Proteomic analysis identifies a signature of disease severity in the plasma of COVID-19 pneumonia patients associated to neutrophil, platelet and complement activation

Fabiola Ciccosanti1, Manuela Antonioli1, Alessandra Sacchi1, Stefania Notari1, Anna Farina2, Alessia Beccacece2, Marisa Fusto2, Alessandra Vergori2, Gianpiero D’Offizi2, Fabrizio Taglietti3, Andrea Antinori2, Emanuele Nicastri2, Luisa Marchioni2, Fabrizio Palmieri2, Giuseppe Ippolito1,5, Mauro Piacentini1,3, Chiara Agrati1,6* and Gian Maria Fimia1,4*

Abstract

Most patients infected with SARS-CoV-2 display mild symptoms with good prognosis, while 20% of patients suffer from severe viral pneumonia and up to 5% may require intensive care unit (ICU) admission due to severe acute respiratory syndrome, which could be accompanied by multiorgan failure.

Plasma proteomics provide valuable and unbiased information about disease progression and therapeutic candidates. Recent proteomic studies have identified molecular changes in plasma of COVID-19 patients that implied significant dysregulation of several aspects of the inflammatory response accompanied by a general metabolic suppression. However, which of these plasma alterations are associated with disease severity remains only partly characterized.

A known limitation of proteomic studies of plasma samples is the large difference in the macromolecule abundance, with concentration spanning at least 10 orders of magnitude. To improve the coverage of plasma contents, we performed a deep proteomic analysis of plasma from 10 COVID-19 patients with severe/fatal pneumonia compared to 10 COVID-19 patients with pneumonia who did not require ICU admission (non-ICU). To this aim, plasma samples were first depleted of the most abundant proteins, trypsin digested and peptides subjected to a high pH reversed-phase peptide fractionation before LC–MS analysis.

These results highlighted an increase of proteins involved in neutrophil and platelet activity and acute phase response, which is significantly higher in severe/fatal COVID-19 patients when compared to non-ICU ones. Importantly, these changes are associated with a selective induction of complement cascade factors in severe/fatal COVID-19 patients. Data are available via ProteomeXchange with identifier PXD036491. Among these alterations, we confirmed by ELISA that higher levels of the neutrophil granule proteins DEFA3 and LCN2 are present in COVID-19 patients requiring ICU admission when compared to non-ICU and healthy donors.

*Correspondence: chiara.agrati@opbg.net; gianmaria.fimia@inmi.it
1 Department of Epidemiology, Preclinical Research and Advanced Diagnostics, National Institute for Infectious Diseases IRCCS “L. Spallanzani”, Rome, Italy
Full list of author information is available at the end of the article

© The Author(s) 2022. Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
Introduction
SARS-CoV-2 infection promotes the development of COVID-19 disease which is characterized by a wide spectrum of clinical symptomsspanning from an asymptomatic state to a life-threatening infection [1]. In many cases the prognosis is favorable but about 20% of patients suffer from respiratory distress requiring different types of oxygen therapy up to mechanical ventilation which can be accompanied by multiorgan failure [2]. Such patients also may develop neurological problems or hematological abnormalities and suffer venous thromboembolism [3]. The mortality rate is higher among the elderly or people with chronic diseases [4, 5]. Various comorbidities contribute to worsen the prognosis in COVID-19 patients including obesity, diabetes, cardiovascular disease, and immunosuppression [6–8].

While the disease etiology of COVID-19 is progressively being unraveled the underlying molecular mechanisms and the associated metabolic alterations remain less understood [9, 10]. This is also due to the fact that in addition to viral factors disease severity appears to depend on host factorssupporting the need to better understand the signature of individuals' responses at a molecular level [11]. For example the molecular changes observed in COVID-19 patients’ sera have highlighted a complex dysregulation of platelet and neutrophil degranulationmacrophage function and the complement system [12–16]. Neutrophil dysregulation is associated with COVID-19 severity [17]. Moreover a rapid decline of lung function observed in patients admitted to intensive care units is associated with a dysregulated immune response—defined “cytokine storm” characterized by increased circulating levels of pro-inflammatory interleukins [18]. The systemic hyperinflammation characterizing severe COVID-19 was associated with different alterations of immune response including lymphopenia, massive bystander T cell activation, T cell exhaustion and cytotoxic lymphocyte impairment [19–21]. In contrast SARS-CoV-2 patients experiencing mild disease a balanced and well-orchestrated immune response was described [22].

Ventilator support is often insufficient for preventing mortality in the most severe cases due to the presence of widespread thrombotic microangiopathy which results in multiorgan failure and death [23, 24].

In this scenario there is therefore an urgent need for more in-depth characterization of COVID-19 induced alterations of host pathways and how they are functionally interrelated with the aim of better deciphering the disease’s complexity by identifying additional factors that can help to interpret the patient’s response to SARS-CoV-2 infection.

Changes in human plasma protein levels have been well recognized as indicators of pathophysiological changes associated with various disease states including viral infections. However a known limitation of proteomic studies of plasma samples is the large difference in the macromolecule abundance with concentration spanning at least 10 orders of magnitude [25]. To improve the coverage of plasma contents our study undertook an in-depth plasma analysis in COVID-19 patients requiring ICU admission by combining the depletion of most abundant proteins with a high pH reversed-phase peptide fractionation before LC–MS analysis.

