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► **To cite this version:**

Marc Romana, Marjorie Leduc, Patricia Hermand, Johanna Bruce, Emilie-fleur Gautier, et al.. Proteomic analysis of neutrophils from COVID-19 patients. *British Journal of Haematology*, 2022, 10.1111/bjh.18347 . hal-03741792

HAL Id: hal-03741792

<https://hal.science/hal-03741792>

Submitted on 1 Aug 2022

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Title: Proteomic analysis of neutrophils from COVID-19 patients

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Data sharing statement: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD033293.

Word count: 1,037

Funding: This work was supported by the Fondation Paralysie Cérébrale, the DIM Thérapie Génique Ile-de-France Région, IBISA, and the Laboratory of Excellence GR-Ex (reference ANR-11-LABX-0051). The Labex GR-Ex is funded by the Investissements d'avenir program of the French National Research Agency (reference ANR-11-IDEX-0005-02).

Contributions: CLK, MR and VB designed the study. MR isolated the neutrophils and prepared the cellular extracts. ML, JB and E-F G performed the proteomic analysis. FM and VB enrolled the patients. PHT and YG performed nucleosome profile analysis. All authors discussed the data, revised and approved the manuscript.

Acknowledgments: The authors thank all the healthy donors and the patients for participating in this study. For those who lost a family member, we express our deep regret.

To the editor:

It was rapidly recognised, after the start of the SARS coronavirus 2 (SARS-CoV-2) pandemic in December 2019, that only a minority of COVID-19 patients develop severe or critical disease, while most SARS-CoV-2-infected patients are either asymptomatic or exhibit mild symptoms.¹ Severe COVID-19 patients may present life-threatening acute respiratory distress syndrome (ARDS) resulting from lower respiratory tract disease, associated with excessive inflammatory response, high pro-inflammatory cytokine production (cytokine storm), and coagulopathy.² Several comorbidities, including hypertension, diabetes, cardiovascular disease and respiratory disease, have been associated with poorer prognosis and increased mortality.³

Increasing evidence suggests that neutrophils, a principal effector of the immune defence in airway infections, may play a major role in severe COVID-19 disease pathophysiology. Indeed, increased neutrophil-to-lymphocyte ratio, in association with high D-dimer levels,¹ the presence of abnormal immature neutrophils⁴, marked infiltration of neutrophils and deposits of neutrophil extracellular traps (NETs) in the pulmonary microvasculature⁵ have been reported in patients. To gain further insight into the involvement of neutrophils in COVID-19 clinical expression, we performed quantitative proteomic analysis of neutrophils from COVID-19 patients and from two non-COVID-19 groups, composed of healthy subjects and ARDS patients hospitalized in an intensive care unit (ICU). All patients were from a homogeneous population attended at the University Hospital of Guadeloupe.

Fourteen COVID-19 patients were included in the study, 6 hospitalized in the ICU (3 of them in ARDS condition) and 8 in other hospital departments. In addition, 4 non-infected patients in ARDS and 5 healthy controls were included. Supplemental Table 1 presents a summary of clinical data. Diagnosis of SARS-CoV-2 infection was established by reverse-transcriptase polymerase chain reaction, in accordance with current standards.

Neutrophils were isolated from EDTA-blood samples, within 1 hour of sampling, using the MACSexpress whole blood neutrophil isolation kit and MACSexpress erythrocyte depletion kit (Miltenyl Biotec GmbH, Germany), according to the manufacturer's instructions. Quantitative proteomics was performed as described in Supplemental data.

Neutrophil proteomic analysis identified 6,205 proteins. Principal component analysis (PCA) (Figure 1) revealed striking differential segregation among the 3 groups (COVID-19 and non-COVID-19 ARDS patients and healthy controls), with the exception of one non-COVID-19 ARDS patient who had repeatedly tested negative for SARS-CoV-2. Importantly, no distinct cluster was observed for ICU-hospitalized COVID-19 patients, as opposed to those in other hospital departments. Moreover, no

difference was detected regarding haematological and biochemical parameters, co-morbidities and survival rate between these two groups (Supplemental Table 1). Altogether, these data strongly suggest that these patients exhibited similar clinical severity. Considering Guadeloupe as one of the major COVID-19 hot spots in France, with limited hospital capacity, we speculate that local medical support capacity was overridden. Regardless of causes, and based on medical outcomes, we grouped these COVID-19 patients for further analyses.

