient was classified as having severe or nonsevere disease every 7 days, where severe disease was defined as illness that resulted in death, the receipt of high-flow nasal cannula, noninvasive or invasive positive pressure ventilation, or extracorporeal membranous

oxygen, and nonsevere disease was defined as receiving no oxygen or low-flow oxygen (≤ 6 L/min) via nasal cannula. This label was adapted from the ordinal scale defined in early COVID-19 clinical trials (9) and was chosen because it is a clinically relevant separation that likely captures nonventilated ARDS (22) and identifies patients who need higher intensity multidisciplinary care. Additional phenotyping included for bacterial coinfection, which was deemed present if the subject had any positive bacteriologic culture or molecular assay from blood, respiratory secretions, urine, or stool in the 5-day window centered around the blood draw (days –2 to +2), and for duration of COVID-19 symptoms prior to the blood draw. Mortality was assessed at 90 days.

Human subjects or their surrogates provided informed consent for the Molecular Epidemiology of SepsiS in the ICU Cohort, protocol 808542 approved initially June 2, 2008, and most recently March 24, 2022. All procedures were followed in accordance with the ethical standards of the University of Pennsylvania Institutional Review Board and with the Helsinki Declaration of 1975. Our validation cohort was recruited at the Massachusetts General Hospital as is described here (23) and was shared publicly by the investigators. We used the plasma Olink results from day 3 in the validation data, as this most closely approximated our samples.

Sample Processing and Proteomic Assay

Whole blood was spun within 2 hours of blood collection (3,000 rpm, 15 min), and plasma was collected, aliquoted, and frozen at –80°C until assay. Plasma was not immunodepleted. We used the Olink Proximity Extension Assay to measure 713 unique proteins. In this assay, oligonucleotide-labeled monoclonal or polyclonal antibodies are used to bind each protein target in a pairwise manner upon which the paired oligonucleotides hybridize (24, 25). The unique hybridization product is amplified by PCR, and multiplex detection occurs in a high throughput fluidic chip system. On each plate, a common interplate control of pooled "healthy" plasma acquired and processed at Olink facilities (24) was used for normalization resulting in a semiquantitative measurement for each protein on $\log_2$-transformed scale referred to as the normalized protein expression (NPX).

Proteomic Signature Identification and Validation

Our signature identification pipeline was divided into two distinct steps. We had significantly more features than subjects; to overcome this and limit the size of our potential candidate feature set, we first identified biological pathways associated with severe COVID-19 disease at admission to the hospital. For each protein feature, we performed logistic regression to estimate its association with severe disease at admission adjusted for age, sex, race, cardiovascular comorbidity, and time from symptom start. These covariates were selected a priori based on the published literature demonstrating associations with COVID-19 outcome (1, 2, 26) and given potential relationships between proteins and these factors which could confound the severity relationship. Comorbidity was modeled as the presence of diabetes mellitus, hypertension, coronary artery disease, or chronic renal insufficiency and was selected as they have been shown to be associated with severe disease and could be associated with the distribution of the protein features (26). We applied the Benjamini-Hochberg procedure with a false discovery rate (FDR) of 5% and then applied Ingenuity Pathway Analysis to identify pathways that enrich in early severe disease (27). Proteins that were members of pathways which enriched with a p value of below 10E–4, to select the most parsimonious group that strongly associated with severity, were advanced to the next step.

We then applied elastic net logistic regression (ENLR) to this feature set using severe disease or death on day 28 as the outcome variable (28). Hyperparameters were selected using 10-fold cross validation in the discovery cohort. Before the model fitting procedure, each feature was centered around its mean and scaled by its SD; when applied in the validation cohort, the center and scaling variables from the discovery cohort were applied. No imputation step was applied, and patients with missing features were excluded from the model fitting procedure.

Discrimination of the signature was assessed using the area under the receiver-operator characteristic curve (AUROC), and we report AUROC values in the discovery and validation cohorts. We quantified uncertainty in our AUROC estimates using 95% CIs derived from a bootstrap procedure with 10,000 bootstrapped datasets. To further understand the proteins associated

with severe COVID-19, we performed univariate regression of protein features on age greater than or equal to 60 and male sex applying the Benjamini-Hochberg procedure with FDR less than 5% to declare significance.

Heterogeneity of Treatment Effect

To examine our signature's potential use in the development of subphenotypes for predictive enrichment, we applied the signature to classify patients into low- and high-risk groups and evaluated for a heterogeneous treatment effect for corticosteroid administration on 90-day mortality in our cohort. We used the coefficients from the trained ENLR model to calculate the probability of severe disease or death on day 28 to classify patients low- or high-risk using the scikit-learn default cutoff of 0.5 (29).

