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Angiopoietin 2 is associated with vascular necroptosis induction in COVID-19 acute respiratory distress syndrome

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Conflict of Interest Statement

AMKC is a cofounder, stock holder and serves on the Scientific Advisory Board for Proterris, which develops therapeutic uses for carbon monoxide. AMKC also has a use patent on CO. The spouse of MEC is a cofounder and shareholder, and serves on the Scientific Advisory Board of Proterris, Inc. EJS reports consulting fees from Axle Informatics for work assisting NIAID with COVID-19 related vaccine clinical trials. All other authors declare no competing interest.

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ABSTRACT

Vascular injury is a well-established, disease modifying factor in acute respiratory distress syndrome (ARDS) pathogenesis. Recently, COVID-19-induced injury to the vascular compartment has been linked to complement activation, microvascular thrombosis, and dysregulated immune responses. We sought to assess whether aberrant vascular activation in this prothrombotic context was associated with the induction of necroptotic vascular cell death. To achieve this, proteomic analysis was performed on blood samples from COVID-19 subjects at distinct timepoints during ARDS pathogenesis (hospitalized “at risk”, N=59, “ARDS”, N=31, and “recovery”, N=12). Assessment of circulating endothelial markers in the “at risk” cohort revealed a signature of low vascular protein abundance that tracked with low platelet levels and increased mortality. This signature was replicated in the “ARDS” cohort and correlated with increased plasma angiopoietin 2 (ANGPT2) levels. COVID-19 ARDS lung autopsy immunostaining confirmed a link between vascular injury (ANGPT2) and platelet-rich microthrombi (CD61) and induction of necrotic cell death (phosphorylated mixed lineage kinase domain-like, pMLKL). Among recovery subjects, the vascular signature identified patients with poor functional outcomes. Taken together, this vascular injury signature was associated with low platelet levels and increased mortality and could be used to identify ARDS patients most likely to benefit from vascular targeted therapies.
INTRODUCTION

Vascular injury has been recognized for decades as a key element in the pathogenesis of acute respiratory distress syndrome (ARDS)\(^1\), but this has not translated into vascular targeted therapies for ARDS. This may in part be related to heterogeneity in the vascular response to injury among ARDS subjects, as well as to difficulty in selecting patients most at risk for ARDS vascular injury. Blood proteomics has been proposed as a novel translational approach to better match patients to precision therapies for ARDS\(^2\). A better understanding of the blood proteomic changes associated with ARDS vascular injury could therefore help identify patients likely to benefit from vascular therapies.

Previous targeted studies of circulating vascular proteins have greatly enhanced the understanding of ARDS vascular injury. For example, measurement of the plasma angiocrine factor angiopoietin 2 (ANGPT2) in patients at the early stages of ARDS demonstrate that vascular injury likely precedes mechanical ventilation\(^3\) and is associated with ARDS disease mortality\(^4\). However, these ANGPT2 mediated vascular disruptions can be countered. In mice, systemic administration of platelet derived pericyte chemokines such as angiopoietin-1 (ANGPT1) and platelet derived growth factor B (PDGFB) counter ANGPT2 mediated vascular disruption, demonstrating the homeostatic potential of the blood vascular proteome\(^5\). Improved understanding of the blood proteomic changes in ARDS subjects with high and low vascular injury could build on these prior observations and shed further light onto disease pathogenesis and identify protein targets for further investigation.

More recently, vascular injury has been associated with COVID-19 acute respiratory distress syndrome (ARDS)\(^6,7\), including the vascular complications of inflammation and thrombosis. In this context, COVID-19-induced injury to the vascular compartment has been
associated with complement activation and microvascular thrombosis, systemic thrombosis, and with dysregulated immune responses. However, this focus on inflammation and thrombosis limits our insights into other disruptions associated with aberrant vascular activation, including angiogenesis, junctional barrier integrity, the role of activated platelets in vascular injury, and induction of vascular cell death, including specialized RIPK3-mediated necrotic cell death. Specifically, while ANGPT2 mediated vascular disruption has been documented in COVID-19, the association between ANGPT2 and induction of vascular cell death remains largely unexplored in ARDS investigations.

The purpose of this study was to assess whether aberrant vascular activation in COVID-19 was associated with the induction of necroptotic vascular cell death. To this aim, blood proteomics was performed in three independent COVID-19 cohorts, which enrolled patients at distinct timepoint in disease pathogenesis and included non-COVID-19 ARDS control samples as well. Protein expression was linked to relevant clinical outcomes and vascular injury and cell death markers in COVID-19 autopsy lung tissue.

MATERIALS AND METHODS

Study design
This study is an exploratory analysis of three cohorts that independently enrolled COVID-19 subjects at New York Presbyterian Weill Cornell Medical Center (WCM) between March 15 and August 17, 2020. Additional historic non-COVID-19 ARDS samples from influenza and bacterial ARDS patients prospectively enrolled into the Weill Cornell Biobank of Critical Illness (BOCI) from October 20, 2014 until May 24, 2020 were included as part of the ARDS cohort as ARDS
controls. The three COVID-19 cohorts were identified according to ARDS status at enrollment, yielding an early hospitalization at risk cohort, termed “at risk”, an intensive care unit cohort with ARDS, termed “ARDS”, and a recovery cohort in early convalescence outside the ICU, termed “recovery”.

Cohort descriptions

The at risk cohort included 59 adult (>18) non-pregnant COVID-19 subjects admitted to the general wards of WCM with serum available and who did not meet ARDS criteria at study enrollment. The ARDS cohort included adult (>18) non-pregnant COVID-19 (N=31) and historic non-COVID ARDS (N=29) subjects admitted to the intensive care unit (ICU) at WCM. For the ARDS cohort, only study subjects meeting ARDS criteria and with blood sampling within 10 days of ICU admission were considered for analysis. The recovery cohort included 12 adult (>18) non-pregnant COVID-19 ARDS subjects with plasma samples available from both the time of ICU care and the subsequent recovery period to allow for longitudinal analyses.