Cluster analysis of differentially expressed proteins in neutrophils of the three patient groups did not provide evidence of a common biological pathway for down-regulated proteins in COVID-19 patients (*data not shown*). A 2D enrichment analysis test using GO, KEGG and keyword annotation databases⁶ using (PMID: 23176165) identified several pathways for up-regulated proteins in the COVID-19 patient group, compared to the two others. Indeed, neutrophil proteins from COVID-19 patients were significantly enriched for biological processes associated with type I interferon (IFN) signalling, antigen processing and presentation, and neutrophil aggregation, as shown in Figure 2, panels A, B and C respectively. In addition, we detected higher levels of proteins involved in chromosome condensation and segregation belonging to the condensin and the structural maintenance of chromosome (SMC) protein complexes in COVID-19 patients and non-COVID-19 ARDS patients, compared to healthy controls (Figure 2D).

Activation of the type I IFN pathway in the COVID-19 group is demonstrated by the high expression level of major Interferon signalling proteins (ISP) such as IFIT1, IFIT2, IFIT3, OAS1, OAS2 and OAS3, confirming previous observations of the significance of this pathway in the innate immune response during viral infection. Interestingly, we also detected an increased level of Transporter associated with Antigen Processing (TAP) proteins, known to be up-regulated by IFN and involved in antigen transport into the endoplasmic reticulum for loading onto class I major histocompatibility complex molecules. Although originally thought to be protective, these new data suggest a more balanced impact of the type I IFN pathway on the COVID-19 clinical course. While mutated proteins involved in the type I IFN pathway⁷ or production of anti-IFN-I autoantibodies⁸ have been linked to severe forms of COVID-19, an early IFN-I production was recently shown to be protective, whereas a late and intense IFN-I response leads to an exacerbated and deleterious pro-inflammatory state.⁹ As regards the potential therapeutic use of IFN-I in COVID-19 patients, further studies are warranted to better describe the activation time course of this pathway.

Consistent with our results, neutrophils from COVID-19 patients reportedly exhibit an increase in proteins associated with fibrin clot formation, such as fibrinogen alpha and fibrinogen beta,⁵ and display augmented aggregate formation between neutrophils and platelets. Such aggregates could

directly contribute to vascular damage by impairing neutrophil transmigration and promoting microthrombi formation in patients' lung vasculature.

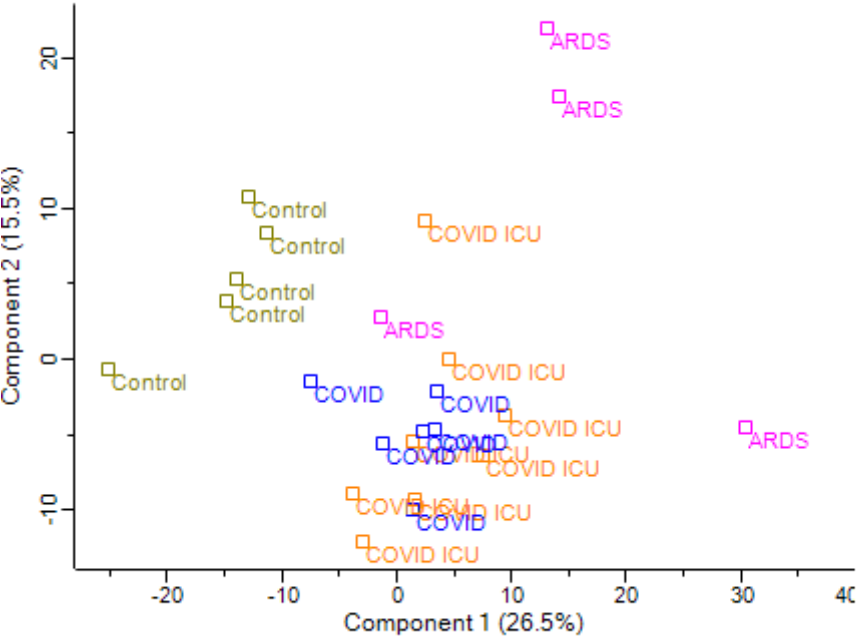
The abnormal expression of proteins involved in chromosome condensation and segregation observed in neutrophils of COVID-19 and non-COVID-19 patients with ARDS was puzzling, as dysregulation of these proteins in terminally differentiated cells is unexpected; however, two studies have previously reported the presence of a low-density inflammatory neutrophil (LDN) population in patients with these two conditions.^{5,10} LDNs, considered as immature neutrophils, exhibit several abnormal properties, including abundant spontaneous formation of NETs.¹⁰ It may be postulated that this high expression level of proteins regulating the organisation of genetic material could be linked to the unravelling of DNA during NET formation. We determined the plasma concentration of nucleosome, a known marker of NETosis, in 13 controls, 33 COVID-19 patients and 4 ARDS patients. As shown in Figure 2E, higher nucleosome concentrations were detected in COVID-19 and in non-COVID-19 patients with ARDS, compared to controls. Further experiments are clearly warranted to confirm these relationships and to decipher the molecular mechanism involved.