Because our cohort was recruited before the publication of randomized trials demonstrating the benefit of corticosteroids, corticosteroid administration was at the discretion of physicians caring for the patients, driven largely by illness severity. We therefore modeled the probability of receiving steroids as a function of severity of illness using the Acute Physiology and Chronic Health Evaluation (APACHE) III score (30), presence of cardiovascular comorbidity as described above, and presence or absence of severe COVID-19 at admission. As a sensitivity analysis, we also fit propensity models that included age, sex, and race, although these features were not expected to significantly predict the probability of receiving steroids. We used this model to estimate inverse probability weights for treatment assignment. We then modeled the outcome of 90-day mortality as a function of corticosteroid administration and risk group and included an interaction term between corticosteroid administration and risk group; subjects in this model were weighted by their inverse probability weight to estimate a marginal structural model (31). Covariate balance before and after weighting was checked graphically. Because weighted regression violates the assumption of heteroscedasticity, SEs were estimated robustly using a sandwich estimator of variance, and these robust errors were used for null hypothesis testing. To visualize this heterogeneity, we fit a second inverse probability weighted regression model using the probability output from the model as a continuous feature in place of the calculated risk group and calculated the marginal risk of 90-day mortality across the predicted probability of severe disease at day 28 stratified by corticosteroid administration; we then plotted these curves with respect to the protein-predicted probability of severe disease. As a comparison, we applied to the same modeling procedures to assess for an interaction with disease severity as estimated by APACHE III (modeled as both continuous and in tertiles) as well as the presence or absence of severe disease at admission to the hospital.

Software, Code, and Data Availability

Analyses were carried out in a combination of Python 3.8 (Python Software Foundation) and R 4.0 (R Foundation). A description of the packages used in each environment and all code is hosted publicly on Github (https://github.com/MESSI-Group/COVID-PDSS). An anonymized version of the dataset is made available on the github above.

RESULTS

Details of our discovery cohort are reported in **Table 1**. Follow-up for 90-day mortality was available in 164 patients (98.2%). Ninety-one patients (54%) were classified as having severe disease on presentation. Although patients with severe disease had significantly higher APACHE III scores (mean score 64.8 vs 33.6; $p < 0.001$) and 90-day mortality (26 expired vs 5; $p = 0.001$), they did not differ significantly with respect to demographics or burden of comorbidity (Table 1).

Of the 167 patients enrolled, 161 had all 713 proteins measured; the six remaining had missing values for a small subset of their features. For each protein, all subjects were included in the enrichment analysis, except where a subject did not have a measurement and was excluded and therefore the majority of regressions included all patients with at most six patients excluded. We identified 145 proteins with significantly increased expression in severe COVID-19 and 49 proteins with significantly decreased expression after correcting for multiple comparisons (**Fig. 1**; and **eTable 1**, http://links.lww.com/CCX/B90). Ingenuity pathway analysis applied to these 194 proteins identified 62 pathways of which 24 were statistically significant after adjustment for multiple comparisons (**eTable 2,** http://links.lww.com/CCX/B90).

TABLE 1.
Discovery Cohort Characteristics

Baseline Characteristics	Total Population (*N* = 167)	Nonsevere (*N* = 76)	Severe (*N* = 91)
Age, yr, mean ± SD	58.9 ± 14.6	57.1 ± 15.6	60.4 ± 13.6
Female sex, *n* (%)	76 (45.5)	36 (47.4)	40 (44.0)
Race, self-reported, *n* (%)			
Black	112 (67.1)	56 (73.7)	56 (61.5)
White	46 (27.5)	18 (23.7)	23 (30.8)
Asian	7 (4.2)	2 (2.6)	5 (5.5)
Other or more than one	2 (1.2)	0 (0)	2 (2.2)
Duration of COVID symptom days, median (interquartile range)	9 (6–14)	10 (7–15)	9 (6–14)
Bacterial coinfection, *n* (%)	16 (10)	6 (8)	10 (11)
Cardiovascular comorbidity, *n* (%)	126 (75.4)	59 (77.6)	67 (73.6)
Corticosteroids prior to blood draw, *n* (%)	60 (36.4)	9 (12.2)	51 (56.0)
World Health Organization Ordinal Scale at enrollment, *n* (%)			
3 (hospitalized, no O_2)	33 (19.8)	33 (43.4)	
4 (hospitalized, $O_2 \leq 6$ L nasal cannula)	43 (25.7)	43 (56.5)	
5 (hospitalized, O_2 by high flow or noninvasive O_2)	42 (25.1)		42 (46.2)
6 (hospitalized, mechanical ventilation)	49 (29.3)		49 (53.8)
Acute Physiology and Chronic Health Evaluation III score, mean ± SD	50.6 ± 30.8	33.6 ± 17.6	64.8 ± 32.4
Vasoactive medications, *n* (%)	50 (40.7)	3 (6.0)	47 (64.4)
Mortality at 90 d, *n* (%)	31 (18.6)	5 (6.6)	26 (28.6)

Bacterial coinfection was determined by positive blood, respiratory, urine, or stool culture or molecular test in the 5-d window from 2 d prior to blood draw until 2 d following blood draw, with blood draw considered day 0. Cardiovascular comorbidity was defined as the presence of any of the following chronic conditions: diabetes mellitus, hypertension, coronary artery disease, or chronic renal insufficiency.

