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Proteomic Analysis of Cerebrospinal Fluid from Severe COVID-19 Patients Reveals Prognostic Biomarkers Associated with Disease Outcome

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ABSTRACT

Purpose: Coronavirus disease 2019 (COVID-19) has highlighted significant neurological complications in severe cases. Cerebrospinal fluid (CSF) proteomics could reveal biomarkers related to clinical outcome among critically ill patients.

Experimental Design: We performed high-resolution proteomic analyses of CSF samples from 29 intensive care unit (ICU) patients with severe COVID-19 and 19 controls. Differentially expressed proteins and associated pathways were identified through bioinformatic and statistical analyses.

Results: Proteomic analysis identified 488 significantly altered proteins between COVID-19 patients and controls. Proteins linked to coagulation, inflammation, and blood-brain barrier dysfunction (e.g., SERPINC1, KNG1, PLG) were elevated in patients who survived ICU admission. Conversely, proteins associated with metabolic disruption, cellular stress, and neuroinflammation (e.g., FABP3, PDIA4) were upregulated in non-survivors. Pathway enrichment analyses confirmed involvement of immune activation, inflammatory responses, and coagulation cascades.

Conclusions and clinical relevance: CSF proteomics in severe COVID-19 patients reveals potential biomarkers predictive of patient outcomes. These findings support the involvement of systemic inflammation and blood-brain barrier disruption in COVID-19 pathophysiology, suggesting novel targets for personalized intervention strategies.

1 | Introduction

The COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has prompted extensive research into its effects beyond the respiratory system. To access all possible affected systems, including the nervous system, all biological fluids including cerebrospinal fluid (CSF) have been extensively investigated.

Harry Alexopoulos and Martina Samiotaki contributed equally to this work

Clinical Significance

Our study addresses an ongoing need to identify reliable biomarkers predictive of COVID-19 clinical outcomes, particularly beyond respiratory involvement. By employing advanced high-resolution proteomics, we analyzed cerebrospinal fluid (CSF) from ICU-admitted COVID-19 patients and identified distinct protein signatures associated with patient survival and mortality. We believe our findings further contribute to the understanding of COVID-19 pathophysiology, highlighting the involvement of systemic inflammation, coagulation dysfunction, and metabolic disruptions. Suggestive biomarkers may hold clinical promise for early prognosis of outcome and personalized therapeutic interventions in severe COVID-19 cases.

Recent studies on CSF in COVID-19 patients, mostly highlight the pathophysiology of neurological complications associated with the virus. A systematic review emphasized the rarity of SARS-CoV-2 detection in CSF, suggesting that neurological complications are not primarily due to direct viral neuroinvasion [1]. This is corroborated by a multicenter study involving 150 lumbar punctures, which identified Blood-CSF barrier dysfunction as the most frequent pathological finding without direct evidence of SARS-CoV-2 presence in the CSF [2].

The investigation of CSF through proteomics in COVID-19 patients has provided significant insights into the sequelae of SARS-CoV-2 infection. A study aimed at characterizing the inflammatory response in the CSF of COVID-19 patients. Through proteomics, enzyme-linked immunoassays, and RNA sequencing, the study delineated inflammatory proteins and explored the transcriptome. Results indicated attenuated inflammatory changes in the CSF of COVID-19 patients compared to those with herpes simplex virus encephalitis (HSVE), including downregulation of specific proteins. This study provides evidence that certain inflammatory mediators found in the CSF may originate from systemic circulation rather than being produced intrathecally, offering a different perspective on the CNS involvement in COVID-19 [3].

Further, comparative proteomic studies have shown distinct differences in the CSF profiles of mild vs. severe COVID-19 cases. In severe cases, there is a notable increase in proteins related to blood-brain barrier disruption, oxidative stress, and neuronal damage, which are less pronounced or absent in milder cases. These findings underscore the importance of proteomic analysis and the discovery of novel reliable biomarkers in monitoring disease progression and severity [4]. By identifying specific protein signatures associated with severe COVID-19, healthcare providers can better predict patient outcomes.

Our study aims to extend the utility of CSF studies, with a focus on clinical outcome among critically ill patients. To this end 29 CSF samples from hospitalized patients with severe COVID-19 and 19 controls were analyzed using high resolution proteomics to understand the inflammatory environment in the intrathecal compartment and how this could lead to prognostic biomarkers.