Materials and methods
Patient selection
This study was approved by the National Institute for Infectious Diseases L. Spallanzani IRCCS Ethics CommitteeRome, Italy. Written informed consent for plasma donation was obtained from all patients and healthy donors (HDs). SARS-CoV-2 infected patients (n = 32) were diagnosed with COVID-19 by SARS-CoV-2 RT-PCR performed on nasopharyngeal and oropharyngeal swabs. We enrolled SARS-CoV-2 positive patients all with pneumoniarevealed with high resolution chest computed tomography, without other infections such as HCV, HBV, MTB, and others; one patient in each group tested positive for HIV. Plasma samples were collected in the first few days of clinical hospitalization. Partial pressure of oxygen/inspired oxygen concentration ratio (PaO2/FiO2 ratio) which represents a valuable clinical measure of the patient’s respiratory status for the evaluation of assisted oxygenation were recorded during the hospitalization. Demographicclinical baseline characteristics were used to determine whether the patients were admitted to ICU. Changes in human plasma protein levels have been well recognized as indicators of pathophysiological changes associated with various disease states including viral infections. However a known limitation of proteomic studies of plasma samples is the large difference in the macromolecule abundance with concentration spanning at least 10 orders of magnitude [25]. To improve the coverage of plasma contents our study undertook an in-depth plasma analysis in COVID-19 patients requiring ICU admission by combining the depletion of most abundant proteins with a high pH reversed-phase peptide fractionation before LC–MS analysis.

Plasma separation from peripheral blood sampling
Peripheral blood in K2-Ethylenediaminetetraacetic acid (EDTA) BD Vacutainer® blood collection tubes (BD Biosciences Franklin Lakes, NJUSA) was centrifuged at 1400
RPM for 10 min to obtain the plasma and aliquoted and stored at –80 °C until use.

**Plasma protein purification peptide purification and LC-MS/MS analysis**

To remove high abundance proteins, 100ul of plasma for each sample pre-treated with Triton-X100 (Merck Life Science) at a final concentration of 1% to make the samples safer were processed with High-select Top14 Abundant Protein Depletion Resin columns (Thermo Fisher Scientific) following the manufacturer's protocol. The depleted samples were eluted in 10 mM PBS and 0.02% sodium azide pH 7.4.

From each sample 14ug of purified proteins were boiled at 95 °C and treated with DL-dithiothreitol (10 mM at 56°C Merck Life Science) and iodoacetamide (55 mM at room temperature Merck Life Science) for disulfide bond reduction and alklylation respectively. Then the samples were diluted 10 times by adding 50 mM NH4HCO3 (Merck Life Science) 1 M urea (Merck Life Science) and digested with trypsin (0.4 μg/sample Promega) at 37 °C overnight.

The peptide mixture was acidified with trifluoroacetic acid (TFA final concentration 0.1% Fluka) and fractionated based on the peptides hydrophobicity using Pierce High pH Reverse Phase Peptide Fractionation Kit (Thermo Fisher Scientific) following the manufacturer's protocol.

Peptides in each fraction (8/sample) were dried suspended in 2.5% acetonitrile (ACN Fluka) 0.1% TFA and 0.1% formic acid (Fluka) and then analyzed twice technical replicates by ultra-high performance liquid chromatography coupled with high resolution mass spectrometry using Thermo Scientific Q Exactive Plus Orbitrap as described [26]. In particular the peptides were separated by nano liquid chromatography (Ultimate 3000 RSLC nano-LC system Thermos Fisher Scientific) loaded onto a 75 μm C18 column (ES800-PepMap™ RSLC C18150 mm × 75 μmThermo Fisher Scientific) using a 100 min linear multistep gradient elution (from 4 to 90% eluent B80% ACN 0.1% formic acid with a constant flow rate of 0.3 μL/min) and were analyzed by Q Exactive plus™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific) with this run condition: electrospray ionization (ESI) voltage 2.0 kV and MS data were acquired in a positive mode using data-dependent mode selecting the 15 most intense ions with full scan MS spectra range from m/z 350.0 to m/z 1700.0 resolution of 70,000 injection time 100 ms AGC target 3 × 106 isolation window ± 2.0 m/z charge exclusion 1,7,8 > 8 and the dynamic exclusion 20 s. For HCD fragmentation resolution was set to 17,500 AGC target to 10000 and injection time of 80 ms.

**Quantification and statistical analysis**

The raw data from the mass spectrometric analysis were processed using the MaxQuant software v.1.5.5.1 and quantified using the iBAQ algorithm [27]. Data were analyzed with Perseus software v.1.6.15 [28] (http://www.perseus-framework.org). Briefly data were filtered based on categorical rows; only identified by sidereverse and potential contaminants were removed. Calculated iBAQ were log2 converted and grouped to compare HD non-ICU and ICU and filtered based on proteins identified in at least one group with a coverage of 90%. Each set was normalized by calculating the median of log2-transformed iBAQ intensities for each MS run and subtracting it from each log2-transformed iBAQ intensity in the same run. Missing values were imputed from normal distribution based on total matrix (width 0.3 and down shift 1.3). The obtained matrix of 216 identifications was used to calculate the Principal Component Analysis (2 components FDR < 0.05) and to generate a volcano plot for HD vs COVID-19 non-ICU HD vs COVID-19 ICU/F groups and COVID-19 non-ICU vs COVID-19 ICU/F groups with 250 randomizations based on two sides t-test (FDR < 0.05S0). Hierarchical clustering of identified proteins was performed after Z-score normalization then significantly different proteins among groups were calculated by multiple-sample test (ANOVA) and Post hoc Tukey’s HSD test of one-way ANOVA (FDR < 0.05).

Euclidean distance were used to compare different groups and clusters were extracted using n = 4 and n = 15 to allow a better discrimination of lower abundant proteins.

Plasma proteins that underwent significant fold changes were considered for the classification of biological dysregulated processes. Only processes relevant for plasma proteins were included in the list. The network analysis and the visualization of plasma proteins significantly modulated in COVID-19 non-ICU and COVID-19 ICU/F was performed using the application of String-DB specifically developed for Cytoscape (v3.8.2) (StringApp) [29]. Functional enrichment analysis was performed for each of identified cluster and significant GO process were reevaluated (FDR < 0.05). Graph pad Prism 8 was used to generate reported plots.