There were 75 proteins shared across these latter pathways; we focused on the 31 unique proteins shared across pathways whose *p* value was below 1E–4. There was substantial correlation among these features suggesting that a more parsimonious model could be derived that retained similar information (**eFig. 1,** http://links.lww.com/CCX/B90). Two patients were missing one of these protein features and were excluded from this portion of the analysis. ENLR with 10-fold cross-validation yielded a model with 14 protein features (**Table 2**). Concordant with their model coefficients, tenascin C (TNC), C-C motif chemokine 7 (CCL7), IL 1 receptor like 1 (IL1RL1), matrix metalloproteinase (MMP) 7, transforming growth factor alpha (TGFA), IL-17C, programmed death ligand 1 (PD-L1, also known as cluster of differentiation (CD274) CD274), galectin-9 (LGALS9), and osteopontin (SPP1) were enriched in patients with more severe disease, and IL-12 beta (IL-12B), tumor necrosis factor superfamily 11 (TNFSF11), apolipoprotein M (APOM), MMP9, and lymphotoxin alpha (LTA) were enriched in patients with less severe disease (**eFigs. 2** and **3**, http://links.lww.com/CCX/B90). This 14-protein model achieved an AUROC of 0.88 (0.83–0.93) in our discovery cohort and an AUROC of 0.92 (0.87–0.95) in our external validation cohort. Applying a 5% FDR, no proteins were significantly associated with male sex and five proteins were associated with age greater than or equal to 60 (**eTable 3,** http://links.lww.com/CCX/B90).

Applying the default risk threshold of 0.5 to the model's output probability to classify patients identified a high-risk subgroup whose membership significantly

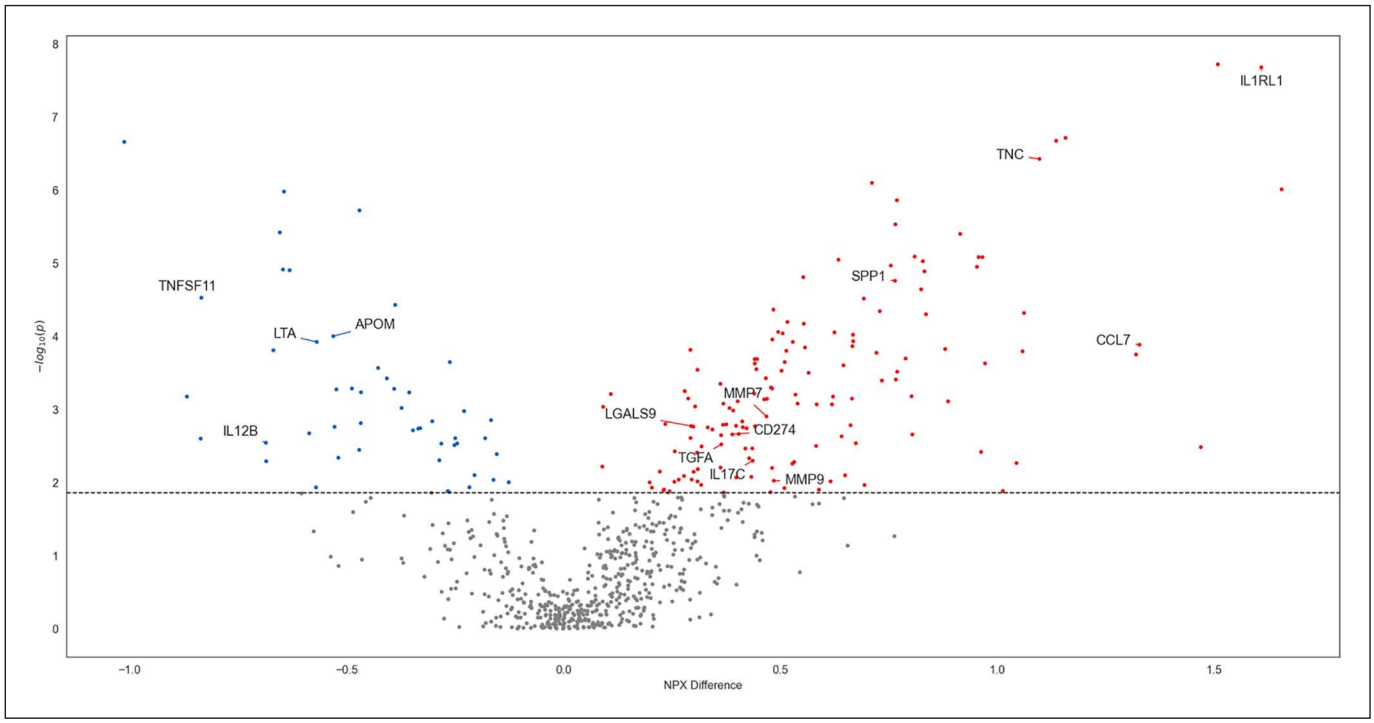

Figure 1. Volcano plot for all proteins comparing nonsevere versus severe COVID-19 at admission in the discovery population. The proteins composing our final proteomic signature, including differential expression by false discovery rate, membership in a differentially expressed pathway, and selection by elastic net logistic regression model, are labeled on the plot. APOM = apolipoprotein M, CCL7 = C-C motif chemokine 7, CD274 = cluster of differentiation 274, IL1RL1 - interleukin-1 receptor like 1, IL12B = interleukin 12B, IL17C = interleukin 17C, LGALS9 = galectin-9, LTA = lymphotoxin alpha, MMP7 = matrix metalloproteinase 7, MMP9 = matrix metalloproteinase 9, NPX = normalized protein expression, SPP1 = osteopontin, TGFA = transforming growth factor alpha, TNC = tenascin C, TNFSF11 = tumor necrosis factor superfamily 11.

interacted with corticosteroid administration (p = 0.006). Neither APACHE III (p = 0.33 modeled as continuous, p = 0.97 modeled as tertiles) nor severe disease on presentation (p = 0.55) significantly interacted with corticosteroid administration. These results were robust to sensitivity analyses including demographic features as additional potential confounders (protein risk group interaction p = 0.005). Modeling the interaction using the probability of severe disease as a continuous variable did not identify a statistically significant interaction with corticosteroid administration (p = 0.173). However, plotting the marginal risk of mortality as a function of the protein signature derived risk stratified by corticosteroid administration provided insight into the identified interaction when using the model to classify patients (**Fig. 2**). This plot suggested that steroids are associated with increased mortality at the lowest end of the risk spectrum and trend toward a benefit as the risk of severe disease as predicted by protein signature increases, albeit the analysis is underpowered to demonstrate a statistically significant benefit even in the highest risk patients.