In conclusion, although our patient populations were small, our quantitative proteomic study of neutrophils confirms the activation of the type I IFN pathway and identifies targets of IFN, such as TAP proteins, specifically in COVID-19 patients, but not in non-COVID-19 ARDS patients, and describes modifications of the neutrophil proteome potentially associated with ARDS.

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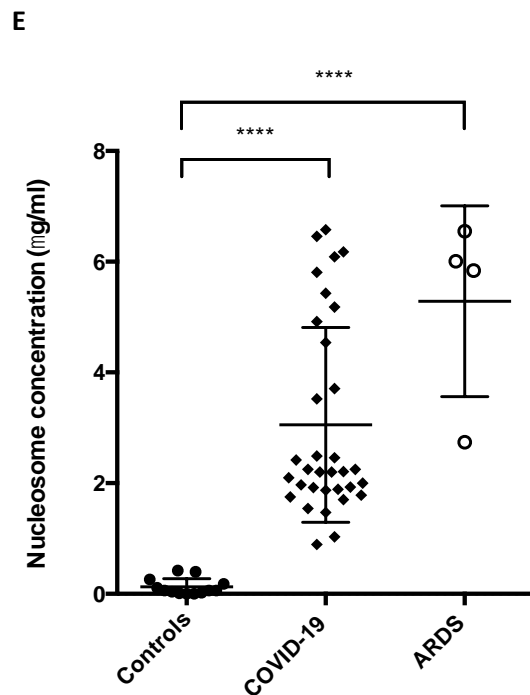
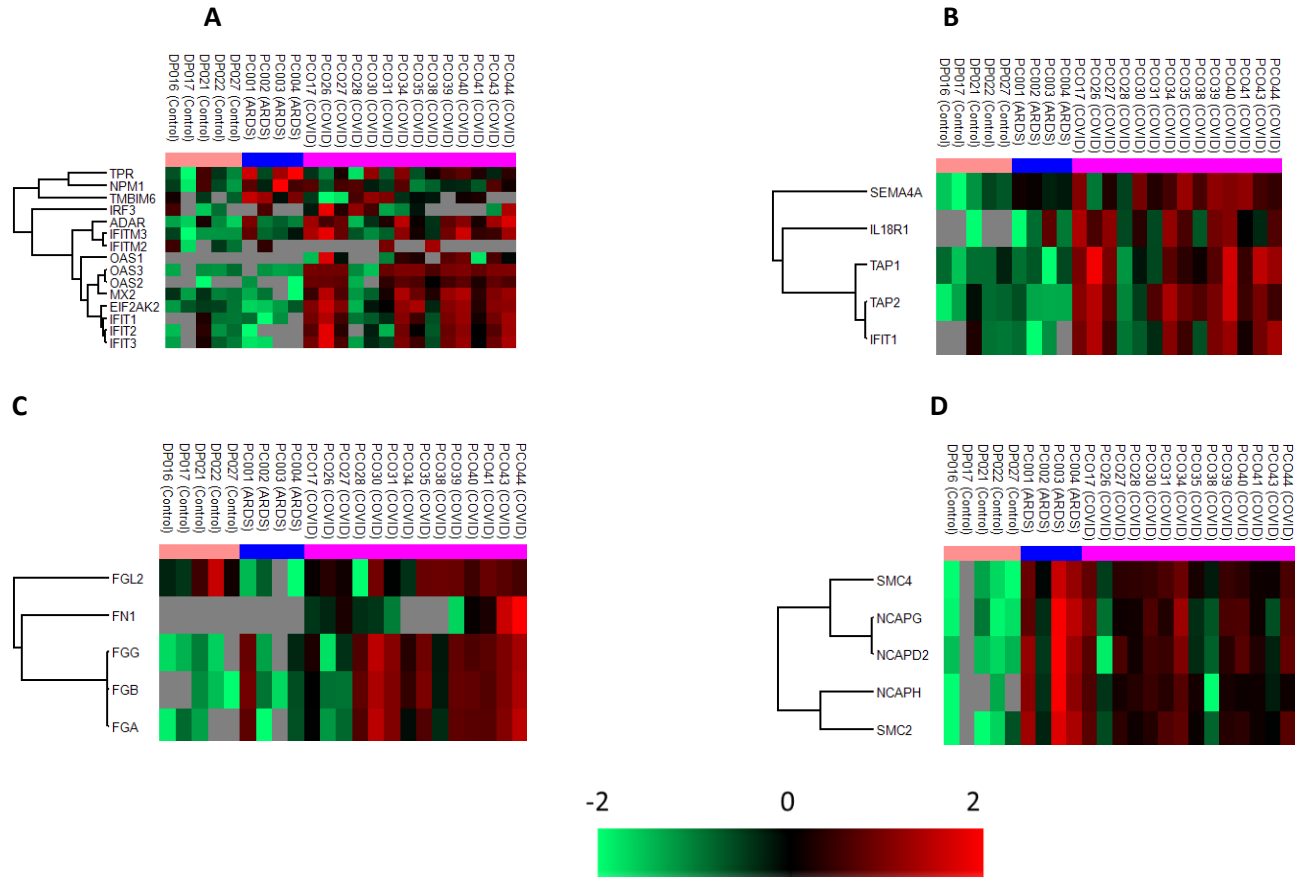
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Figure 1: Principal component analysis of log2 (label-free quantification intensity) of the neutrophil proteome