**ELISA assay**

Plasma protein levels were measured using a Human ELISA kit (MyBioSource) following the manufacturer's protocol. Plasma was diluted 1:9 for the Human MPO kit assay (MBS175830) 1:2 for the Human DEFA3 kit assay (MBS2705383) and 1:2 for the Human NGAL kit assay (MBS268158).

Statistical analyses were performed using Kruskal–Wallis one-way analysis of variance (ANOVA) with
Dunn’s post hoc analysis. Statistics were carried out using GraphPad Prism 5.00. P-values less than 0.05 were considered significant.

**Results**

**Patients and experimental design**

Blood samples were collected from a cohort of 20 patients with COVID-19 pneumonia admitted to INMI L. Spallanzani (Rome, Italy) including 8 patients with severe pneumonia who required ICU admission (ICU), 2 patients who died before ICU admission (F = fatal) and 10 COVID-19 patients with pneumonia who did not require ICU admission (non-ICU) (Fig. 1A). Severity of COVID-19 was graded using the WHO ordinal outcome scale of clinical improvement (Fig. 1B, https://www.who.intteams/blueprint/covid-19). Among COVID-19 ICU patients 5 died and 3 had survived and been discharged from the hospital. Among COVID-19 non-ICU patients 6 required non-invasive oxygen therapy (non-invasive ventilation (NIV)/continuous positive airway pressure mask (CPAP) n = 3; large-reservoir Venturi masks (Ventimask)/nasal cannula (NC) n = 3) while 4 did not require any of these devices. Most of the COVID-19 patients suffered from at least one chronic condition (Fig. 1C).
disease (ICU/F: 9/10; non-ICU: 6/10) (Additional file 1: Table S1).

Blood samples from 10 healthy donors (HD) collected before SARS-CoV-2 pandemic (before January 2020) were analysed for comparison. Samples were collected early after hospitalization: interquartile range (IQR) 2.5 days for COVID-19 non-ICU (from 1.25–3.75) interquartile range (IQR) 2.75 days for COVID-19 ICU/F (from 2–4.75). In the latter groupsamples were collected either before ICU admission (4 patientsfrom 1 to 6 days) or after ICU admission (4 patientsfrom 0 to 8 days).

To improve the coverage of plasma contents14 highly abundant proteins including albumin IgG and fibrinogen were depleted by affinity chromatography. Proteins were then digested with trypsin (Fig. 1A). Peptides from each sample were then subjected to high pH reversed-phase chromatography and eluted in eight fractions. Each peptide mixture was analyzed by liquid chromatography with tandem mass spectrometry (LC–MS/MS) using a Q-Exactive Plus instrument.

**Proteomic profiling of plasma from COVID-19 patients**

Each sample was run twice as technical replicates. MaxQuant software [27] identified about 6100 peptides in totalwith a number of peptides ranging from 1,426 to 3,807 per patient (Additional file 2: Table S2). Considering only proteins matched by 2 independent peptides449 human proteins were identified in totalwith 194 proteins quantified in all samples (40.2%) indicating a good reproducibility of the proteomic profiling (Additional file 3: Table S3). To ensure data qualityonly 216 proteins mutually quantified in > 90% samples were considered for protein level comparison. For each proteinmultivariate normal imputation (MVNI) was applied to impute the missing values [30].

The principal-component analysis (PCA) of the 30 samples was performed by using raw MS/MS values from 216 proteins with normalized expression values across samples (Fig. 1C). Samples from COVID-19 and HD were unambiguously distinguished. Samples from ICU/F and non-ICU COVID-19 samples were not completely separated. InterestinglyCOVID-19 non-ICU patients that overlap with the COVID-19 ICU/F ones are mainly those who required non-invasive oxygen therapies. On the other handthe COVID-19 ICU patient who clusters closer to the COVID-19 non-ICU ones had a blood sam-ple collected 6 days before entering in ICU suggesting that main changes in proteomic profile were not yet present.

Prompted by this initial evidence that proteomic plasma alterations may correlate with the disease statuswe investigated more in details which specific pathways account for the classification of COVID-19 cases.

**Proteomic alterations in plasma of severe vs non-severe COVID-19 patients**

Signatures of COVID-19 were identified by analyzing plasma proteins that underwent significant fold changes (FCs) among different groups (log2 (FC) > 0.5; unpaired two-sided Welch’s t test; p < 0.05). A total of 120 differentially expressed proteins (DEPs) were identified in COVID-19 ICU/F vs HD91 DEPs in COVID-19 non-ICU vs HD and 98 DEPs in COVID-19 ICU/F vs non-ICU (Fig. 2; Additional file 4: Table S4). InterestinglyDEPs that discriminate COVID-19 ICU/F from non-ICU were all upregulated in the first group (Fig. 2C) suggesting that the alterations of plasma proteins became more extensive in compromised conditions.

The DEPs were then subjected to Gene Ontology (GO) [31] enrichment and network analyses. In all comparisons the GO terms highly enriched in processes are related to inflammationplatelet and neutrophil degranulation and coagulation cascades (Fig. 3A, C and Additional file 5: Table S5). Proteins involved in lipoprotein remodeling are mainly observed in COVID-19 non-ICU patientswhile importantly complement activation become relevant in the COVID-19 ICU patients when compared to both COVID-19 non-ICU and HD suggesting that dysregulation of this process may account for the more severe forms of COVID-19 leading to ICU and/or death. Although all processes are strictly interrelatedas indicated in the STRING maps a dense network of interactions is observed between inflammation and neutrophil factoras well as between coagulation and platelet factors (Fig. 3B and D), which further overlaps with complement proteins in COVID-19 ICU patients (Fig. 3D and F).

**Cluster analysis of biological processes dysregulated in severe vs non-severe COVID-19 patients**

Since a complex combination of dysregulated processes contribute to the severe forms of COVID-19 we evaluated which pathways are concomitantly altered when COVID-19 ICU/F versus COVID-19 non-ICU plasma proteomes were compared by a hierarchical clustering method and results were visualized in a heat map (Fig. 4A). Thirteen different clusters were identified by using two thresholds (n = 4 and n = 15) which were applied to better separate groups either with high or low levels of variations. These clusters were then analysed in relation to the processes to which these proteins play a role (Additional file 6: Table S6).