Blood sampling

In the at risk cohort, between 1 and 3 consecutive daily samples were obtained from the central lab after routine processing to obtain serum. To obtain serum, blood collected in serum separator tubes (SST) was processed within 2 hours of venipuncture. Whole blood was centrifuged at 1,500 g for 7 minutes. The serum layer was aliquoted and stored at -80°C. These samples were obtained with a waiver of informed consent. In this cohort, samples collected after patient intubation were excluded from the analysis. In the ARDS and recovery cohorts, plasma was isolated from study subjects according to existing plasma isolation protocols. To obtain
plasma, blood collected in EDTA tubes was processed within 6 hours of venipuncture. Whole
blood was centrifuged at 490 g for 10 minutes. The plasma layer was removed in 200 uL aliquots
and stored at -80. ARDS samples were obtained from patients in the intensive care unit while
Recovery blood samples were obtained from patients convalescing in the hospital rehabilitation
floors, as well as from the New York Presbyterian Weill Cornell Medicine Post-ICU recovery
clinic.

Clinical evaluation

Baseline clinical parameters and outcomes were extracted from the electronic medical
record (EMR) as described previously. Baseline comorbidities were manually extracted from
the EMR. Baseline clinical data (labs, severity of illness, ventilator data) were measured at time of
blood sampling in both the at risk cohort and ARDS cohort. In the recovery cohort, baseline clinical
data was measured from the ICU timepoint to allow for direct comparison with the ARDS cohort.
Severity of illness was defined by the sequential organ failure assessment score (SOFA). ARDS
was determined according to the Berlin definition with ARDS severity capped at mild for subjects
on non-invasive ventilation. Two critical care investigators independently adjudicated the ARDS
diagnosis. In all study subjects, COVID-19 was diagnosed if a subject had a syndrome compatible
with COVID-19 and a nasopharyngeal (NP) swab positive for SARS-CoV-2 by reverse
transcriptase polymerase chain reaction (RT-PCR).

Recovery Evaluation

The EQ-5D-3L was used to assess recovery at 12 months from ICU admission. The EQ-5D-3L
is a self-assessment of the patient recovery, and considers 5 distinct domains, namely
mobility, self-care, usual activities, pain or discomfort, and anxiety or depression. Each domain was scored 0, 1, or 2 depending on whether the patient reported no, some, or extensive limitations in each respective domain. For each patient, a final score was defined as the sum of the scores across the five domains and treated as an ordinal variable in the statistical analysis. Maximal functional limitation would have a score of (2*5=)10 while an optimal recovery would be scored 0.

**Autopsy studies**

Twenty autopsies performed between March 19 and June 30, 2020 with pre-mortem nasopharyngeal swabs positive for SARS-CoV-2 were considered for lung tissue staining. Hematoxylin and eosin (H&E), ANGPT2, CD61, and phosphorylated mixed lineage kinase domain-like (pMLKL) staining were performed in all autopsy subjects. Additionally, CD31 and ANGPT2/ERG co-staining were performed on the four autopsy subjects highlighted in the manuscript.

All autopsies were performed in a negative pressure ventilation room with full personal protective equipment including N-95 masks. No bone saw was used to prevent aerosolized dusts and, as such, brain examination was not performed. All tissues were immediately fixed in formalin for a minimum of 24 hours. All tissues for RNA studies were immediately immersed in Trizol for its viricidal effects. To minimize exposure, only two individuals were allowed in the suite during the autopsy and the room was disinfected before and after each case. Lung tissue specimens were fixed in 10% formalin for 48–72 hours. Hematoxylin and eosin staining were performed for all cases. Immunohistochemistry was carried out for angiopoietin-2 (sc-74403, Santa Cruz, TX, 1:100), CD-61 (CD61 clone 2F2, Leica Biosystems, IL), ERG (ab92513, Abcam, Cambridge, UK).
1:100), CD31 (PA0250, Leica Biosystems, IL, ready to use), and phosphorylated mixed lineage kinase domain-like (pMLKL, MAB91871, NOVUS Biologicals, CO, 1:750 with casein for background reduction). Specimens were scanned by whole-slide image technique using an Aperio slide scanner with a resolution of 0.24 μm/pixel. Quantification of ANGPT2 and CD61 was performed on four random 20X images selected using a random overlay of points and excluding fields with large vessels or airway. All twenty autopsies were analyzed using Immunohistochemistry profiler\textsuperscript{24} as a plugin for Image J (https://imagej.nih.gov Version 1.52a, National Institutes of Health, USA, last accessed 3/10/2022). After deconvolution of the 20X images, both area of expression (e.g., number of pixels) and intensity of expression (e.g., intensity of pixel) were measured and combined into a single score according to the equation score=\{(number of pixels in a zone x score of the zone)/total number of pixels in image. High, intermediate, low, and overall percent positive was averaged over the four measurements. The median ANGPT2 quantification was used to define the high (>median) and low (<median) ANGPT2 staining. The association between CD61 and ANGPT2 was then calculated based on CD61 quantification in the low and high ANGPT2 groups using Mann-Whitney U test.

O-link Plasma Proteomics

To quantify the circulating vascular proteome, plasma and serum samples from the at risk, ARDS and recovery cohorts were profiled using O-Link through the Proteomics Core of Weill Cornell Medicine-Qatar. The O-link assays were performed using Inflammation (v.3021), Cardiovascular II (v.5005), and Cardiovascular III (v.6113) panels (O-link, Uppsala, Sweden).

EDTA plasma and serum samples were heat-inactivated at 56 degrees for 15 mins. The protein measurements were performed with the Proximity Extension Assay technology (PEA)
according to manufacturer’s instructions. In summary, high throughput real-time PCR of reporter DNA linked to protein specific antibodies was performed on a 96-well integrated fluidic circuits chip (Fluidigm, San Francisco, CA). Signal quantification was carried out on a Biomark HD system (Fluidigm, San Francisco, CA). Each sample was spiked with quality controls to monitor the incubation, extension, and detection steps of the assay. Additionally, samples representing external, negative and inter-plate controls were included in each analysis run. From raw data, real time PCR cycle threshold (Ct) values were extracted using the Fluidigm RT-PCR analysis software at a quality threshold of 0.5 and linear baseline correction. Ct values were further processed using the O-link NPX manager software (Version 5.0, O-link, Uppsala, Sweden). Here, log2-transformed Ct values from each sample and analyte were normalized based on spiked-in extension controls and scale-inverted to obtain normalized log2 scaled Protein eXpression (NPX) values. NPX values were further adjusted based on the median of inter plate controls (IPC) for each protein and intensity median scaled between all samples and plates. As an external validation of the O-link platform, plasma protein levels of CD40LG and ANGPT1 were measure by enzyme-linked immunosorbent assays (ELISA, see methods below) in high and low expression patient samples and correlation measured between O-link and ELISA protein values.