2 | Materials and Methods

2.1 | Patients and Study Design

Consecutive patients admitted to the ICU of Evangelismos Hospital, Athens, Greece between January first, 2021 and May 30th, 2021 ($n = 29$) were included in this study based on the following criteria: (a) RT-PCR diagnosed SARS-CoV-2 infection, (b) age >18 years old, and (c) critical COVID-19 illness based on the NIH classification criteria necessitating intubation and subsequent mechanical ventilation. At the time (late 2020) the predominant SARS-CoV-2 variants were B.1.1.177 and B.1.258 followed by the Alpha (Lineage B.1.1.7) predominance (early mid 2021). CSF samples for anti-SARS-CoV-2 antibody testing and proteomic analysis were obtained upon admission of patients in the ICU. Patients were not vaccinated. Clinical characteristics such as age, sex and outcome were noted. Control CSF samples ($n = 19$) were obtained from subjects negatively tested for inflammatory markers and autoantibodies in the Department of Pathophysiology routine immunology laboratory. The present study was approved by the Ethics Committee of School of Medicine, NKUA, Athens, Greece (protocol no: 456) and was conducted according to the principles of the Helsinki Declaration and the GDPRs of the European Union. All patients or their legally authorized representatives provided written informed consent.

2.2 | Detection of Antibodies Against SARS-CoV-2

Anti-SARS-CoV-2 IgG antibodies against the S1 domain of SARS-CoV-2 spike protein were detected in the CSF using an FDA-approved ELISA method, reliable for all variants (Euroimmun, Lübeck, Germany), according to manufacturer's instructions. The cut-off positive threshold was >1.1, after calculating the ratio of optical density (OD) of samples measured at 450 nm, divided by the OD value provided by the calibrator.

2.3 | Proteomic Analysis

Proteomic sample preparation using the single-pot, solid-phase-enhanced sample preparation Sp3-mediated protein digestion protocol [5]. The CSF sample (50 µL) was incubated in a lysis buffer consisting of 4% SDS, 0.1 M DTT and 0.1 M Tris pH 7.4. The samples were subjected to heating for 3 min at 99°C followed by a centrifugation step for 15 min at 17000 x g. The supernatant was collected and processed according to the Sp3 protocol including an alkylation step in the dark for 15 min in 10 mg/mL iodoacetamide (Acros Organics). 20 ug of beads (1:1 mixture of hydrophilic and hydrophobic SeraMag carboxylate-modified beads, GE Life Sciences) were added to each sample in 50% ethanol. The proteins were allowed to bind to the beads for 15 min followed by repeated steps of protein clean-up using a magnetic rack. The beads were washed two times with 80% ethanol and once with 100% acetonitrile (Fisher Chemical). The captured-on beads proteins were digested overnight at 37°C under vigorous shaking (1200 rpm, Eppendorf Thermomixer) with 0.5 ug Trypsin/LysC (MS grade, Promega) prepared in 25 mM ammonium bicarbonate. Next day, the supernatants were collected, and the peptides were purified using a modified Sp3 clean up protocol. The peptide mixture

(~30 ul) was further purified by addition of 20 ul magnetic bead mixture in 1.2 mL acetonitrile. The mixture was inverted several times and after 30 min the tube was placed on the magnetic stand, and the supernatant was removed. The beads with the bound peptides were washed twice with 200 ul 100% acetonitrile. Finally, 50 ul of water was added to elute the peptides from the magnetic beads. The eluates were dried down using a speedvac. Finally, the dried peptides were solubilized in Buffer A (0.1% formic acid in water), sonicated and the peptide concentration was determined through absorbance at 280 nm measurement using a nanodrop instrument.

2.4 | LC-MS/MS Analysis

Samples were run on a liquid chromatography tandem mass spectrometry (LC-MS/MS) setup consisting of a Dionex UltimateRSLC online with a Thermo Q Exactive HF-X Orbitrap mass spectrometer Thermo Scientific). Peptide samples were directly injected and separated on a 25 cm-long analytical C18 column (PepSep, 1.9µm3 beads, 75 µm ID, Bruker) using a 1 h long run, starting with a gradient consisting of 7% Buffer B (0.1% Formic acid in 80% Acetonitrile) to 35% for 40 min and followed by an increase to 45% in 5 min and a second increase to 99% in 0.5 min and then kept constant for equilibration for 14.5 min. The flow rate was set to 400 nL/min in the sample loading phase and lowered to 250 nL.min in the main phase of sample analysis, A full MS was acquired in profile and positive mode using the mass spectrometer, operating in the scan range of 375–1400 m/z using 120K resolving power with an AGC of 3×106 and max IT of 60 ms followed by data independent analysis using 8 Th windows (39 loop counts) with 15K resolving power with an AGC of 3×105 and max IT of 22 ms and a normalized collision energy (NCE) of 26. At least two technical replicas were acquired per sample.

2.5 | Data and Statistical Analysis

Orbitrap raw data was analyzed in DIA-NN 1.8.1 (Data-Independent Acquisition by Neural Networks) Demichev through searching against the reference proteomes of Homo sapiens (HUMAN_UP000005640_9606) retrieved from Uniprot database (containing 20583 proteins) in the library free mode of the software, allowing up to two tryptic missed cleavages [6]. A spectral library was created from the DIA runs and used to reanalyze them. DIA-NN default settings have been used with oxidation of methionine residues and acetylation of the protein N-termini set as variable modifications and carbamidomethylation of cysteine residues as fixed modification. N-terminal methionine excision was also enabled. The match between runs (MBR) feature was used for all analyses and the output (precursor) was filtered at 0.01 FDR and finally the protein inference was performed on the level of genes using only prototypic peptides. The software directLFQ was applied for additional sample normalization and the calculation of protein intensities [7].