DISCUSSION

We performed a high-dimensional sampling of the proteome in 167 patients with COVID-19 and used pathway analysis and regularized logistic regression to identify a parsimonious protein signature which strongly discriminates severe disease in both our discovery cohort and an external validation cohort. Our protein signature may also identify heterogeneity in the effect of corticosteroid treatment on 90-day mortality in patients with COVID-19. Although multiple high-quality plasma proteomic studies have been reported in COVID-19 (23, 24, 32), we believe ours is notable for using a high dimensional platform while identifying a parsimonious protein signature capable of predicting severe disease and death when applied to hospitalized inpatients. Our validation in an external, geographically distinct hospitalized population, our representation of African American subjects, and our enrichment for critical illness are unique strengths of our study.

TABLE 2.
Elastic Net Logistic Regression–Selected Proteins

Feature (Protein)	Coefficient	OR
Tenascin C	0.414	1.513
C-C motif chemokine 7	0.329	1.390
IL 1 receptor like 1	0.317	1.373
MMP7	0.189	1.208
Transforming growth factor alpha	0.161	1.175
IL17C	0.113	1.120
CD274	0.083	1.087
Galectin-9	0.048	1.049
Osteopontin	0.039	1.040
IL12B	−0.011	0.989
Tumor necrosis factor superfamily 11	−0.031	0.970
Apolipoprotein M	−0.122	0.885
MMP9	−0.166	0.947
Lymphotoxin alpha	−0.235	0.790

CD = cluster of differentiation, IL = interleukin, MMP = matrix metalloproteinase, OR = odds ratio.
Feature coefficients from the final elastic net logistic regression model presented on the log-odds and OR scale, quantifying the estimated association between the feature and risk of severe disease at day 28. Because the input data were normalized, these coefficients can be directly compared and serve as a direct measure of relative feature importance of each protein to the predictive model.

The proteins identified are implicated in a variety of biological processes including proinflammatory immune activation (IL1RL1, IL-17C, CCL7, IL-12B), dampening of the immune response (CD274 [PD-L1], LTA, TNFSF11, LGALS9), extracellular remodeling and fibrosis (TNC, MMP7, MMP9, TGFA, SPP1), and endothelial barrier integrity (APOM). Many of these proteins have been implicated in sepsis and ARDS due to SARS-CoV-2 or other etiologic agents (32–40). Although this study was not designed to identify causal relations, these proteins and their associated pathways represent biologically credible targets that warrant further evaluation.

In particular, our analysis suggested a strong association between COVID-19 severity and the IL-33 receptor protein IL1RL1, which is also known as suppression of tumorigenesis-2 (ST2). The IL-33/IL1RL1 axis has been implicated in myocardial infarction, heart failure, community acquired pneumonia, asthma, and ARDS (41–44). It has more recently been demonstrated as a biomarker associated with persistent ventilator dependence, and our findings replicate others identifying ST2 as a prognostic biomarker in COVID-19 (45). It exists in a membrane-bound form and as a soluble splice variant (sST2) with the former facilitating IL-33 signal transduction and the latter acting as a potential dummy receptor that dampens IL-33 signaling. Although the cellular source of sST2/IL1RL1 during COVID-19 is not known, sST2 is released by type II pneumocytes in response to strain and by regulatory T cells in response to stimulation by IL-33 (33, 44). The varied roles of IL-33 include promoting T helper 2 (Th2) cell differentiation and expanding regulatory T-cell expression; thus, the relative balance of IL-33, membrane expression of ST2 across various cell types, and secretion of sST2 may represent a crucial axis linking epithelial damage to the tuning of the adaptive immune response (46), particularly in light of data linking T-cell activation to severe COVID-19 (3).

It is also noteworthy that markers associated with repair and fibrosis were implicated despite how early in the disease process the plasma samples were collected. MMP7, MMP9, TNC, and SPP1 have all been implicated as biomarkers in fibrotic interstitial lung disease (47–49). For example, SPP1 can be used in a three-protein index to discriminate idiopathic pulmonary fibrosis (IPF) from other interstitial pneumonias and is elevated in hepatic, retroperitoneal, and myocardial fibrosis (49); recent mechanistic work in mice suggests a causal role in driving pulmonary fibrosis (50). TNC emerged as a plasma marker elevated in IPF that is also overexpressed at the transcript and protein level in human alveolar epithelial-like cells with telomere dysfunction (51). Although these potential markers of fibrosis might be nonspecific indicators of extracellular matrix injury, the accelerated fibrosis that appears to complicate severe COVID at a high rate (52) might relate to novel profibrotic programs being activated (53). High plasma MMP7 was correlated with a decline in forced vital capacity and diffusion capacity of carbon monoxide among subjects with COVID-19 followed longitudinally, suggesting a link between immunofibrotic drivers and loss of lung function, although study subjects did not have lung imaging (53). Further work is needed to understand the specificity of these markers for COVID-related lung injury and whether they predict fibrotic complications post COVID.