The at risk cohort was profiled in two separate runs. The second run included a total of 11 samples, among which 5 bridge samples were used to scale this batch toward the first one, as recommended by O-link. First, for each bridge sample, the pairwise difference between the first and second batch was computed. An overall batch adjustment factor was then derived as the median of these pairwise differences and subtracted to the values in the second batch. Subsequently, protein levels were exponentiated, normalized using probabilistic quotient normalization and log2-retransformed. Missing values were imputed using a k-nearest neighbors
approach\textsuperscript{26} (k=10). 10 proteins were measured across multiple panels and, therefore, their duplicated values were averaged, leaving a total of 266 unique proteins. Protein values were standardized prior to statistical analysis. The preprocessed O-link and validation ELISA proteomics data for the three cohorts presented in this paper are available at https://doi.org/10.6084/m9.figshare.19341536.v1 (accessed 3/10/2022).

ELISA measurements

Plasma samples from the ARDS and Recovery cohorts were used for enzyme-linked immunosorbent assays (ELISA) according to manufacturer recommendations. Human ANGPT2 (R&D, CAT#DANG20) and receptor interacting protein kinase 3 (RIPK3, Cusabio, CAT#CSB-EL019737HU) kits were used to measure plasma protein levels. Additionally, CD40LG (R&D, CAT#: DCDL40) and ANGPT1 (R&D, CAT#: DANG10) were measure in high and low expressing patient samples (N=6 each, 12 total) to validate the O-link platform. Plasma samples were diluted (1:8 dilution for ANGPT2, 1:10 for RIPK3, 1:8 for ANGPT1, 1:15 for CD40L) prior to plating. Final sample absorbance was measured at 450 nm with wavelength correction performed at 570 nm. Sample concentrations were calculated from a four-parameter logistic curve created from known standard concentrations. Dilution factors were accounted for to calculate the final sample concentration. Plasma protein values were log10-transformed prior to statistical analysis.

Statistics

In the at risk cohort proteomic analysis, protein associations with death (i.e. whether the patient ended up dying) and platelet count (minimum value across the sampling days) were
computed using a mixed linear effect model, which allows to properly account for the multiple samples collected per patient. The model was formulated as follows: \( \text{protein} \sim \text{outcome} + \text{replicate} + \text{batch} + (1|\text{patient}) \), where \( \text{outcome} \) is either death or platelet count, \( \text{replicate} \) indicated the day of blood sample draw (first, second or third since hospital admission), and \( \text{batch} \) indicated whether the sample was measured in the first or second run. Association p-values were corrected for multiple testing using the Benjamini-Hochberg method for controlling the false discovery rate\textsuperscript{27}. Adjusted p-values less than 0.1 were considered significant.

The protein vascular signature was selected from proteins significantly associated with both outcomes, and proteins significantly associated with either mortality or platelet count and with known, well characterized links to vascular function. TIE2 was additionally included as it is the receptor for ANGPT2.

For all cohorts, patient hierarchical clustering based on the standardized proteomics value was performed using Ward linkage and Euclidean distance. The differential analysis between patient clusters was performed using Mann-Whitney U tests for continuous variables, Kendall’s rank correlation for ordinal variables, and log-rank tests for survival times. The correlation between ANGPT2 and RIPK3 was estimated using Pearson correlation. For these analyses, a p-value less than 0.05 was considered significant.

In the recovery cohort, patients were first divided into two groups based on unsupervised hierarchical clustering (Ward linkage, Euclidean distance) performed on the recovery timepoint. Then, for each patient, a protein abundance difference (\( \text{delta} \)) was calculated between the ICU and recovery timepoints. Finally, for each protein it was determined whether the protein delta was different across the two patient groups using the linear model \( \text{delta} \sim \text{group} \). P-values were corrected for multiple tests using the Benjamini-Hochberg method. Given the small sample size
and validation of protein set in two prior cohort, an adjusted p-value less than 0.25 was considered significant.

All statistical analyses were performed in R 4.0.1 using the maplet package\textsuperscript{28}. The R code used to generate the statistical findings presented in this paper is publicly available at https://github.com/krumsieklab/covid-vascular-injury (accessed 3/10/2022).

**Study Approval**

The study was approved by the institutional review board (IRB) at WCM (20-05022072, 20-03021681, and 1811019771). Written informed consent was received prior to participation by all patients, except when the IRB approved a waiver of informed consent (e.g., for the use of discarded samples and deidentified patient data in the at risk cohort).

**RESULTS**

The hospitalized at risk cohort blood proteome identifies a signature of vascular limitation preceding critical illness. A total of 1,384 subjects were admitted to the medical floors of New York Presbyterian Weill Cornell Medical Center (WCM) during the study period. Fifty nine of the 1,384 hospitalized COVID-19 subjects were profiled (Figure 1). Profiled subjects were more likely to have cancer (15% versus 7.5%, P=0.044) but otherwise were similar to unprofiled subjects (Supplementary Table 1). The median age of profiled subjects in the at risk cohort was 69 years old and was majority male (64% male versus 36% female). Fifty-three percent of the cohort had hypertension and 15% had cancer. Additional cohort characteristics are listed in Supplementary Table 2.
Blood biomarkers in the *at risk* cohort were measured using the O-link proximity extension assay. External validation of protein expression by ELISA showed excellent correlation between O-link and ELISA protein measurements (*Supplementary Figure 1A*, CD40LG\textsubscript{OLink} and CD40LG\textsubscript{ELISA} was $R=0.94$ (P<0.001), ANGPT1\textsubscript{Olink} and ANGPT1\textsubscript{ELISA} was $R=0.94$ (P<0.001)).

To first identify clinically relevant proteins associated with vascular injury and platelet thrombosis, a protein set was defined based on the association of circulating proteins with death and platelet levels (see Figure 2A). Thirteen proteins were significantly associated with both parameters (FDR 0.1): PDGFA, PDGFB, ANGPT1, SORT1, HBEGF, LAP TGFB1, CD84, CXCL5, MMP9, PAI, IL7, IL1RA, and CXCL1. In addition, 8 proteins were selected as they were associated with either death or platelet count (FDR 0.1) and have known vascular functions: ADAMTS13, CD40LG, EGFR, SELP, UPA, VEGFA, GP6, and HO1. TIE2 was additionally included since it is the receptor for ANGPT2\textsuperscript{29}. The final set comprised 22 proteins (see *Supplementary Figure 2*), including proteins related to vascular junctional integrity (ANGPT1, TIE2), angiogenesis (PDGFA, PDGFB), platelet degranulation (CD40LG, GP6), and coagulopathy (ADAMTS13, PAI), highlighting the potential functional significance of the identified proteins. Notably, these representative vascular proteins had lower expression in *at risk* subjects who died (Figure 2B), representing an early signal of vascular limitation in COVID-19 pathogenesis.