The obtained matrix of a total of 3440 proteins was imported in Perseus software (1.6.15.0) [8]. Data transformed to logarithmic scale and row filtering performed based on the presence of each protein in at least 50% of samples in each group. For the rest 1735 proteins, missing values were imputed from normal distribution. Sample 4013 was removed as outlier. The significantly different proteins among groups ($p < 0.05$) were calculated by multiple-sample test (ANOVA) and Post hoc Tukey's HSD test of one-way ANOVA for pairwise comparisons. Proteins with padj < 0.05 (FDR correction) were selected as significant for each comparison. Gene annotation added by transforming the protein symbols to their corresponding ENTREZ IDs with the package org.Hs.eg.db. Enrichment analysis and visualizations were performed in R (v 4.4.2) with the packages clusterProfiler in the databases Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO) and DiseaseGeneNETwork (DisGeNET).

2.6 | Machine Learning Analysis for Biomarker Prediction

A random forest classifier was trained using the R packages randomForest and caret with 5-fold cross-validation repeated 10 times [9]. After optimizing the Area Under the Curve (AUC), the final model was trained on the full dataset (Exit and Death samples) using the ranger implementation of Random Forest (1000 trees). Permutation importance was extracted to quantify each protein's contribution to the prediction performance. Top biomarkers were ranked and visualized.

3 | Results

COVID-19 patients were 65% male and 35% female with an average age of 74.6 years. Control subjects were 53% male and 47% female with an average age of 64.5 years. 14 patients (average age 71.6 years) exited ICU (48.3%) while 15 patients (average age 77.8 years died in ICU (51.7%). 6/19 patients (31.5%) were positive for anti-SARS-CoV 2 antibodies in the CSF. Antibody positivity did not correlate with the outcome (Death vs Exit). Proteomic analysis identified 3440 protein groups and cluster analysis of all samples following data processing, log2 transformation and an ANOVA test, identified 488 proteins with differentiated abundance.

Centering the values on healthy samples revealed expression patterns of higher and lower abundance proteins (Figure 1). More specifically, the first cluster (left) contained mostly control samples, while the second (right) contained mostly CSF samples derived from Death or Exit groups (hospitalized patients with severe COVID-19). Supporting Figure presents a Volcano plot of Healthy vs COVID which represents the universal proteomic footprint of severe COVID-19. It reveals, as expected, ubiquitous markers of inflammation and blood-brain barrier (BBB) disruption common to all critically ill patients such as complement and coagulation cascades activation. Both analyses designate a clear diversification of CSF from COVID-19 patients *vs* our control donors. Proteins (see below) that are consistently upregulated or downregulated (as compared to Healthy control samples) in CSF from ICU admitted Death or Exit COVID-19 patients can therefore be assessed for validity as biomarkers for disease outcome.

Next, we performed an enrichment analysis of all ANOVA significant proteins. The 488 differentially expressed proteins were mapped using the KEGG, GO, and DisGeNET databases.