Cluster 12 and 13 report proteins that are increased in all COVID-19 patients but at higher levels in ICU/F patients (Fig. 4B) which are mainly constituted by proteins playing a role in acute inflammatory response (CRP, ORM1, ORM2, SAA1, SAA2, S100A8, S100A9, Serpin...
Other highly represented classes are platelet and neutrophil activity (Fig. 4B). In particular, high levels of HIST1H4H and MPO are associated to Neutrophil extracellular traps (NETs) formation while LCN2, PGLYRP1, DEFA3 are neutrophil granule proteins with antimicrobial activity. Importantly, LCN2 were previously reported to be one of the genes predictor for admission to intensive care [17]. Moreover, we detected the pattern recognition receptor CD14 which is expressed by both monocytes and neutrophils and has been proposed as potential target to reduce acute inflammation in COVID-19 patients [32] together with LBP (LPS binding protein) a protein responsible for the potentiation of LPS-mediated activation of monocytes/neutrophils via CD14 [33].

With regard to the platelet activation, platelet granule proteins were detected such as FGA, FGB, FGG, VWF, A1BG and QSOX1, together with LGALS3BP, a multifunctional secreted glycoprotein with prothrombotic and proinflammatory properties [34].

These clusters include only a few members of the complement pathway: C6C9, Serpin A1 and G1 and MBL2 (Mannose-binding lectin 2) a pattern recognition molecule that initiates the lectin pathway of complement activation with polymorphisms associated with COVID-19 severity [35].

Various components of the extracellular matrix (ECM) and plasma membrane proteins were also detected, including the vascular adhesion protein VCAM1, the platelet adhesion CADHERIN-13, the PROTEOGLYCAN 4a protein with anti-inflammatory properties increased in chronic obstructive pulmonary disease [36], TGFBI an ECM factor required for normal alveolar structure and function [37] and EFEMP1 (EGF-containing fibulin-like extracellular matrix protein 1) whose gene is mutated in a macular degeneration disease characterized by complement-trigger protein/lipid deposits [38]. Increased extracellular matrix proteins were previously associated with pulmonary fibrosis [39], with TGFBI having potential roles in extracellular matrix remodeling [40], including lung and cardiac fibrosis [41, 42]. Moreover, the increase in the hemoglobin proteins HBA1 and HBB, the hemoglobin binding protein (HP) and Carbonic Anhydrase 1 (CA1) both likely indicative of hemolysis as previously reported [43].
GO-based enrichment analysis of DEPs COVID-19 non-ICU vs HD

Network analysis of DEPs COVID-19 non-ICU vs HD

GO-based enrichment analysis of DEPs COVID-19 ICU/F vs HD

Network analysis of DEPs COVID-19 ICU/F vs COVID-19 non-ICU

Fig. 3 (See legend on previous page.)
Cluster 9 and 10 show proteins that are significantly upregulated only in COVID-19 ICU/F patients (Fig. 4C). The majority of them play a role in the complement cascade including proteins involved in the induction of the different activation pathways (classical pathway: e.g. C1q, C1r, C1s; alternative pathway, e.g. C3, CfrH1, CfrH2; lectin pathway: e.g. ColeC11, MasP1, MasP2 and FcN3). An increase was also detected for proteins involved in coagulation (F5, F9, F10, F11, ProS1, SerPin C1, SerPin A10, SerPin F2, HABP2) inflammation-induced proteins involved in different types of amyloid diseases (Saa1, Saa2-Saa4, ApcS, ApmA, B2M, Cst3) and extracellular matrix organization (Fn1, Vtn, Ith3, Ith4). In addition we detected an increase in VLDL/LDL lipoprotein (ApoC3, APOE, ApOL1) and interestingly in two multifunctional proteins Gpx3 and MST1/Msp both playing a role in the regulation of inflammation and lipid metabolism [44, 45].

The clusters reporting proteins that are decreased in COVID19 patients include mainly proteins involved in lipid transport and platelet components (Fig. 5). In particular we observed a decrease in the HDL components Apoa4 and Apoc1 in both COVID-19 non-ICU and ICU/F patients (cluster 3, Fig. 5A) while Apoa2, Apod, Apoh, Apom and the HDL-associated PON1 protein appear to be decreased mainly in COVID-19 non-ICU patients (cluster 7, Fig. 5B). Related to lipid metabolism we also observed a reduction in CETP (cluster 4, Fig. 5A) which is involved in cholesterol exchange between LDL and HDL [46], Pcox1 (cluster 5, Fig. 5A) a pro-oxidant enzyme associated to LDL [47] and the GPI degrading enzyme GPLD1 (cluster 7, Figure 5B) [48]. Concerning platelet function both COVID-19 ICU and non-ICU patients show a reduction in structural proteins such as Vcl, Sparc, Plek, Tln1, Tagln2, Flna (all present in cluster 4 Figure 5A) and Tmsb4x (cluster 3, Fig. 5A), a possible indication of thrombocytopenia already reported in COVID-19 patients [49], while a reduction of secreted platelet factor such as Pf4 and PpbP was observed only in COVID-19 non-ICU patients. Other processes that are altered mainly in COVID-19 non-ICU patients (cluster 7, Fig. 5B) refer to endothelial cell integrity such as VasNa factor downregulated during vessel repair [50], the vascular endothelial cadherin CDH5 and the regulation of glucose metabolism by the adipose tissue including Adiponectin (AdipoQ) and Retinol-Binding Protein 4 (Rbp4).

Interestingly we also detected a small set of proteins whose levels are decreased in COVID-19 non-ICU patients and increased in COVID-19 ICU/F which play a role in the complement (C8g, C8b, Cfb, C1qB and C1qC, cluster 12 Fig. 5C) and platelet/coagulation (Serpin A6, Serpin D1, F2 in cluster 12 and Kng1, Plg in cluster 6 Fig. 5C) cascades confirming that these processes may be differently modulated depending on disease severity.