Patient clustering based on this protein set identified three distinct patient groups (clusters A, B, and C in Figure 2C), with mortality and low platelets progressively enriched. Interestingly, this mortality and low platelet enrichment was associated with lower mean abundance of the 22 proteins (P<0.001, *Supplementary Figure 3A*) and higher age (P=0.016, *Supplementary Figure 3B*). Baseline cancer status (P=0.042, *Supplementary Table 3*) as well as creatinine (P=0.011,
Supplementary Table 3) were additionally enriched across the patient clusters, identifying additional patient characteristics associated with low protein abundance.

Loss of circulating vascular proteins is associated with low platelets, mortality, and plasma ANGPT2 in ARDS. Next, the vascular protein signature was evaluated in the ARDS cohort. A total of 439 COVID-19 ARDS subjects were admitted to the WCM ICUs during the study period. Thirty-one COVID-19 ARDS subjects were profiled, together with 29 non-COVID-19 ARDS controls selected from 57 total non-COVID-19 ARDS subjects in the ICU biobank (Figure 1). Profiled COVID-19 ARDS subjects were younger (62 versus 67 year old, P=0.032), but otherwise were similar to unprofiled COVID-19 ARDS subjects (Supplementary Table 4). There were no baseline differences between profiled and unprofiled non-COVID-19 ARDS control subjects (Supplementary Table 4). Among all profiled ARDS subjects (N=60), there were no significant age, sex or race differences between COVID-19 ARDS (N=31) and non-COVID-19 ARDS subjects (N=29) in the cohort (Supplementary Table 2). Cancer was over-represented in the non-COVID-19 ARDS control subjects (48.0% versus 3.2% in COVID-19 ARDS). There were also notable differences in respiratory physiology. COVID-19 ARDS was associated with more severe hypoxemia (PaO2:FiO2 ratio, P:F ratio 84 versus 193 in non-COVID-19 ARDS) but lower ventilator ratio (1.65 vs 2.89 in non-COVID-19 ARDS).

First, the vascular protein set was investigated in only COVID-19 ARDS subjects (Supplementary Figure 4A). Confirming the protein results from the COVID-19 at risk cohort, low mean protein abundance of the protein set was associated with worse survival (P=0.026, Supplementary Figure 4B), low platelet count (P<0.001, Supplementary Figure 4C), and older patient age (P=0.035, Supplementary Figure 4D). The addition of non-COVID-19 ARDS patients (bacterial sepsis and influenza ARDS), lead to a similar trend (Figure 3A) with survival
(P=0.020, Figure 3B) and low platelets (P<0.001, Supplementary Figure 5A) associated with the low mean vascular protein abundance cluster (P<0.001, Supplementary Figure 5B). Similar to the at risk cohort, baseline cancer status (P<0.001, Supplementary Table 3) and elevated baseline creatinine (P=0.022, Supplementary Table 3) were more frequent in ARDS subject in the low mean vascular protein abundance cluster. Importantly, this low abundance signature did not reflect the relative expression of these proteins compared to healthy controls. As an example, CD40LG and ANGPT1 had opposite expression patterns in ARDS compared to healthy controls, with CD40LG being increased in ARDS compared to control while ANGPT1 expression was similar to healthy control (Supplementary Figure 1B). Despite the opposite expression patterns of the ARDS biomarkers compared to healthy controls, lower expression of both CD40LG and ANGPT1 was associated with the low platelet, worse clinical outcomes cluster.

Similar to the at risk cohort, the junctional integrity proteins ANGPT1 and TIE2 had lower expression in the low platelet, high mortality cluster B (Figure 3A). As ANGPT2 is known to negatively regulate ANGPT1 and TIE2 we measured plasma ANGPT2 in the ARDS cohort. Notably, plasma ANGPT2 was higher in the low mean protein abundance cluster (P=0.001, Figure 3C and Supplementary Figure 4E), linking low vascular protein abundance and plasma ANGPT2 in diverse ARDS subjects.

Interestingly, when COVID-19 ARDS was considered alone (Supplementary Figure 4), this higher vascular injury signature was present in 39% (12 of 31) of COVID-19 ARDS subjects, yet when all three infection types were considered (Figure 3), only 13% (4 of 31) of COVID-19 ARDS were in the higher vascular injury cluster compared to 58% (14 of 24) of bacterial sepsis ARDS and 80% (3 of 4) of influenza ARDS subjects, demonstrating that COVID-19 ARDS may be associated with less ANGPT2-associated vascular injury than bacterial sepsis and influenza
related ARDS. This finding is supported by a lower ventilator ratio in COVID-19 ARDS subjects compared to non-COVID-19 ARDS subjects (Supplementary Table 2), a physiologic surrogate for vascular injury in ARDS\textsuperscript{30}. This is also consistent with previous investigations showing higher platelet counts and less platelet consumption in COVID-19 compared to bacterial sepsis ARDS\textsuperscript{31}.

**Angiopoietin 2 is correlated with CD61 staining microthrombi in COVID-19 ARDS subjects.** The observed link between platelet activation and ANGPT2 was then explored in COVID-19 ARDS lung tissue. Twenty COVID-19 ARDS lung autopsy specimens were stained for the lung injury marker ANGPT2 and the activated platelet stain CD61. Representative sections from a high and low ANGPT2 subject are shown in Figure 4A. ANGPT2 staining was pronounced in the microvasculature and was mirrored by CD61 positive microthrombi in a similar distribution, linking vascular injury and platelet-rich microvascular microthrombi in COVID-19 ARDS. ANGPT2 and endothelial nuclear stain ERG co-staining confirmed endothelial origin of the ANGPT2 staining (Supplementary Figure 6A). Importantly, staining for CD31 as a constitutive endothelial marker (Supplementary Figure 6B) showed that differences in ANGPT2 and CD61 staining in these subjects was not due to differences in preservation of endothelium between autopsy subjects. Quantification of ANGPT2 and CD61 staining showed that high ANGPT2 protein was associated with increased CD61 (P=0.005, Figure 4B). Blood proteomics was performed in 3 of the autopsy subjects (Figure 4A and 4C; subjects labelled as P1, P2, and P3). Consistent with the ARDS cohort, low protein abundance of our protein set, including lower expression of angiopoietin axis proteins ANGPT1 and TIE2 (Figure 4C, red font), was associated with increased vascular ANGPT2 staining (Figure 4C).