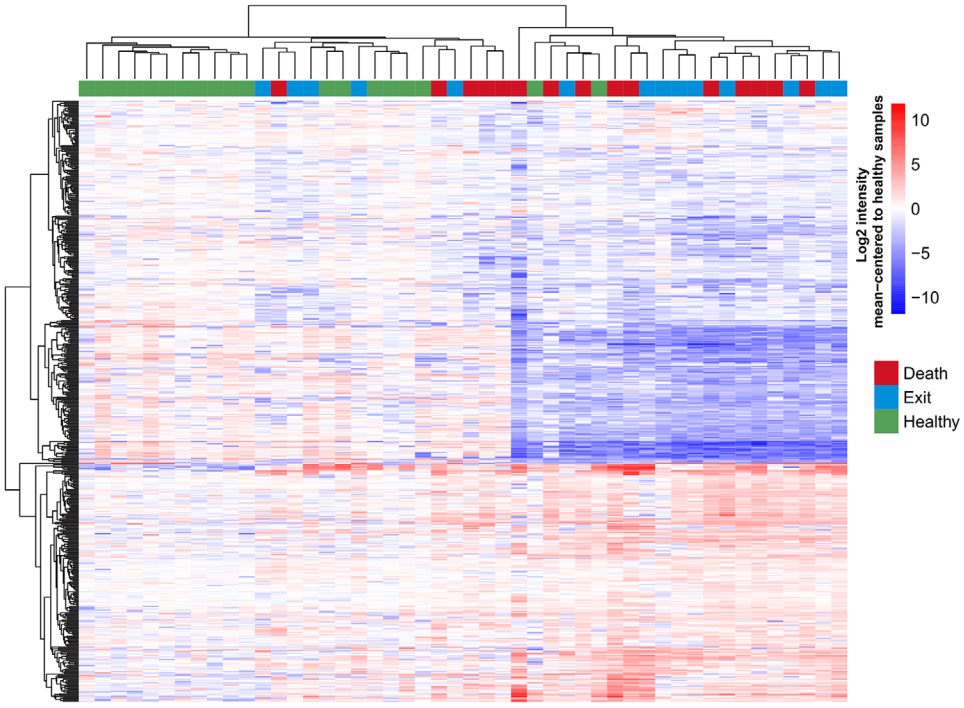

FIGURE 1 | Heatmap with hierarchical clustering of the 488 ANOVA significant proteins with mean-centering on healthy samples. **Red**: Indicates higher than the Healthy mean expression levels. **Blue**: Indicates lower than the Healthy mean expression levels.

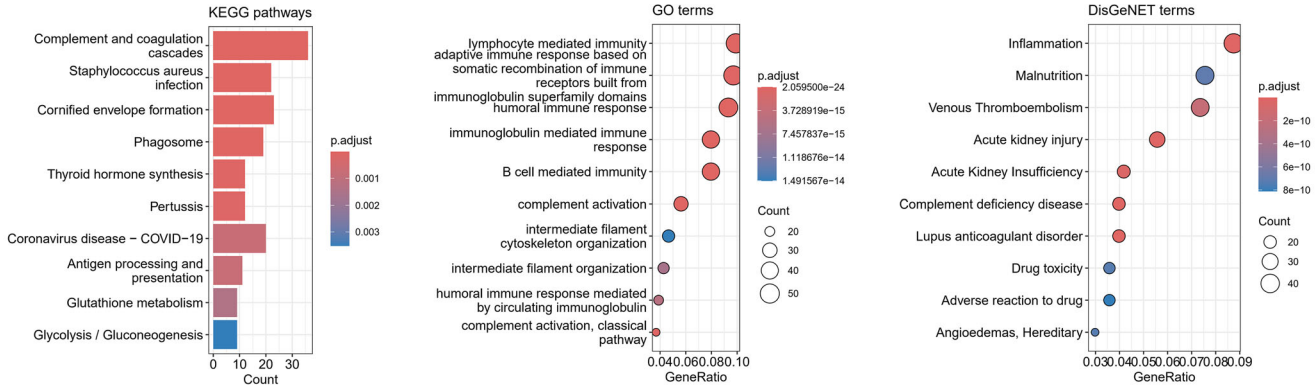

FIGURE 2 | Enrichment analysis, KEGG pathways, Gene Ontology (GO) terms, and Disease Gene Network (DisGeNET) terms.

Upregulated and downregulated proteins were analyzed as separate input lists and/or using rank-based metrics that account for fold-change directionality. KEGG pathways showed a broad range of processes, from immune response to metabolic pathways, indicating the complex nature of COVID-19. GO terms highlighted key biological processes such as immune responses and complement activation and DisGeNET pointed to strong enrichment of inflammatory responses and nutritional status (Figure 2).

We further investigated the pairwise differentially abundant proteins. Post-hoc Tukey HSD analysis identified 259 significantly different (padj < 0.05) proteins between Healthy and Exit samples, 385 between Healthy and Death samples and 69 between Exit and Death. The volcano plots of the pairwise comparisons highlight the differentially expressed proteins (Figure 3A). Proteins such as CRP, SERPINA3 and RNASE2 show significant upregulation in the Death group compared to the Healthy group, while proteins like TSPYL1 and KRT81; KRT85; KRT86 are significantly downregulated in the Death group. Proteins such as CRP, SERPINA3, and SAA1 are significantly upregulated in the Exit group compared to the Healthy group while proteins PLAUR, WSB1 and KRT81; KRT85; KRT86 are significantly downregulated in the Exit group.

Next, we analyzed our data to visualize intersections via a Venn diagram. The intersection of Death *vs* Healthy and Exit *vs* Healthy represents the shared severe signature. The proteins unique to the Death vs Exit comparison are prognostic candidate biomarkers. The only 2 proteins that were statistically different between the 3 groups were SERPINA4 and HRG (Figure 3B).