Validation of alterations related to neutrophil hyper-activity.
ELISA was used to confirm some of the changes observed in the proteomic analysis focusing on the hyper-activation of neutrophils. For this analyses we compared independent groups of 6 COVID-19 patients who required ICU admission and 6 COVID-19 patients who did not require it. Six healthy donors were also included in the analysis. Demographicsclinical baseline characteristicstherapy and cause of death are shown in Additional file 1: Table S1. When compared to the cohort of patients used for the proteomic analysis major differences for COVID-19 ICU patients were the number of deceased patients (1 vs 8) and oxygen saturation median (96 vs 89.5) while for COVID-19 non-ICU patients the sample collection time after hospitalization (IQR 4.5 days vs 2.55 days). In particular we focused on 3 neutrophil granule proteins (MPO, Defa3 and Lcn2/ngal) whose plasma levels were increased in non-ICU and ICU/F patients at a higher extent in latter group (Mpo: 1.3 vs 2.4, Defa3: 2.9 vs 3.6, Lcn2/ngal 2.1 vs 2.9). Changes in the levels of the 3 proteins discriminate between COVID-19 ICU patients and HD (Fig. 6) while variance with proteomic results no significant difference was observed between COVID-19 non-ICU patients and HD, suggesting that the variability in the levels of these proteins in the non-ICU group is higher than what estimated in the proteomic analysis. Conversely, Defa3 and Lcn2/ngal shows significant differences between COVID-19 ICU and non-ICU...
Hierarchical cluster analysis of DEPs

GO-based enrichment analysis of DEPs upon clustering (upregulated proteins)

- Acute inflammatory response
- Complement activation, classical pathway
- Platelet degranulation
- Immune response
- Acute-phase response
- Platelet degranulation
- Complement activation, lectin pathway
- Response to wounding
- Complement activation, alternative pathway
- Blood coagulation, fibrin clot formation
- Response to nutrient levels
- Antimicrobial humoral response

Fig. 4 (See legend on previous page.)
patients (Fig. 6) confirming that neutrophil activation could be a hallmark of COVID-19 severity.

**Discussion**

Here we reported a proteomic analysis of plasma of COVID-19 patients with severe/fatal pneumonia compared to COVID-19 patients with pneumonia not requiring ICU admission with the aim of identifying molecular features associated to disease progression.

Our data show that several proteins belonging to the complement cascade are mainly increased in COVID-19 patients requiring ICU admission in combination with a series of proteins playing a role in acute inflammatory responseplatelet and neutrophil function that are progressively increased in ICU/F versus non-ICU COVID-19 patients. Our results are highly consistent with the plasma/sera proteomic signatures of severe COVID-19 patients published to date [12–16, 51–61]. Noteworthysome of the identified proteins involved in the inflammatory responseplatelet and complement cascades such as CRP, B2M, CST3, SERPIN A3, PLG, C1R, were recently identified as predictors of future worsening of the clinical condition and CD14, B2M, SERPIN D1, C1QB, C1QC, SERPIN A3 were predictive of a longer need for inpatient treatment [62].

The complement system generates a highly regulated innate immune response mediated by both the perturbation of target membranes and the generation of a proinflammatory response through a proteolytic cascade that is initiated by three different pathways (classical, alternative and lectin) [63]. We observed an increase in the levels of several complement proteinsmost of which are exclusively induced in severe COVID-19 patients while upregulation of the lectin receptor MBL2 was also detected in COVID-19 non-ICU patients and further increased in COVID-19 ICU/F. Interestingly Gutmann and colleagues reported complement activation as a core component of the dysregulated immune response to COVID-19 with elevated MBL2 being a predictor of 28-day ICU mortality [51]. Moreover induction of complement factors has been reported in both proteomic and transcriptomic studies of blood and lung samples from patients with severe COVID-19 [58, 64, 65]. Of note, several lines of evidence support that SARS-CoV-2 can directly activate complement through the alternative and the lectin pathways which are both highly represented in our proteomic analysis [66–68]. However several inflammatory processes can account for the indirect activation of complement cascades.

Among the different class of plasma proteins whose levels are altered in COVID-19 ICU/F patients we have focused our interest to neutrophil markers because these cells are key players in the innate immune system [69] and an important contribution of a dysregulated activity of these cells to COVID-19 disease severity is emerging from a plethora of evidence [52, 70, 71]. In particular a strict functional interaction between neutrophils and the complement cascade has been described which is bidirectional and if not tightly regulated may establish a self-amplifying loop which can lead to hyper-activation of the immune system and thrombotic microangiopathy [72–74]. ELISA of an independent group of patients have confirmed that MPO, LCN2 and DEFA3 are increased in COVID-19 ICU/F patients when compared to HD. And observed that LCN2 and DEFA3 can discriminate ICU/F from non-ICU COVID-19 patients.

Neutrophils contribute to the innate immune response through both pathogen phagocytosisinflammation and release of NETextracellular traps composed of neutrophil-derived chromatin and microbicidal proteins [69, 75]. Lung-recruited neutrophils are characterized by exacerbated production of IL-8IL-1βIL-6 and CCL3/4 along with elevated levels of neutrophil elastase and MPO. The compartmentalization of transcriptionally active and highly inflammatory neutrophils in the lung participates in driving acute respiratory distress syndrome (ARDS) [75]. The increase in the levels of histone H4, MPO, LCN2, PGLYRP1 and DEFA3 detected in our proteomic analysis strongly support the observation that excessive NET formation occurs in severe COVID-19 patients [72, 76–81]. Accordingly histological detection of NETs has been observed in the microvasculature of lung, kidney and heart of these patients [82–84]. NETs also activate platelets and the clotting cascade are integral part of vascular thrombi [85, 86]. On the other hand platelet-derived and complement factors are known to trigger NET formation underling how each process may promote the others [86, 87]. NETosis a regulated form of neutrophil death has been implicated in the pathogenesis of COVID-19 [88]. Opsonized pathogens may
A. GO-based enrichment analysis of DEPs upon clustering (downregulated proteins)