We found it striking that the protein signature seemed to predict harm from corticosteroids among subjects with low protein severity scores and only marginally and nonsignificantly predicted improvement

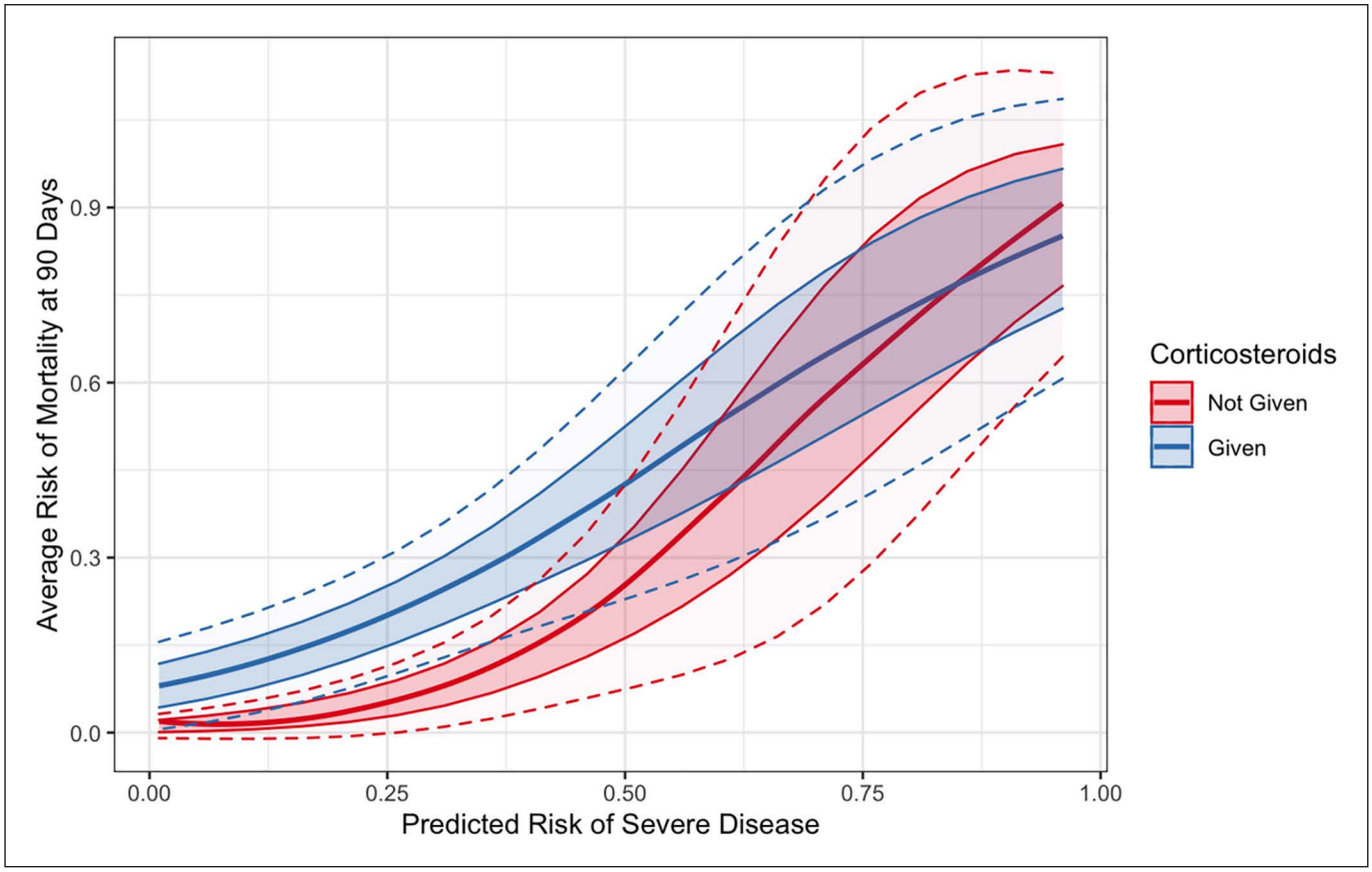

Figure 2. Average risk of mortality as a function of protein-derived risk stratified by steroid administration and adjusted for demographic factors, comorbidity, and disease severity. The *solid line* reflects the marginal estimate, with the *darker* and *lighter shaded areas* reflecting 1 and 2 SDs, respectively. Although the analysis is underpowered, the risk of mortality for patients with low protein derived risk is estimated to be higher for those receiving steroids compared with those not receiving steroids, but the balance of benefit versus harm reverses as the protein score predicted risk increases.

with corticosteroids in the high protein severity score group. There are prior examples, in both observational and randomized settings, of molecular phenotypes predicting harm from corticosteroids (14, 54, 55). It may be that low protein severity score indicates a more regulated host response to infection (3), and perturbing this favorable response has untoward consequences. Alternatively, since subjects who received corticosteroids in our observational cohort commonly received methylprednisolone rather than dexamethasone or hydrocortisone, perhaps we are observing drug-specific effects. We also acknowledge the strong threat of residual confounding despite our adjustment for our measured confounders (eFig. 3, http://links.lww.com/CCX/B90) and that this shortcoming may bias our apparent treatment interaction. However, it is encouraging that we did not detect interaction in the steroid treatment effect for severity of illness as measured by the APACHE III score nor for the enrollment level of COVID-19 severity.