**Induction of vascular cell death in ANGPT-2 associated vascular injury.** Having validated the vascular injury signature in diverse ARDS populations and COVID-19 ARDS
autopsy tissue, it was investigated whether ANGPT2-associated vascular injury could be associated with genetically regulated necrotic cell death, known as necroptosis. Increased expression of plasma RIPK3, a vital necroptosis protein\textsuperscript{32}, was first demonstrated in high ANGPT2 ARDS subjects (P=0.020, Figure 5A). Plasma RIPK3 was also correlated with plasma ANGPT2 (r=0.40, P=0.003, Figure 5B), supporting the existence of a link between circulating necroptosis proteins and ANGPT2-associated vascular injury. High ANGPT2 staining/low vascular protein abundance autopsy subjects (Figure 4A; NA and P3) demonstrated diffuse microvascular staining for pMLKL, a terminal protein in necrotic cell death execution downstream of RIPK3 (Figure 5C). Conversely, the low ANGPT2 staining/high vascular protein abundance autopsy subjects (Figure 4A; P1, P2) were negative for pMLKL. Taken together, this data link ANGPT2-associated vascular injury to necroptosis induction in COVID-19 ARDS.

Among COVID-19 ARDS recovery subjects, longitudinal plasma proteomics identifies a stable protein trajectory associated with good functional recovery. The 22-protein set was further investigated with respect to its predictive ability during COVID-19 ARDS recovery. Among 276 recovery subjects during the study period, 12 COVID-19 ARDS subjects had plasma available from both their ICU and recovery time point and were profiled longitudinally (Figure 1). Profiled recovery subjects were younger (47 versus 62 years old, P=0.002) than unprofiled recovery subjects (Supplementary Table 1). Among the profiled recovery subjects, the median age of this cohort was 47 years old and was majority male (67% versus 33% female, Supplementary Table 2).

Patient clustering based on the recovery plasma protein set revealed two distinct clusters (Figure 6A). Again, the low protein abundance cluster was associated with platelet level (P=0.048, Supplementary Figure 7A) and higher age (P=0.048, Supplementary Figure 7B). One year
follow up functional recovery data based on the EQ-5D-3L questionnaire was available on 11 of these 12 recovery individuals (top annotation in Figure 6A, Methods for details). Notably, the cluster of patients with lower abundance of the protein set (P=0.004, Supplementary Figure 7C) displayed worse functional recovery 12 months after admission from the ICU, while higher vascular protein abundance was associated with better functional recovery (P=0.027, Figure 6B). To test whether the protein trajectory from ICU to recovery was different between good and poor functional recovery subjects, the differences in protein abundances was compared between the two timepoints in the two patient clusters (see Methods). For proteins representative of junctional barrier integrity (TIE2, P=0.20), angiogenesis (PDGFA, P=0.20), platelet degranulation (GP6, P=0.20), and coagulopathy (PAI, P=0.20), good functional recovery was associated with stable protein trajectory (Figure 6C), as opposed to the large protein changes among the poor recovery subjects. This stable trajectory among good functional recovery subjects was similar for platelet levels (P=0.086, Supplementary Figure 7E) and ANGPT2 (p=0.083, Supplementary Figure 7F).

DISCUSSION

In this study, we trace a vascular protein signature through the natural history of COVID-19 ARDS from hospital admission to either recovery or death. Reflected in both the blood proteome and lung tissue, we demonstrate the clinical relevance of low abundance of circulating vascular proteins with known vascular functions and reveal a link between ANGPT2 and vascular cell death, and in particular specialized necroptotic cell death.

This vascular phenotype is notably present in certain COVID-19 subjects prior to ICU admission. While vascular injury spans the COVID-19 disease continuum from asymptomatic blue toes to catastrophic thromboembolic disease and ARDS-associated microangiopathy, the identification of broad loss of vascular protein expression in early severe disease generalizes this
vascular dysfunction to the large population of hospitalized COVID-19 subjects. The loss of vascular proteins could result from SARS-CoV-2 endothelial infection\textsuperscript{6,7}, although this remains controversial and thus far only reproducible in artificially engineered endothelial cell\textsuperscript{33}, while primary human endothelial cell appear resistant to infection\textsuperscript{34}. Alternatively, in common with bacterial sepsis\textsuperscript{35,36} and influenza infection\textsuperscript{37}, unrestrained COVID-19 related inflammatory signaling\textsuperscript{13} could similarly induce vascular cell death. Indeed, induction of genetically regulated necrotic cell death mediator (pMLKL) was demonstrated in the microvasculature of high ANGPT2-associated vascular injury COVID-19 autopsy subjects. Diverse upstream mediators previously linked to COVID-19 (e.g. TNF-alpha\textsuperscript{38}, interferons\textsuperscript{39,40}) can induce necroptosis\textsuperscript{32}, providing a crucial link between SARS-CoV-2 infection and both direct (virus) or indirect (TNF, interferons) induction of vascular cell death in COVID-19 subjects. Vascular cell death in COVID-19 ARDS is also supported by lung imaging mass cytometry (IMC) studies that show a reduction in the absolute number of endothelial cells in late COVID-19 autopsy tissue which could reflect disease related vascular cell death\textsuperscript{41}.

The role of activated platelets in vascular injury and repair is also apparent in the data. Activated platelets amplify immune responses in early ARDS but also play an essential role in vascular repair. The consistently low platelet levels across the cohorts and the extensive microthrombi observed in the autopsy subjects implies a circulating milieu of platelet consumption. This milieu of platelet consumption is supported by a blood signature of ongoing thrombolysis (high UPA and low PAI) and low levels of platelet derived proteins (low SELP, and GP6) in the high ANGPT2-associated vascular injury subjects. Relative loss of ADAMTS13, linked to secondary microangiopathy in COVID-19\textsuperscript{42}, is similarly deficient in the high ANGPT2 subjects, linking platelet consumption with microangiopathy in severe COVID-19. Low platelets
have previously been linked to ARDS mortality and the data suggest this may be related to depletion in platelet related angiogenic and junctional barrier factors. Consistently low circulating angiogenic (low PDGFA and PDGFβ) and barrier protein (low ANGPT1) in the higher ANGPT2-and low platelet subjects imply limitations in these essential reparative processes.