- high-density lipoprotein particle remodeling
- regulation of hydrolase activity
- platelet degranulation
- positive regulation of small molecule metabolic process
- regulation of endocytosis
- negative regulation of peptidase activity

B. GO-based enrichment analysis of DEPs upon clustering (upregulated proteins)

- regulation of plasma lipoprotein levels
- phosphatidylcholine metabolic process
- response to lipid
- lipid transport
- platelet degranulation
- regulation of cholesterol transport
- protein activation cascade
- response to immune system
- regulation of cytolysis
- small molecule metabolic process
- response to stress
- regulation of macrophage differentiation
- negative regulation of inflammatory response
- vitamin transmembrane transport
- regulation of neutrophil chemotaxis
- negative regulation of hydrolase activity
- response to toxic substance
- monocarboxylic acid metabolic process
- triglyceride metabolic process
- negative regulation of catabolic process
- regulation of angiogenesis

C. GO-based enrichment analysis of DEPs upon clustering (upregulated proteins)

- regulation of protein activation cascade
- negative regulation of platelet degranulation
- complement activation, alternative pathway
- negative regulation of fibrinolysis
- response to estradiol
- response to carbohydrate

Fig. 5 (See legend on previous page.)
trigger NETosis through the engagement of neutrophils complement receptors and complement components are able to stabilize NETs by preventing DNase I mediated clearance [89]. On the other hand neutrophils may activate the C3-convertase through proteins present on the neutrophil surface [90] and in the NETs [91]. Moreover myeloperoxidase and neutrophil granule proteases may activate properdin and potentiate complement cascade on NETs [92]. Related to this we also detected an increase of VCAM and various ECM components which could be indicative of vessel damage as well as red blood proteins as possible indication of hemolysis.

Limitations of the study
The main limit of our study is the small number of patients that were analysed. To counter this problem we have also analysed by ELISA some of changes in the level of neutrophil granule proteins detected by proteomic analysis in an independent small cohort of patients.

A further limitation is the difference in median age of healthy donors with respect to COVID-19 patients (HD: 43 non-ICU: 67.5 ICU/F: 65) as well as the absence of morbidity which may have influenced the proteomic results independently of SARS CoV-2 infection. Age and comorbiditiessuch as obesity are well-known risk factors for severity progression in COVID-19 with obesity being associated with neutrophil activation [93]. However our study was mainly focused on the comparison between ICU/F and non-ICU COVID-19 patients and the HD group was included just a control arm. The two groups of COVID-19 patients have homogeneous clinical baseline characteristics and in this case comorbidities should not have contributed in a relevant manner to the observed proteomic differences of plasma profiles.

Conclusion
We have provided an in-depth analysis of proteomic changes in the plasma of COVID-19 patients in relation to the disease severity which may help to elucidate the molecular basis of COVID-19 severe pathogenesis and provide a framework for studies aimed at identifying biomarkers with prognostic values for the COVID-19 outcome.

Abbreviations
A1BG: Alpha-1-B glycoprotein; ADIPOQ: Adiponectin; APCS: Serum amyloid P-component; ADIPOQ: Adipocyte plasma membrane associated protein APOA1, Apolipoprotein A1; APOA4: Apolipoprotein A4; APOC1: Apolipoprotein C1; APOC3: Apolipoprotein C3; APOD: Apolipoprotein D; APOE: Apolipoprotein E; APOH: Apolipoprotein H; APOL1: Apolipoprotein L1; APOM: Apolipoprotein M; ARDS: Acute respiratory distress syndrome; B2M: Beta-2-microglobulin; CA1: Carbonic anhydrase 1; C1Q: Complement component 1q; C1QB: Complement C1q subcomponent subunit B; C1QC: Complement C1q subcomponent subunit C; C1R: Complement C1r subcomponent; C1S: Complement C1s subcomponent; C3: Complement component C3; C6: Complement component C6; C9: Complement component C9; CBB: Complement component C8 beta chain; CBG: Complement component C8 gamma chain; CCL3/4: C–C motif chemokine ligand 3/4; CD14: Monocyte differentiation antigen CD14; CDH5: Cadherin-5; CETF: Cholesteryl ester transfer protein; CFH: Complement factor H; CFHR1: Complement factor H-related protein 1; CFHR2: Complement factor H-related protein 2; COLEC11: Collectin-11; CRP: C-reactive protein; CST3: Cystatin-C; DEFA3: Neutrophil defensin 3; ECM: Extracellular matrix; EFEMP1: EGF-containing fibulin-like extracellular matrix protein 1; ELISA: Enzyme linked immunosorbent assay; F10: coagulation factor
The online version contains supplementary material available at https://doi.org/10.1186/s12014-022-09377-7.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12014-022-09377-7.

Additional file 1: Tables S1: Demographics, clinical baseline characteristics, therapy and cause of death of COVID-19 patients.

Additional file 2: Tables S2: MaxQuant software analysis of peptides detected by mass spectrometry from plasma of COVID-19 patients.

Additional file 3: Tables S3: MaxQuant software identification of proteins from plasma of COVID-19 patients.

Additional file 4: Tables S4: Proteins with statistically different levels in COVID-19 patients.

Additional file 5: Tables S5: Analysis of biological processes associated to the proteins altered in COVID-19 patients.

Additional file 6: Tables S6: Cluster analysis of proteins modulated in the plasma of COVID-19 patients.

**Author contributions**

GMF, GI, MP and CA and conceived the study. FC performed the mass spectrometry experiments. MA conceived the data analysis approach. MA, FC and GMF analysed the data. AS and NS contributed to sample collection and storage. AF, AB, MF, AI, GDO, FT, AA, EN, LM, FP contributed to patients’ recruitment and clinical evaluation. GMF and CA wrote the manuscript. All authors reviewed and approved the manuscript.