Despite these promising implications of our signature, our study has significant limitations and multiple barriers to overcome before operationalizing the signature to select trial participants or specific treatments. Foremost, Olink is a discovery platform with protein expression measured on the NPX scale normalized to the samples generated from pooled control plasma. This precludes direct quantitative comparisons between samples for the same protein or between these results and methods that return an absolute quantitative value. For the same reason, calibration of our model was not directly assessed. Our model identifies a discriminative, parsimonious feature set, and future work could focus on developing biomarker detection techniques that are rapid, reliable, and informative. These features could then be used in an individual manner or as part of a multivariable model, both of which will require thoughtful probability calibration. We assessed proteins at only one early time point and validated using day 3 values in the replication

population. Although values early during admission have face validity for prognostication, we cannot address the stability of the protein signature over time nor whether the signature varies with clinical course to represent a treatment response indicator. Although we did sample blood at 1 week, we observed significant informative missingness at the later time point due to competing risks of death or discharge. Last, the SARS-CoV-2 virus has continued to evolve as has the landscape of the host response with the introduction of various vaccines and early therapies, and therefore, we will need to confirm that features of this signature have remained significant in more current populations. Our study was conducted during the first Alpha variant, in the era of randomized trials for remdesivir and preceding widespread use of other immunomodulators, monoclonal antibodies, or convalescent plasma; thus, we cannot determine whether those treatments or different virus strains might influence our results.

CONCLUSIONS

This work, which began with a high-dimensional sampling of the proteomic milieu and concluded with an externally validated, parsimonious protein signature, suggests important biomarker features of severe COVID-19. We find that this signature performs well in disease prognostication and may interact with immune-focused therapy on risk of mortality. Our work adds to the growing literature that disentangle heterogenous critical illness syndromes into subgroups of host response, characterized largely by variation in host inflammatory processes. Future studies seeking to develop treatments for severe COVID-19 and other critical illness syndromes will benefit from methods that deconvolute this underlying heterogeneity allowing for the precise targeting of therapeutics to those most likely to benefit.

ACKNOWLEDGMENTS

We are grateful for the patients and families who agreed to participate and for the many nurses, respiratory therapists, pharmacists, physicians, and hospital staff who worked tirelessly to provide outstanding care during challenging circumstances. In addition, we acknowledge the UPenn COVID Processing Unit who were instrumental in processing biosamples and the authors of reference 23, whose generous data sharing made replication possible and facilitates knowledge transfer.

1 *Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.*

2 *Department of Epidemiology, Biostatistics, and Informatics, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.*

3 *Institute for Immunology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.*

4 *Department of Systems Pharmacology and Translational Therapeutics, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.*

5 *Division of Hematology/Oncology, Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.*

6 *Parker Institute for Cancer Immunotherapy, Philadelphia, PA.*

7 *Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA.*

8 *Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.*

9 *Department of Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.*

10 *Center for Translational Lung Biology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.*

11 *Immune Health Project, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.*

12 *Lung Biology Institute, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.*

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (http://journals.lww.com/ccejournal).

Supported, in part, by National Institutes of Health (NIH) HL137006, HL137915, and HL155804 (to principal investigator [PI] Dr. Meyer) and NIH AI082630, AI108545, and AI155577 (to PI Dr. Wherry). Work in the Wherry lab is also supported, in part, by the Parker Institute for Cancer Immunotherapy.

Dr. Wherry has consulting agreements with and/or is on the scientific advisory board for Merck, Roche, Pieris, Elstar, and Surface Oncology. He is a founder of Surface Oncology and Arsenal Biosciences. EJW has a patent licensing agreement on the PD-1 pathway with Roche/Genetech. He is an inventor on U.S. patent number 10,270,446 submitted by Emory University that covers the use of programmed death ligand 1 blockade to treat infections and cancer. Dr. Meyer receives funding to her institution from Quantum Leap Healthcare Consortium, Athersys, Inc, Biomarck Inc, and the Marcus Foundation for work unrelated to this article. The remaining authors have disclosed that they do not have any potential conflicts of interest.

For information regarding this article, E-mail: nuala.meyer@pennmedicine.upenn.edu