The validation of the vascular phenotype across diverse causes of ARDS broadens the relevance of the reported findings. In linking low platelets, vascular function, and mortality in COVID-19, bacterial sepsis, and influenza ARDS, we hint at a common final pathway of vascular injury that is more disease- (ARDS) than cause- (COVID-19) specific. While we acknowledge that matching COVID-19 and non-COVID-19 ARDS subjects would also have strengthened any comparison between these groups, the fact that there were similarities in protein expression despite stark differences in clinical parameters, including a marked difference in baseline cancer prevalence (14/29, 48% in non-COVID-19 ARDS vs 1/31, 3% COVID-19 ARDS), remains a strength of the analysis as it suggests that different causes of platelet depletion (e.g. failure of production is cancer patients with non-COVID-19 ARDS and consumption in COVID-19 ARDS patients) leads to the same protein expression pattern and worse clinical outcomes. Of note is that this vascular injury pattern may be related to a reduced baseline vascular resilience in the high ANGPT2-associated vascular injury subjects. Consistently, the high vascular injury subjects are older, have worse baseline renal function, and are more likely to have cancer, all variables known to be associated with vascular disease.

The identification of this severe vascular phenotype across infectious causes of ARDS also presents an opportunity for targeted vascular therapies in ARDS, including those that have shown promise in COVID-19, ARDS generally, and in exciting preclinical and early human experimental therapies, including ANGPT1 supplementation trial currently underway in COVID-
19 subjects (Available from: https://clinicaltrials.gov/ct2/show/NCT04737486, accessed 3/5/2022). And while a ANGPT2 neutralizing antibody study in hospitalized patient with COVID-19 was stopped for futility in October 2020 (Available from: https://clinicaltrials.gov/ct2/show/NCT04342897, accessed 3/5/2022), the presented data could improve patient selection for similar trials in the future, including the use of platelet levels to identify subjects with vascular limitation.

Finally, the identification of a vascular recovery proteome is novel. Nearly 4.5 million patients have been hospitalized in the United States since the start of the COVID-19 pandemic (Available from: https://www.cdc.gov/coronavirus/2019-ncov/covid-data/covidview/index.html, accessed 3/5/2022), with countless more hospitalized worldwide. But even in recovery, patients remain at risk for disease related morbidity and mortality. We demonstrate that a stable circulating vascular proteome is important for functional recovery. This association between vascular stability, platelet levels, and functional recovery could also support platelet levels as a novel biomarker in ARDS recovery. Larger studies will be needed to validate this observation.

We recognize that our study has limitations. While we describe a milieu of platelet consumption in ARDS subjects with increased vascular injury, we cannot rule out alternative mechanisms of platelet depletion in these subjects, including decreased bone marrow production, particularly in the ARDS subjects with malignancies. Platelet depletion also tracks with the vascular protein signature in the three cohorts, yet we do not establish cohort-specific platelet value that clinicians can use to identify these subjects. Also, while we suspect that endothelial cells, pericytes, and platelets represent the likely cellular origin of the vascular signature, the lack of spatial proteomic or transcriptomic data allows for the possibility that some identified proteins are from non-vascular sources, including immune cells. Future murine cell-specific knockout studies
will be needed to resolve the cellular origin of the signature. Finally, while we show an observed association between ANGPT2 and vascular cell death proteins RIPK3 and pMLKL, additional in vivo and in vitro studies will be needed to determine the critical crosstalk between vascular injury and vascular cell death proteins.

In summary, we identify a vascular injury signal in COVID-19 ARDS that has predictive value in early disease through to recovery and well as in bacterial sepsis and influenza ARDS and could improve patient selection and timing of vascular targeted therapies in ARDS.

**Author contributions**

DRP, EB share the first author position. DRP is listed first based on higher total effort to the project. DRP, EB, JK, AMKC, and SR designed the study. DRP, ACR, and ACB performed the autopsy staining analyses. LGE, SAM, AC, CNP, AR, JGC, SZJ processed samples and organized the patient clinical data. EB, HS, RB, MB, KC, FS, JK analyzed the proteomic data. KLH and IE provided statistical support for patient clinical data. EL, KW, CNP, LL perform functional assessment of recovery subjects. DRP, EB, JK, AMKC, RB, FS, JGC, EJS, ACR, HOR, JCL, MEC, and SR critically appraised the final dataset. DRP, EB wrote the manuscript. All authors approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Study Design. (A) Flow chart of study subjects selected for blood proteomic profiling in the “at risk”, “ARDS”, and “recovery” cohorts. (B) Venn diagram showing the overlap among COVID-19 subjects across the study cohorts. (C) Schematic of study subject sampling in relation to hospital admission (“at risk” cohort), ICU admission (“ARDS” cohort) and ICU discharge (“recovery” cohort). (D) Characteristics of study cohorts including total subjects (“N”), blood sample type, existence of longitudinal samples, sample timing, and whether proteomic profiling, angiopoietin 2 (ANGPT2) and receptor interacting protein kinase 3 (RIPK3) measurements were performed.

Figure 2. The hospitalized at risk cohort (N=59) blood proteome identifies a signature of vascular limitation preceding critical illness. (A) Overview of the associations of the protein set to death and platelets. All displayed proteins were included in the final protein set. Proteins in green associated with platelets, proteins in red associated with death, and proteins in black associated with both. Tie2 was additionally included as it is the receptor for ANGPT1 and ANGPT2. (B) Box plots demonstrating the association between proteins of vascular junctional integrity, angiogenesis, platelet degranulation, and coagulopathy to mortality in the at risk cohort after adjusting for multiple comparisons. Boxes indicate the interquartile range (IQR) of the data distribution, the line in the box represents the median value and the whiskers extend for 1.5 times the range of the IQR. Dots indicate the protein level in individual patients. (C) Heatmap of protein set abundance in the at risk COVID-19 subjects. Hierarchical clustering was performed using Ward linkage and Euclidean distance. Age, platelet count and death are overlaid at the top. Mean
abundance of the 22 protein set are displayed at the bottom. Mean protein abundance is progressively lower from cluster A to B to C.