Overall proteomic analysis of neutrophils isolated from healthy controls (controls), non-COVID-19 ARDS patients (ARDS), COVID-19 patients hospitalized in the ICU (COVID-ICU) and COVID-19 patients hospitalized in other medical departments (COVID).

Figure 2: Cluster analysis of Z-score (label-free quantification intensity) of differentially enriched proteins between COVID-19 patients, non-COVID-19 ARDS patients (ARDS) and healthy controls: type I interferon (IFN) signalling (panel 2A); antigen processing and presentation (panel 2B); neutrophil aggregation (panel 2C); chromosome condensation and segregation (Panel 2D) and intergroup comparison of nucleosome concentrations (panel 2E)



2A to 2D: Heatmaps for proteins overexpressed in the neutrophils of COVID-19 patients, as highlighted by 2D enrichment analysis. Annotations out of the diagonal corresponding to differential expression in healthy controls (controls), non-COVID-19 ARDS patients (ARDS) or COVID-19 patients (COVID) are indicated. The colour scale indicates the level of protein expression.

2E: Plasma nucleosome concentrations in the 3 groups controls, COVID-19 patients and ARDS patients. ****: $P < 0.0001$.

Supplementary data

Patients and controls

All patients and healthy controls included in the present study provided informed written consent in accordance with the regional ethic committee (CPP Sud/Ouest-Mer III, Bordeaux, France, registration number: 2020-401908-31). Their demographical and clinical characteristics are shown and compared in the following Table (Sup Table 1).

Sup Table 1. Demographical and clinical characteristics of studied patients

	ICU COVID-19			
n				
Age, median (IQR)				
Female/Male				
Hospital duration, median (IQR)				
Survival to date. n				
Leukocytes ($10^9/L$)				
$10^9/L$				
$10^9/L$				
$10^9/L$				
$10^9/L$				

Kruskal-Wallis test followed by Dunn’s multiple comparisons test

Quantitative proteomic analysis of neutrophils

Eluted peptides, obtained from 50 micrograms of lysed neutrophils, were fractionated using strong cation exchange (SCX) StageTips in 5 fractions as previously described (Kulak N A et al., 2014) and vacuum-dried while centrifuging in a Speed Vac (Eppendorf). Mass spectrometry (MS) analyses were then performed on a Dionex U3000 RSLC nano-LC system (Thermo Fisher Scientific) coupled to a

TIMS TOF Pro mass spectrometer (Bruker Daltonik GmbH). Peptides from each SCX fraction were solubilized in 0.1% trifluoroacetic acid containing 10% acetonitrile and separated on an Aurora C18 column (1.6-mm particle size, 75-mm inner diameter, 25-cm length) connected with a Captive nanoSpray Ionization module (IonOpticks). The MS data were analysed using MaxQuant software version 1.6.17.0 (Cox J et al., 2009), using the Uniprot-Swissprot reviewed database (Uniprot-Swissprot, release 2020-03) and a protein false discovery rate of 0.01. Functional analyses of data from MaxQuant were done with Perseus software (Tyanova S et al., 2016).

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Quantification of plasma nucleosomes

Plasma was collected from 13 controls, 33 COVID-19 patients and 4 ARDS Non COVID-19 patients and stored at -80°C until nucleosome quantification. All samples were performed in duplicate and diluted 4-fold except for controls. Plasma nucleosome levels were evaluated using NU.QTM H3.1 Assay Kit (ACTIVE MOTIF Waterloo, Belgium) according to the manufacturer's instructions.