REFERENCES

1. Zhou F, Yu T, Du R, et al: Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: A retrospective cohort study. *Lancet* 2020; 395:1054–1062
2. Feng Y, Ling Y, Bai T, et al: COVID-19 with different severities: A multicenter study of clinical features. *Am J Respir Crit Care Med* 2020; 201:1380–1388
3. Mathew D, Giles JR, Baxter AE, et al; UPenn COVID Processing Unit.: Deep immune profiling of COVID-19 patients reveals distinct immunotypes with therapeutic implications. *Science* 2020; 369:eabc8511
4. Vella LA, Giles JR, Baxter AE, et al; UPenn COVID Processing Unit.: Deep immune profiling of MIS-C demonstrates marked but transient immune activation compared to adult and pediatric COVID-19. *Sci Immunol* 2021; 6:eabf7570
5. Lucas C, Wong P, Klein J, et al: Longitudinal analyses reveal immunological misfiring in severe COVID-19. *Nature* 2020; 584:463–469
6. Merad M, Blish CA, Sallusto F, et al: The immunology and immunopathology of COVID-19. *Science* 2022; 375:1122–1127
7. Hammond J, Leister-Tebbe H, Gardner A, et al: Oral nirmatrelvir for high-risk, nonhospitalized adults with COVID-19. *N Engl J Med* 2022; 386:1397–1408
8. Westendorf K, Żentelis S, Wang L, et al: LY-CoV1404 (bebtelovimab) potently neutralizes SARS-CoV-2 variants. *Cell Rep* 2022; 39:110812
9. Horby P, Lim WS, Emberson JR, et al; RECOVERY Collaborative Group: Dexamethasone in hospitalized patients with COVID-19. *N Engl J Med* 2021; 384:693–704
10. Gordon AC, Mouncey PR, Al-Beidh F, et al; REMAP-CAP Investigators: Interleukin-6 receptor antagonists in critically ill patients with COVID-19. *N Engl J Med* 2021; 384:1491–1502
11. Kalil AC, Patterson TF, Mehta AK, et al; ACTT-2 Study Group Members: Baricitinib plus remdesivir for hospitalized adults with COVID-19. *N Engl J Med* 2021; 384:795–807
12. Prescott HC, Calfee CS, Thompson BT, et al: Toward smarter lumping and smarter splitting: Rethinking strategies for sepsis and acute respiratory distress syndrome clinical trial design. *Am J Respir Crit Care Med* 2016; 194:147–155
13. Seymour CW, Kennedy JN, Wang S, et al: Derivation, validation, and potential treatment implications of novel clinical phenotypes for sepsis. *Jama* 2019; 321:2003–2017
14. Antcliffe DB, Burnham KL, Al-Beidh F, et al: Transcriptomic signatures in sepsis and a differential response to steroids: From the VANISH randomized trial. *Am J Respir Crit Care Med* 2019; 199:980–986
15. Calfee CS, Delucchi K, Parsons PE, et al; NHLBI ARDS Network: Subphenotypes in acute respiratory distress syndrome: Latent class analysis of data from two randomised controlled trials. *Lancet Respir Med* 2014; 2:611–620
16. Calfee CS, Delucchi KL, Sinha P, et al; Irish Critical Care Trials Group: Acute respiratory distress syndrome subphenotypes and differential response to simvastatin: Secondary analysis of a randomised controlled trial. *Lancet Respir Med* 2018; 6:691–698
17. Maddali MV, Churpek M, Pham T, et al; LUNG SAFE Investigators and the ESICM Trials Group: Validation and utility of ARDS subphenotypes identified by machine-learning models using clinical data: An observational, multicohort, retrospective analysis. *Lancet Respir Med* 2022; 10:367–377
18. Bos LD, Schouten LR, van Vught LA, et al; MARS Consortium.: Identification and validation of distinct biological phenotypes in patients with acute respiratory distress syndrome by cluster analysis. *Thorax* 2017; 72:876–883
19. Sinha P, Furfaro D, Cummings MJ, et al: Latent class analysis reveals COVID-19-related ARDS subgroups with differential responses to corticosteroids. *Am J Respir Crit Care Med* 2021; 204:1274–1285
20. Sinha P, Delucchi KL, McAuley DF, et al: Development and validation of parsimonious algorithms to classify acute respiratory distress syndrome phenotypes: A secondary analysis of randomised controlled trials. *Lancet Respir Med* 2020; 8:247–257
21. Bowman WS, Newton CA, Linderholm AL, et al: Proteomic biomarkers of progressive fibrosing interstitial lung disease: A multicentre cohort analysis. *Lancet Respir Med* 2022; 10:593–602
22. Ware LB: Go with the flow: Expanding the definition of acute respiratory distress syndrome to include high-flow nasal oxygen. *Am J Respir Crit Care Med* 2022; 205:380–382
23. Filbin MR, Mehta A, Schneider AM, et al: Longitudinal proteomic analysis of severe COVID-19 reveals survival-associated signatures, tissue-specific cell death, and cell-cell interactions. *Cell Rep Med* 2021; 2:100287
24. Su Y, Chen D, Yuan D, et al; ISB-Swedish COVID19 Biobanking Unit: Multi-omics resolves a sharp disease-state shift between mild and moderate COVID-19. *Cell* 2020; 183:1479–1495.e20
25. Protein Biomarker Discovery - Olink [Internet]. Available at: https://www.olink.com. Accessed July15, 2021
26. Wingert A, Pillay J, Gates M, et al: Risk factors for severity of COVID-19: A rapid review to inform vaccine prioritisation in Canada. *BMJ Open* 2021; 11:e044684
27. Krämer A, Green J, Pollard J, Jr, et al: Causal analysis approaches in ingenuity pathway analysis. *Bioinformatics* 2014; 30:523–530
28. Zou H, Hastie T: Regularization and variable selection via the elastic net. *J R Statist Soc B* 2005; 67:301–320
29. Pedregosa F, Varoquaux G, Gramfort A, et al: Scikit-learn: Machine learning in python. *J Machine Learning Res* 2011; 12:2825–2830
30. Knaus WA, Wagner DP, Draper EA, et al: The APACHE III prognostic system. Risk prediction of hospital mortality for critically ill hospitalized adults. *Chest* 1991; 100:1619–1636
31. Robins JM, Hernán MA, Brumback B: Marginal structural models and causal inference in epidemiology. *Epidemiology* 2000; 11:550–560
32. Gisby J, Clarke CL, Medjeral-Thomas N, et al: Longitudinal proteomic profiling of dialysis patients with COVID-19 reveals markers of severity and predictors of death. *Elife* 2021; 10:e64827
33. Griesenauer B, Paczesny S: The ST2/IL-33 axis in immune cells during inflammatory diseases. *Front Immunol* 2017; 8:475
34. Wong JJM, Tan HL, Zhou J, et al: Large scale cytokine profiling uncovers elevated IL12-p70 and IL-17A in severe pediatric acute respiratory distress syndrome. *Sci Rep* 2021; 11:14158