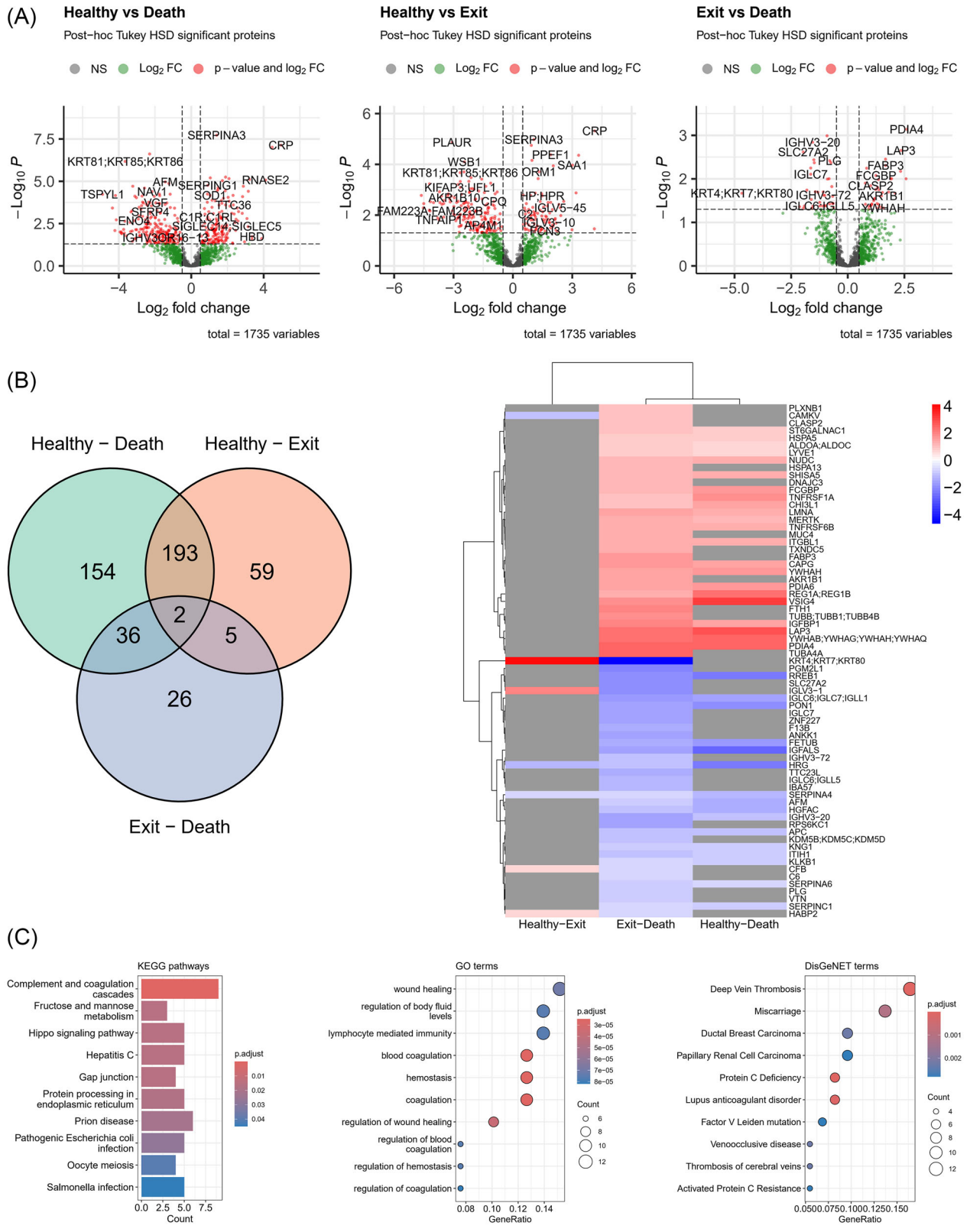

FIGURE 3 | **(a)** Volcano plots comparing our three defined groups. Green (Log2 FC): Variables that show significant changes in log2 fold change but are not statistically significant in p-value. Red (*p*-value and log2 FC): Variables that are significant in both log2 fold change and p-value. Gray: Non-significant values based on Tukey HSD with low log2FC values. (**b**). Protein intersections among the studied groups. Left: Venn diagram indicating the number of intersections of statistically significant proteins per comparison. Two proteins (HRG and SERPINA4) were found to be statistically different within all pairwise comparisons. Right: Heatmap of all Exit-Death proteins with overlap to Healthy-Death or Healthy-Exit (left). Heatmap of all 69 Exit-Death differentially abundant proteins (right). Color scale indicates the Log2FC. Gray tiles: No statistical significance detected. (**c**). KEGG, GO and DisGeNET pathway analysis on the comparison Death vs. Exit.

Specific analysis of only the Exit vs Death comparison again clearly discriminated the two groups and further highlighted significantly differentially regulated proteins associated with risk of dying and survival (Figure 3B). Especially, associated with survival the proteins standing out include SLC27A2, FETUB, SERPINC1, KNG1, PLG, KRT4; KRT7; KRT80, PGM2L1, IGLV3-1 and RREB1. On the contrary, proteins associated with death risk included FCGBP, SHISA5, LAP3, PDIA4, FABP3, HSPA5, YWHAB; YWHAG; YWHAH; YWHAQ, FTH1, IGFBP1, VSIG4, and TUBA4A. Looking deeper into the pairwise comparisons, we can distinguish that KRT4; KRT7; KRT80 which is the top downregulated protein in the Exit vs. the Death samples, is also significantly more abundant compared to the healthy samples. On the contrary, this protein does not have statistically significant deference between the Healthy and Death samples, making it a specific signature of the Exit samples. Two proteins already mentioned (HRG and SERPINA4) were present in all comparisons. Their levels were significantly lower in COVID-19 patients (Exit and Death) and lower in the Death than the Exit group. The rest 26 proteins have statistically significant difference in their protein levels between Exit and Death samples but compared to the Healthy group we did not detect any changes in their abundance (Figure 3B).