**Funding**

This work was supported in part by grants from the Italian Ministry of Health (Ricerca Corrente line 1, COVID-2020-1237173S and COVID-2020-12371817), donation from "Avvocati e Procuratori dello Stato", "Beyond Borders" University of Rome "Tor Vergata" and Regione Lazio (Gruppi di ricerca E56C18000460002 and from the European Commission—Horizon 2020 (European Virus Archive GLOBAL—871029 and EU project 101003551—EXCALATE4CoV).

**Availability of data and materials**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [94] partner repository with the dataset identifier PXD036491.

**Declarations**

**Ethics approval and consent to publish**

The study was approved by the institutional review board (approval number: 9/2020) and signed written informed consent was obtained from patients.

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Author details**

1 Department of Epidemiology, Preclinical Research and Advanced Diagnostics, National Institute for Infectious Diseases IRCCS “L. Spallanzani”, Rome, Italy. 2 Infectious Disease–Clinical Department, National Institute for Infectious Diseases IRCCS “L. Spallanzani”, Rome, Italy. 3 Department of Biology, University of Rome “Tor Vergata”, Rome, Italy. 4 Department of Molecular Medicine, University of Rome “Sapienza”, Rome, Italy. 5 Present Address: General Directorate for Research and Health Innovation, Italian Ministry of Health, Rome, Italy. 6 Present Address: Department of Hematology/Oncology and Cell and Gene Therapy, Bambino Gesù Children Hospital, IRCCS, Rome, Italy.

Received: 13 June 2022 Accepted: 26 October 2022 Published online: 08 November 2022