35. Huang X, Venet F, Wang YL, et al: PD-1 expression by macrophages plays a pathologic role in altering microbial clearance and the innate inflammatory response to sepsis. *Proc Natl Acad Sci USA* 2009; 106:6303–6308
36. Guerrini MM, Okamoto K, Komatsu N, et al: Inhibition of the TNF family cytokine RANKL prevents autoimmune inflammation in the central nervous system. *Immunity* 2015; 43:1174–1185
37. Okada T, Suzuki H: The role of Tenascin-C in tissue injury and repair after stroke. *Front Immunol* 2020; 11:607587
38. Madtes DK, Rubenfeld G, Klima LD, et al: Elevated transforming growth factor-alpha levels in bronchoalveolar lavage fluid of patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1998; 158:424–430
39. Christoffersen C, Nielsen LB: Apolipoprotein M--A new biomarker in sepsis. *Crit Care* 2012; 16:126
40. Morrell ED, Bhatraju PK, Sathe NA, et al: Chemokines, soluble PD-L1, and immune cell hyporesponsiveness are distinct features of SARS-CoV-2 critical illness. *Am J Physiol Lung Cell Mol Physiol* 2022; 323:L14–L26
41. Alladina J, Levy SD, Cho JL, et al: Plasma soluble suppression of tumorigenicity-2 associates with ventilator liberation in acute hypoxemic respiratory failure. *Am J Respir Crit Care Med* 2021; 203:1257–1265
42. Borovac JA, Glavas D, Susilovic Grabovac Z, et al: Circulating sST2 and catestatin levels in patients with acute worsening of heart failure: A report from the CATSTAT-HF study. *ESC Heart Fail* 2020; 7:2818–2828
43. Watanabe M, Takizawa H, Tamura M, et al: Soluble ST2 as a prognostic marker in community-acquired pneumonia. *J Infect* 2015; 70:474–482
44. Zhao J, Zhao Y: Interleukin-33 and its receptor in pulmonary inflammatory diseases. *Crit Rev Immunol* 2015; 35:451–461
45. Alladina JW, Giacona FL, White EB, et al: Soluble suppression of tumorigenicity-2 associates with ventilator dependence in coronavirus disease 2019 respiratory failure. *Crit Care Explor* 2021; 3:e0480
46. Peine M, Marek RM, Löhning M: IL-33 in T cell differentiation, function, and immune homeostasis. *Trends Immunol* 2016; 37:321–333
47. Andersen GN, Nilsson K, Pourazar J, et al: Bronchoalveolar matrix metalloproteinase 9 relates to restrictive lung function impairment in systemic sclerosis. *Respir Med* 2007; 101:2199–2206
48. Estany S, Vicens-Zygmunt V, Llatjós R, et al: Lung fibrotic tenascin-C upregulation is associated with other extracellular matrix proteins and induced by TGFβ1. *BMC Pulm Med* 2014; 14:120
49. White ES, Xia M, Murray S, et al: Plasma surfactant protein-D, matrix metalloproteinase-7, and osteopontin index distinguishes idiopathic pulmonary fibrosis from other idiopathic interstitial pneumonias. *Am J Respir Crit Care Med* 2016; 194:1242–1251
50. Kumar A, Elko E, Bruno SR, et al: Inhibition of PDIA3 in club cells attenuates osteopontin production and lung fibrosis. *Thorax* 2022; 77:669–678
51. Sullivan DI, Jiang M, Hinchie AM, et al: Transcriptional and proteomic characterization of telomere-induced senescence in a human alveolar epithelial cell line. *Front Med (Lausanne)* 2021; 8:600626
52. King CS, Mannem H, Kukreja J, et al: Lung transplantation for patients with COVID-19. *Chest* 2022; 161:169–178
53. Chun HJ, Coutavas E, Pine AB, et al: Immunofibrotic drivers of impaired lung function in postacute sequelae of SARS-CoV-2 infection. *JCI Insight* 2021; 6:e148476
54. Wong HR, Cvijanovich NZ, Allen GL, et al: Corticosteroids are associated with repression of adaptive immunity gene programs in pediatric septic shock. *Am J Respir Crit Care Med* 2014; 189:940–946
55. Wong HR, Cvijanovich NZ, Allen GL, et al: Validation of a gene expression-based subclassification strategy for pediatric septic shock. *Crit Care Med* 2011; 39:2511–2517