**Figure 3. Loss of circulating vascular proteins is associated with low platelets, mortality, and plasma ANGPT2 in the ARDS cohort (N=60).** (A) Heatmap of 22 protein set abundance in diverse ARDS subjects, divided into two clusters. Hierarchical clustering was performed using Ward linkage and Euclidean distance. Age, log10(ANGPT2), platelet count, mortality, and ARDS etiology are overlayed at the top. Mean protein abundance of the 22 protein set is overlayed at the bottom. (B) Kaplan-Meier survival analysis for the two heatmap clusters showing worse survival for the high ANGPT2 ARDS cluster B. X-axis was capped at 60 days. The table at the bottom indicates the number of patients at risk at each timepoint in the two clusters. (C) Log10(ANGPT2) values in the two clusters demonstrating higher ANGPT2 expression in low vascular protein abundance cluster B. Differential statistic was assessed with a two-sided Mann-Whitney U test. The boxes indicate the interquartile range (IQR) of the data distribution, the line in the box represents the median value and the whiskers extend for 1.5 times the range of the IQR. Dots indicate the protein level in individual patients across the different ARDS categories: COVID-19 (orange), bacterial sepsis (brown) and influenza (mustard).

**Figure 4. Angiopoietin 2 is correlated with CD61 staining microthrombi in COVID-19 ARDS subjects.** (A) Representative hematoxylin and eosin (H&E), Angiopoietin-2 (ANGPT2) and CD61 staining in COVID-19 ARDS subjects. H&E demonstrates alveolar septal wall thickening across displayed autopsy subjects. (arrow). Increased ANGPT2 (open arrowhead) and CD61 (closed arrowhead) immunostaining is seen in subjects NA and P3 in a vascular distribution. (B) Lung autopsy specimens from 20 COVID-19 ARDS subjects were stained for ANGPT2 and platelet
activation stain CD61. High ANGPT2 (N=10) corresponds to autopsy subjects with ANGPT2 quantification above the median of the autopsy cohort while low ANGPT2 (N=10) represents the low ANGPT2 cohort. High ANGPT2 was associated with increased CD61 staining (P=0.005). (C) Blood proteomic data from autopsy subject P1, P2 (both low ANGPT2/low CD61 staining) and subject P3 (high ANGPT2, high CD61) demonstrate that low expression of the vascular protein set is associated with high ANGPT2 and high CD61 staining. Angiopoietin axis proteins ANGPT1 and Tie2 highlighted in red. NA = no blood proteomic data available for the autopsy subject. Scale bars = 50 µm.

**Figure 5. Induction of vascular cell death in ANGPT2-associated vascular injury.** (A) Plasma receptor interacting protein kinase 3 (RIPK3) in ARDS by heatmap cluster (Figure 3A, N=60). RIPK3 is associated with high ANGPT2 (P=0.020). COVID-19 (orange), bacterial sepsis (brown) and influenza (mustard). (B) Correlation of plasma RIPK3 and plasma ANGPT2 in the ARDS cohort (Fig. 3a, N=60). r indicate the Pearson correlation coefficient of the two variable and P its corresponding p-value. The black line represents the linear regression line and the gray area indicates the 95% confidence interval of the fit. Dots indicate the protein level in individual patients across the different ARDS categories: (C) Phosphorylated mixed lineage kinase domain-like (pMLKL) staining in COVID-19 ARDS autopsy subjects P1, P2 (both low ANGPT2/low CD61 staining) and subjects NA, P3 (high ANGPT2, high CD61) demonstrating increased expression of pMLKL (open arrowhead) in high ANGPT2 staining autopsy subjects. NA = no blood proteomic data available for the autopsy subject. Scale bars = 50 µm.

**Figure 6. Among COVID-19 ARDS recovery subjects (N=12), longitudinal plasma proteomics identifies a stable protein trajectory associated with good functional recovery.**
(A) Heatmap of COVID-19 recovery subjects. Functional recovery, age, platelet count and 12-month recovery scores are overlaid at the top. Hierarchical clustering was performed with Ward linkage and Euclidean distance. (B) Follow-up recovery scores at 12 months after ICU admission in the two heatmap clusters. Differential statistic was assessed with a two-sided Mann-Whitney U test. The boxes indicate the interquartile range (IQR) of the data distribution, the line in the box represents the median value and the whiskers extend for 1.5 times the range of the IQR. Dots indicate the protein level in individual patients. High scores indicate worse functional recovery. (C) Trajectory of vascular proteins from ICU to recovery time points by functional recovery group. The boxes indicate the interquartile range (IQR) of the data distribution, the line in the box represents the median value and the whiskers extend for 1.5 times the range of the IQR. Dots indicate the protein level in individual patients in the two timepoints. Values from the same patient are linked by a line and colored according to the corresponding heatmap cluster: A (cream) or B (red). Differential statistic of the protein trajectories between the two patient clusters was computed with a linear model. All displayed trajectory differences were significant to an adjusted p-value < 0.25.
COVID-19 Hospitalized: N=1384
Unprofiled: N=1325
At Risk: N=59

COVID-19 ARDS: N=439
Unprofiled: N=408

Historic ICU Biobank: N=405
No ARDS: N=348
Non-COVID-19 ARDS: N=57
Non-COVID-19 ARDS: N=28

COVID-19 Recovery: N=276
Unprofiled: N=264

At Risk Cohort
ARDS Cohort
Recovery Cohort

Cohort | N | Sample | Longitudinal Samples | Sample Timing | Proteomics | ANG2 | RIPK3
---|---|---|---|---|---|---|---
At Risk | 59 | Serum | No | Hospital Day 1-3 | Yes | No | No
ARDS | 60 | | Yes | Yes | Yes | Yes | Yes
COVID-19 | 31 | Plasma | No | ICU Day 1-10 | Yes | Yes | Yes
Non-COVID-19 | 29 | Plasma | No | ICU Day 1-10 | Yes | Yes | Yes
Recovery | 12 | Plasma | Yes | ICU: Day 1-10 | Yes | Yes | Yes

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Significant Associations

- Death only
- Platelets only
- Both death and platelets
- TIE2
- Non-significant

Vascular Junction Integrity

| Gene   | Platelets | Death | Adj. P  |
|--------|-----------|-------|---------|
| ANGPT1 |           |       | 0.004   |
| TIE2   |           |       | 0.289   |