Pathway analysis on the comparison between the Death and Exit groups based on the KEGG, GO and DisGeNET databases showed that differentially expressed proteins mainly map on known pathways being de-regulated in severe COVID-19 (Figure 3C). KEGG analysis reveals **complement and Coagulation Cascades which are** highly enriched, indicating significant involvement in coagulation and immune response. The GO Term enrichment analysis identified pathways of **activation of** wound healing, regulation of body fluid levels and lymphocyte mediated immunity. The emergence of pathways of **hemostasis and body fluid regulation** highlights the role of proteins in blood clotting and maintaining blood flow. Finally, our Disease Gene Network (DisGeNET) Enrichment Analysis showed involvement of Deep Vein Thrombosis pathways whose strong enrichment suggesting involvement of blood clotting, then of Miscarriage and of the Venous Thromboembolism and Thrombophilia pathway which again highlights genes associated with a blood clotting disorder.

Additional machine learning analysis on the Death and Exit samples identified the top biomarkers with possible prognostic value (Figure 4). SLC27A2 and FETUB are at the top of the candidate list. We had prior identified both these proteins to be among the ones with statistically higher abundance in Exit vs. Death samples. In addition, FTH1, VSIG4, HSPA5, PDIA4 were also identified to be higher in the Death than in the Exit samples. The proteins CD163, C7 were both differentially expressed in the Death and Exit groups compared to the Healthy samples, but the difference in protein levels of the Exit vs. Death was not statistically significant. Finally, DGKH, HSD17B4 were not among the statistically significant findings of the ANOVA test.

4 | Discussion

Since understanding COVID disease pathophysiology, many efforts have been geared towards identifying prognostic biomarkers of disease outcome. For COVID patients with neurological

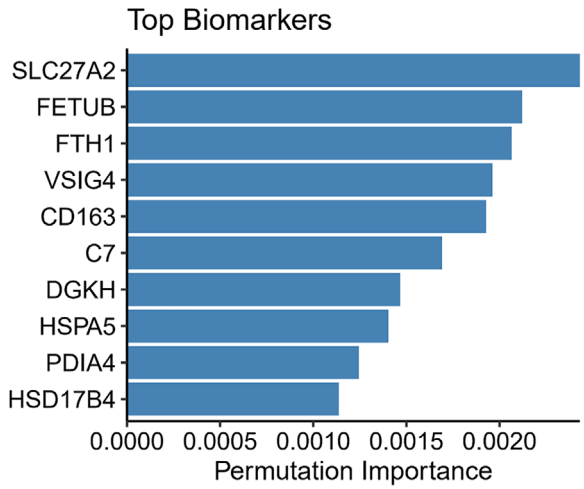

FIGURE 4 | Top protein biomarkers from the final Random Forest, ranked by Permutation Importance.

acute disease the presence of anti-SARS-CoV antibodies in CSF and disrupted BBB was proposed [10] and validated as a biomarker [11]. Our study aimed to elucidate whether proteomic CSF analysis could discriminate between patients with severe COVID requiring ICU care that eventually died vs. patients that exited ICU. Indeed, hierarchical cluster analysis clearly defined the two groups suggesting that analysis of CSF inflammatory milieu is of importance. Our enrichment and pathway analysis, either in our 3 groups (healthy vs. death vs. exit) or in 2 groups (death vs. exit) revealed that KEGG Pathway Analysis highlighted pathways involved in immune response, coagulation, and viral processes, that the GO Term Analysis highlighted key biological processes such as immune response activation, blood coagulation, and wound healing and that the DGN Analysis identified diseases associated with the proteins, particularly those related to blood clotting disorders and immune response. The involvement of these pathways is well established in COVID-19 and our results suggest that the abundance of these proteins in the CSF is a result of leakage from the periphery due to blood brain disruption. We were also able to distinguish proteins, where the preservation of physiological levels was associated with survival, whereas their depletion correlates with mortality. These proteins were also ranked as candidate biomarkers.