**References**

1. Hu B, Guo H, Zhou P, Shi ZL. Characteristics of SARS-CoV-2 and COVID-19. Nat Rev Microbiol. 2020;19:141-51.
2. Attaway AH, Scheraga RG, Bhimraj A, Biehl M, Hatopo Lu. Severe covid-19 pneumonia: pathogenesis and clinical management. BMJ. 2021. https://doi.org/10.1136/bmjn436.
3. Gupta A, Madhavan MV, Sehgal K, Nair N, Mahajan S, Sehrawat TS, et al. Extrapulmonary manifestations of COVID-19. Nat Med. 2020. https://doi.org/10.1038/s41591-020-0968-3.
4. Williamson DJ, Walker AJ, Bhaskaran K, Bacon S, Bates C, Morton CE, et al. Factors associated with COVID-19-related death using OpenSAFELY. Nature. 2020;584:430–6.
5. Dessie ZG, Zewotir T. Mortality-related risk factors of COVID-19: a systematic review and meta-analysis of 42 studies and 423,117 patients. BMC Infect Dis. 2021. https://doi.org/10.1186/s12879-021-06636-3.
6. Nishiga M, Wang DW, Han Y, Lewis DB, Wu JC. COVID-19 and cardiovascular disease: from basic mechanisms to clinical perspectives. Nat Rev Cardiol. 2020;17:543–58.
7. Stefan N, Birkenfeld AL, Schulze MB. Global pandemics interconnected - obesity/stymepared metabolic health and COVID-19. Nat Rev Endocrinol. 2021;17:135–49.
8. Belsky JA, Tullius BP, Lamb MG, Sayegh R, Stanek JR, Auletta JJ. COVID-19 in immunocompromised patients: a systematic review of cancer hematopoietic cell and solid organ transplant patients. J Infect. 2021;82:329–38. https://doi.org/10.1016/j.jinf.2021.01.022.
9. Vškovski P, Kratzel A, Steiner S, Stalder H, Thiel V. Coronavirus biology and replication: implications for SARS-CoV-2. Nat Rev Microbiol. 2021;19:155–70.
10. Ayres JS. A metabolic handbook for the COVID-19 pandemic. Nat Metab. 2020;2:572–85.
11. Fleiflage T, Boyd DF, Meliopoulos V, Thomas PG, Schultz-Cherry S. Influenza virus and SARS-CoV-2: pathogenesis and host responses in the respiratory tract. Nat Rev. 2021;19:425–41.
12. Shen B, Yi X, Sun X, Bi X, Du L, Zhang C, et al. Proteomic and Metabolomic Characterization of COVID-19 Patient Sera. Cell. 2020;182:59-72.e15.
13. Messner CB, Demichev V, Wendisch D, Michalick L, White M, Freiwald A, et al. Ultra-high-throughput clinical proteomics reveals classifiers of COVID-19 infection. Cell Syst. 2020;11:1-24.e4.
coagulation and complement status as a function of IL-6 Level. J Proteom. 2020;19:4417–27.
59. Halaszmagi L, Salumets A, Rumm AP, Jurgenston M, Krassoehina E, Remm A, et al. Longitudinal proteomic profiling reveals increased early inflammation and sustained apoptosis proteins in severe COVID-19. Sci Rep. 2020. https://doi.org/10.1038/s41598-020-77525-w.
60. Filbin MR, Mehta A, Schneider AM, Kays KR, Guess JR, Gentili M, et al. Longitudinal proteomic analysis of severe COVID-19 reveals survival-associated signature/tissue-specific cell death and cell-cell interactions. Cell Rep. 2021. https://doi.org/10.1016/j.celrep.2021.100287.
61. Lee JS, Han D, Kim SY, Hong KH, Jiang M, Jinkim MJ, et al. Longitudinal proteomic profiling provides insights into host response and proteome dynamics in COVID-19 progression. Proteomics. 2021. https://doi.org/10.1002/pmic.202000278.
62. Demichev V, Tober-Lau P, Lemke O, Nazaenko T, Thibeault C, Whittwell M, et al. A time-resolved proteomic and prognostic map of COVID-19. Cell Syst. 2021;12:780-794.e7.
63. Bajic G, Deen SE, Thiels S, Andersen GR. Complement activation-regulation and molecular basis for complement-related diseases. EMBO J. 2015;34:2735–57.
64. Overmyer KA, Shishkova E, Miller U, Stewart R, Coon JJ, Jaitovich A, et al. Large-scale multi-omic analysis of COVID-19 severity. Cell Syst. 2021;12:23–40.
65. Holter K, Pischke SE, de Boer E, Lind A, Jenum S, Holten AR, et al. Systemic complement activation is associated with respiratory failure in COVID-19 hospitalized patients. Proc Natl Acad Sci USA. 2020;117:25018–25.
66. Yu J, Yuan X, Chen H, Chaturvedi S, Braunstein EM, Brodsky RA. Direct activation of the alternative complement pathway by SARS-CoV-2 spike proteins is blocked by factor D inhibition. Blood. 2020;136:2080–9.
67. Wilk-CM. Coronavirus hijacks the complement system. Nat Rev Immunol. 2020;20:2350.
68. Stavrakis M, Pagani I, Paraboschi EM, Pedotti M, Doni A, Scavelli F, et al. Recognition and inhibition of SARS-CoV-2 by humoral innate immunity pattern recognition molecules. Nat Immunol. 2022;23:275–86.
69. Mayadas TN, Cullere X, Lowell CA. The multifaceted functions of neutrophils. Annu Rev. 2019;49:181–218.
70. Ackermann M, Anders HJ, Bilyy R, Bowlin GL, Daniel C, De Lorenzo R, et al. Longitudinal proteomic profiling reveals increased early inflammasome and sustained apoptosis proteins in severe COVID-19 patients. bioRxiv. 2021. https://doi.org/10.1101/2021.06.02.446468v1.
71. Bautista-Becerril B, Campi-Caballero R, Sevilla-Fuentes S, Hernández-Regino LM, Hanono A, Flores-Bustamante A, et al. Immunothrombosis in COVID-19: implications of neutrophil extracellular traps. Biomolecules. 2021. https://doi.org/10.3390/biom11050694.
72. Mezlish ML, Pine AB, Bishai JD, Goshtas H, Nadelmann ER, Simonov M, et al. Neutrophil activation signature predicts critical illness and mortality in COVID-19. Blood Adv. 2021;5:1164–77.
73. Hazeldine J, Lord JM. Neutrophils and COVID-19: active participants and rational therapeutic targets. Front Immunol. 2021. https://doi.org/10.3389/fimmu.2021.680134.
74. Prévét R, Dupont A, Labrouche-Colomer S, García G, Dewitte A, Rauch A, et al. Plasma markers of neutrophil extracellular trap are linked to survival but not to pulmonary embolism in COVID-19-related ARDS patients. Front Immunol. 2022. https://doi.org/10.3389/fimmu.2022.851497.
75. Zhu Y, Chen X, Liu X. NETosis and neutrophil extracellular traps in COVID-19: immunothrombosis and beyond. Front Immunol. 2022. https://doi.org/10.3389/fimmu.2022.838901.
76. Block H, Zarbock A. A fragile balance: does neutrophil extracellular trap formation drive pulmonary disease progression? Cells. 2021. https://doi.org/10.3390/cells10081932.
77. Leppkes M, Knopf J, Naschberger E, Lindemann A, Singh J, Herrmann I, et al. Vascular occlusion by neutrophil extracellular traps in COVID-19. EbioMedicine. 2020. https://doi.org/10.1016/j.ebiom.2020.102925.
78. Veras FP, Pontelli MC, Silva CM, Toller-Kawahisa J, Ede LM, Nascimento DC, et al. SARS-CoV-2-triggered neutrophil extracellular traps mediate COVID-19 pathology. J Exp Med. 2020. https://doi.org/10.1084/jem.20201129.
79. Nicolai L, Leung A, Brambs S, Kaiser R, Weinberger T, Weigand M, et al. Immunothrombotic dysregulation in COVID-19 pneumonia is associated with respiratory failure and coagulopathy. Circulation. 2020;142:1176–89.
80. Kaiser R, Escaig R, Erber J, Nicolai L. Neutrophil-platelet interactions as novel treatment targets in cardiovascular disease. Front Cardiovasc Med. 2022. https://doi.org/10.3389/fcvm.2021.824112.
81. Martinod K, Wagner DD. Thrombosis: tangled up in NETs. Blood. 2021;132:2768–76.
82. de Bont CM, Boelens WC, Prijn GM. NETosis and neutrophil extracellular traps: a triangualar relationship. Cell Mol Immunol. 2019;16:19–27.
83. Gillot C, Favresse J, Mullier F, Lecompte T, Dognè JM, Doufflis J. NETosis and the immune system in COVID-19: mechanisms and potential treatments. Front Pharmacol. 2021. https://doi.org/10.3389/fphar.2021.708302.
84. Leffler J, Martin M, Guilstrand B, Tydén H, Lood C, Truedsson L, et al. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. J Immunol. 2020;188:3522–31.
85. Carnous L, Roumenina L, Bigot S, Brachemi S, Frémeaux-Bacchi V, Lesavre P, et al. Complement alternative pathway acts as a positive feedback amplification of neutrophil activation. Blood. 2011;117:1340–9.
86. Wang H, Wang C, Zhao MH, Chen M. Neutrophil extracellular traps can activate alternative complement pathways. Clin Exp Immunol. 2015;181:518–27.
87. O’Flynn J, Dixon KO, Faber KMC, Daha MR, Van Kooten C. Myeloperoxidase oxidasizes properdin-mediated complement activation. J Innate Immun. 2014;6:417–25.
88. Xu X, Su S, Wang X, Barnes V, De Miguel C, Owrnby D, et al. Obesity is associated with more activated neutrophils in African American male youth. Int J Obes. 2015;39:26–32.
89. Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu DJ, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res. 2019;47:D442–50.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.