Angiogenesis

| Gene   | Platelets | Death | Adj. P  |
|--------|-----------|-------|---------|
| PDGFB  |           |       | <0.001  |
| PDGFA  |           |       | 0.004   |

Platelet Degranulation

| Gene   | Platelets | Death | Adj. P  |
|--------|-----------|-------|---------|
| CD40LG |           |       | 0.012   |
| GP6    |           |       | 0.105   |

Coagulopathy

| Gene   | Platelets | Death | Adj. P  |
|--------|-----------|-------|---------|
| PAI    |           |       | 0.063   |
| ADAMTS13|          |       | 0.076   |
A

H&E

Angiopoietin 2

CD61

Autopsy Identifier:
P1 P2 P3

P=0.005

P=0.001

B

C

P=0.098

P<0.001

Journal Pre-proof
A

\[ \log_{10}(RIPK3) \]

3.0 3.5 4.0 4.5

\[ \log_{10}(ANGPT2) \]

\[ r=0.40, P=0.003 \]

B

C

\[ \text{COVID-19} \quad \text{Bacterial sepsis} \quad \text{Influenza} \]

Low ANGPT2

High ANGPT2

\[ P=0.020 \]

Autopsy Identifier: P1 P2 NA P3
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: O-link validation and healthy control versus ARDS CD40L and ANGPT1 plasma levels. (A) and (B) CD40L and ANGPT1 levels from 12 ARDS were measured originally by O-link and additionally by ELISA. Correlation plots show excellent correlation CD40LG\textsubscript{OLink} and CD40LG\textsubscript{ELISA} was R=0.94 (P<0.001), ANGPT1\textsubscript{OLink} and ANGPT1\textsubscript{ELISA} was R=0.94 (P<0.001). (C) and (D) Plasma levels of CD40L and ANGPT1 were measured by ELISA in healthy control (N=12) and match ARDS subjects (N=9). (C) CD40L levels were significantly higher in ARDS compared to healthy control but similar (blue circle) among ARDS subjects in the low protein abundance/high ANGPT2 group. (D) ANGPT1 levels were similar among ARDS and healthy controls but lower (blue circles) among ARDS subjects in the low protein abundance/high ANGPT2 group.

Supplementary Figure 2: Protein subset selection. Proteins in bold represent the final protein subset. To identify these proteins, we first considered proteins associated with both death (adjusted p-value < 0.1) and platelet level (adjusted p-value < 0.1), indicated in the overlap region. Additionally, proteins associated with either death or platelet level with known essential vascular functions were included (ADAMTS13, CD40LG, EGFR, SELP, UPA, VEGFA). These proteins are annotated with an asterisk in the figure. Finally, TIE2 was included in the final subset based in its role as the receptor for ANGPT2.

Supplementary Figure 3: Mean protein abundance and age by patient cluster in the at risk cohort (N=59). (A) Successive patient clusters based on the protein set abundance demonstrate significant loss of protein set expression (p-value < 0.001). (B) Patient clusters identify are
characterized by significantly different age distributions (p-value = 0.016). The boxes indicate the interquartile range (IQR) of the data distribution, the line inside the box represent the median value and the whiskers extend for 1.5 times the range of the IQR. Dots indicate values in individual patients. Differential statistic was assessed with a two-sided Wilcoxon rank test.

**Supplementary Figure 4: Loss of essential vascular proteins is associated with low platelets, mortality, and ANGPT2 levels in COVID-19 ARDS (N=31).** (A) Heatmap of protein set abundance in COVID-19 ARDS patients. Age, log10(ANGPT2), platelet count, and mortality are overlayed at the top. (B) Kaplan-Meier plot for the two heatmap clusters. The test statistic was estimated with a log-rank test. The x-axis was capped at 60 days. The table at the bottom indicates the number of patients at risk at each timepoint in the two clusters. (C-E) Platelet count, age, and log10(ANGPT2) values in the two heatmap clusters, respectively. The boxes indicate the interquartile range (IQR) of the data distribution, the line inside the box represent the median value and the whiskers extend for 1.5 times the range of the IQR. Dots indicate values in individual patients. Differential statistic was assessed with a two-sided Wilcoxon rank test.

**Supplementary Figure 5: Platelet count (A), mean protein abundance (B), and age (C) distribution in the two patient clusters defined in the ARDS cohort (N=60).** Both platelet count and mean protein abundance are significantly lower in cluster B (p-value < 0.001). There were no significant age differences between the two patient clusters (p-value – 0.18). The boxes indicate the interquartile range (IQR) of the data distribution, the line inside the box represent the median value and the whiskers extend for 1.5 times the range of the IQR. Dots indicate values in individual
patients across the different ARDS categories: COVID-19 (orange), bacterial sepsis (brown), and influenza (mustard). Differential statistic was assessed with a two-sided Wilcoxon rank test.

Supplementary Figure 6: ANG2/ERG and CD31 staining of ARDS lung tissue: (A) Angiopoietin-2 (ANGPT2)/ERG co-staining in COVID-19 ARDS subjects shows ERG positive ANGPT2 negative endothelial cells (closed arrowhead) in low ANGPT2 staining autopsy subjects and ERG positive ANGPT2 positive endothelial cells (open arrowhead) in high staining ANGPT2 autopsy subjects. (B) CD31 staining shows similar expression (arrow) among COVID-19 ARDS subjects previously shown to have high and low ANGPT2 staining. Scale bars = 50 µm.

Supplementary Figure 7: Differential analysis of clinical parameters in the recovery cohort (N=12). (A-D) Platelet count, age, mean protein abundance, and log10(ANGPT2) level in the two patient clusters defined in the recovery cohort, respectively. The boxes indicate the interquartile range (IQR) of the data distribution, the line inside the box represent the median value and the whiskers extend for 1.5 times the range of the IQR. Dots indicate values in individual patients. Differential statistic was assessed with a two-sided Wilcoxon rank test. E-F Platelet and log10(ANGPT2) trajectories from the ICU timepoint to the recovery timepoint. The boxes indicate the interquartile range (IQR) of the data distribution, the line inside the box represent the median value and the whiskers extend for 1.5 times the range of the IQR. Dots indicate values in individual patients. Values from the same patient are linked by a line, where the color indicates the corresponding heatmap group: cluster A (cream) or cluster B (red). Differential statistic of the protein trajectories between the two patient clusters (good versus poor functional recovery) across the two timepoints was computed with a linear model.