Of relevance, SERPINC1 encodes antithrombin III, a serine protease inhibitor that regulates blood coagulation. Its deficiency can lead to increased risk of thrombosis [12]. Reduced levels of antithrombin have been observed in severe COVID-19 patients, contributing to a prothrombotic state [13]. Elevated SERPINC1 in survivors might suggest its potential protective effect against COVID-19-associated coagulopathies and the maintenance of homeostatic balance. In the group that perished, the lower levels of SERPINC1 likely do not reflect decreased production, but rather consumptive coagulopathy. KNG1 (Kininogen-1) follows a similar pattern, being higher in the Exit group and depleted in the Death group. Kininogen is the precursor protein for bradykinin, a potent vasodilator and inflammatory mediator. Kininogen is also part of the kallikrein-kinin system which can influence inflammation and vascular permeability. Dysregulation of this system, leading to excessive bradykinin, has been

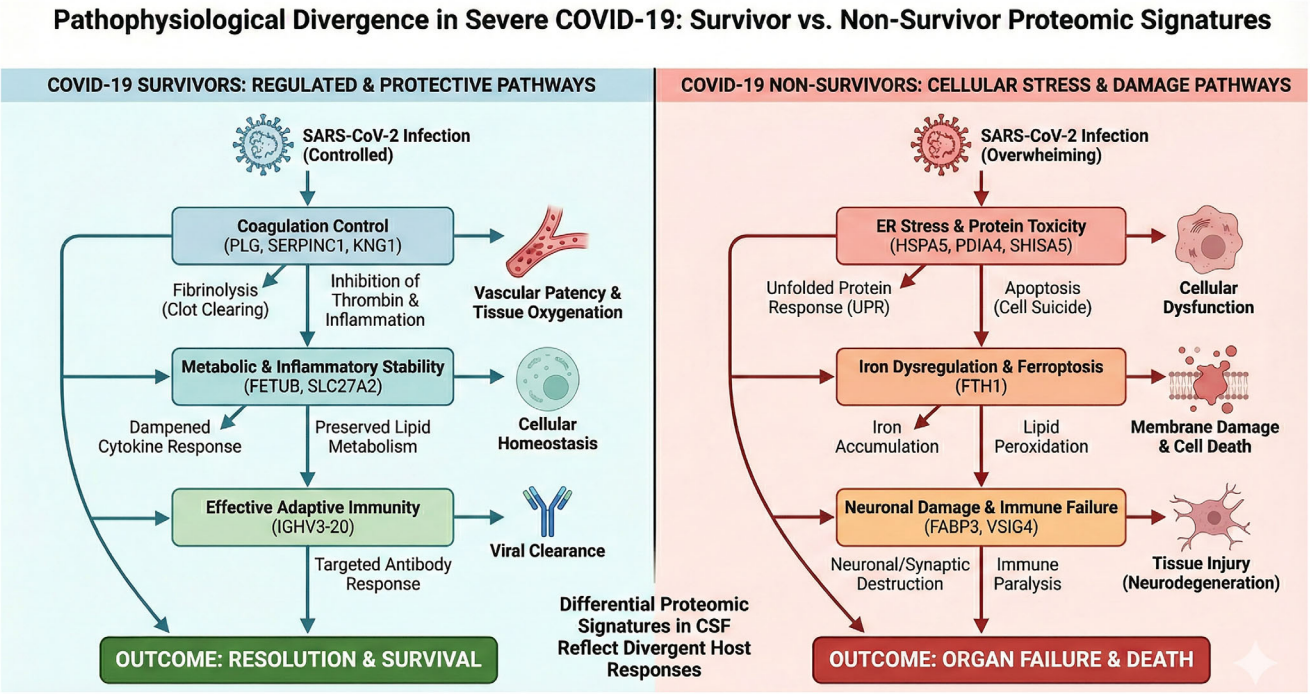

FIGURE 5 | Infographic of the proposed pathophysiological divergence in severe COVID-19.

proposed as a mechanism contributing to the severe inflammation and respiratory distress seen in severe COVID-19 cases. Elevated bradykinin levels have been implicated in the so-called "bradykinin storm" hypothesis, which explains the excessive inflammation and edema in COVID-19 [14]. High KNG1 in survivors suggests a regulated inflammatory response where the precursor is not rapidly depleted, sparing the patient from the catastrophic vascular leakage associated with the bradykinin storm.

Plasminogen (PLG) is a precursor to plasmin, an enzyme that plays a crucial role in fibrinolysis, the breakdown of blood clots [15]. In COVID-19, there is evidence of impaired fibrinolysis alongside hypercoagulability. Elevated plasmin activity has been noted in severe COVID-19 patients, contributing to the breakdown of fibrin clots and potentially exacerbating bleeding tendencies while also facilitating viral spread through tissue matrices [15]. Altered plasminogen levels in CSF have been reported in conditions involving hemorrhage and clotting disorders, such as subarachnoid hemorrhage and traumatic brain injury [16]. The depletion of Plasminogen in the death group again points to consumption or failure of the fibrinolytic system. Survivors maintained adequate Plasminogen levels, allowing them to effectively lyse clots and maintain organ perfusion. FETUB is a liver-derived glycoprotein, a selective inhibitor of certain metalloproteases and part of a protease-inhibitor network that shapes angiogenesis, immune defense and matrix remodeling. It has been associated as a predictor of survival in serum proteomics studies [17].

Our other group of identified proteins, related to non-survival, include FCGBP which is involved in mucosal immunity and has been observed with altered glycosylation patterns in cancer [18] and SHISA5 which is associated with apoptotic processes, potentially linking SHISA5 to the excessive cell death seen in critical cases [19]. LAP3 is involved in protein processing and degradation. It has been implicated in inflammatory processes, which are significantly heightened in severe COVID-19 [20]. PDIA4 is involved in protein folding and the endoplasmic reticulum stress response. Severe COVID-19 cases exhibit significant cellular stress, implicating PDIA4 in the disease's pathology [21] and FABP3 is linked to lipid metabolism and has been associated with cardiac injury, a serious complication in severe COVID-19, suggesting its involvement in the cardiovascular impacts of the virus [22]. FABP3 is also a CSF marker of degeneration in diseases such as Alzheimer's and CJD. It's presence in our cases might indicate cardiac injury or damaged neurons leaking the protein [23].

HSPA5, also known as BiP/GRP78, is a chaperone involved in the unfolded protein response. Its expression is upregulated in response to stress, which is prevalent in severe COVID-19, indicating its role in managing cellular stress and inflammation. This protein is elevated in conditions of cellular stress and is found in higher levels in the CSF of patients with neurodegenerative diseases and traumatic brain injury [24]. Finally, TUBA4A (Tubulin family) is part of a family of structural components of the cytoskeleton. Alterations in cytoskeletal dynamics have been observed in severe COVID-19, potentially linking TUBA4A to the cellular disruptions seen in critical cases [25].

In summary, CSF proteomics in ICU patients with COVID-19 revealed two contrasting pathophysiological states associated with outcome. Survivors displayed a profile dominated by proteins involved in regulated coagulation and fibrinolysis (PLG, SERPINC1, KNG1), negative acute-phase/serpin biology (FETUB), preserved metabolic competence (SLC27A2), and focused humoral immunity (IGHV3-20). Together, these biomarkers are consistent with intact hepatic support, controlled

activation of the coagulation system at the neurovascular interface, and an effective antiviral response in CNS and barrier cells that limits microthrombi formation and secondary ischemic or inflammatory injury. (Figure 5).

By contrast, nonsurvivors exhibited a CSF signature indicative of overwhelming cellular stress, thromboinflammation, and possible neuronal damage. Increased FTH1 suggests iron overload and ferroptosis-like injury [26], while elevated VSIG4 points to hyperactivated, complement-engaged macrophages with concurrent T-cell suppression. High levels of ER-stress and unfolded protein response markers (HSPA5, PDIA4, SHISA5) support a scenario of proteostatic collapse in CNS and vascular cells, with potential promotion of platelet activation and microvascular thrombosis. Concurrently, elevated FABP3, TUBA4A and LAP3 could indicate neuronal injury and interferon-driven catabolic stress within the CNS compartment. (Figure 5).

Our study has limitations. Firstly, the sample size may limit the generalizability of our findings, and larger cohorts are necessary for further validation. Secondly, the cross-sectional nature of the study does not allow the assessment of dynamic changes in protein expression over time or their direct causal relationships with clinical outcomes. Thirdly, the control group included subjects tested negatively for inflammatory markers and neuronal autoantibodies rather than healthy volunteers, which could introduce confounding factors affecting protein comparisons.

To conclude our proteomic analysis proposes potential CSF biomarkers for COVID-19 prognosis, emphasizing the crucial roles of systemic inflammation, coagulation disruption, and metabolic stress. These biomarkers could significantly contribute to the clinical management and outcome prediction in patients suffering severe COVID-19 and warrant further exploration in larger cohorts and appropriate control populations.

Associated Data

Due to ethical concerns related to human subjects, the data supporting the findings of this study are available from the corresponding author upon request and signing of an agreement of confidentiality.

Author Contributions

H.A.: Designed study, performed research, collected and analyzed data, wrote the paper. **M.S.**: Performed research, collected and analyzed data, wrote the paper. **E.G.**: Analyzed data. **S.M.**: Collected data. **K.B.**: collected data. **G.P.**: Analyzed data, **A.T.**: wrote the paper. **E.M.**: collected and analyzed data. **A.K.**: Collected and analyzed data, **I.T.**: designed study, wrote the paper

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Conflicts of Interest

All authors have reviewed and approved this submission and declare no conflicts of interest.

Funding

The authors have nothing to report.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: prca70042-sup-0001-Figure.png *Supplementary Figure:* Volcano plot that demonstrates a clear distinction between COVID and Healthy groups. A metascape analysis revealed 80 downregulated and 50 upregulated proteins from COVID in comparison to healthy subjects. Upregulated pathways, as expected, include complement and coagulation cascades, acute-phase response proteins and hemostasis.