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Ruggeri T, De Wit Y, Schärz N, van Mierlo G, Angelillo-Scherrer A, Brodard J, Schefold JC, Hirzel C, Jongerius I, Zeerleder S

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Immunothrombosis and complement activation contribute to disease severity and adverse outcome in COVID-19

Tiphaine Ruggeri[1,2*], Yasmin De Wit[3*], Noëlia Schärz[1,2], Gerard van Mierlo[3], Anne Angelillo-Scherrer[1,2], Justine Brodard[1], Joerg C. Schefold[4], Cédric Hirzel[5#], Ilse Jongerius[3#], and Sacha Zeerleder[6#]

[1]Department of Hematology and Central Hematology Laboratory, Inselspital, Bern University Hospital, Bern, Switzerland
[2]Department for BioMedical Research, University of Bern, Bern, Switzerland
[3]Department of Immunopathology, Sanquin Research, Amsterdam, the Netherlands
[4]Department of Intensive Care Medicine, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland
[5]Department of Infectious Diseases, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland
[6]Department of Hematology, Kantonsspital Luzern, Lucerne and University of Bern, Switzerland
* Both authors contributed equally to this work
[#] Shared last authorship
Short title: Innate immunity in COVID-19
Corresponding Author: Sacha Zeerleder, MD, PhD
Departement of Hematology and University of Bern
Luzerner Kantonsspital
Spitalstrasse
CH-6006 Luzern
Switzerland
Phone: +41 41 2055147
E-mail: sacha.zeerleder@luks.ch

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Abstract
Severe COVID-19 is characterized by systemic inflammation and multiple organ dysfunction syndrome (MODS). Arterial and venous thrombosis are involved in the pathogenesis of MODS and fatality in COVID-19. There is evidence that complement - and neutrophil activation in the form of neutrophil extracellular traps are main drivers for development of microvascular complications in COVID-19. Plasma and serum samples were collected from 83 patients infected by SARS-CoV-2 during the two first waves of COVID-19, before the availability of SARS-CoV-2 vaccination. Samples were collected at enrollment, day 11, and day 28; and patients had differing severity of disease. In this comprehensive study, we measured cell-free DNA, neutrophil activation, deoxyribonuclease 1 activity, complement activation, and D-dimers in longitudinal samples of COVID-19 patients. We show that all the above markers, except deoxyribonuclease 1 activity, increased with disease severity. Moreover, we provide evidence that in severe disease there is continued neutrophil and complement activation, as well as D-dimer formation and nucleosome release, whereas in mild and moderate disease all these markers decrease over time. These findings suggest that neutrophil and complement activation are important drivers of microvascular complications and that they reflect immunothrombosis in these patients. Neutrophil activation, complement activation, cell-free DNA and D-dimer levels have the potential to serve as reliable biomarkers for disease severity and fatality in COVID-19. They might also serve as suitable markers with which to monitor the efficacy of therapeutic interventions in COVID-19.

INTRODUCTION

Infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease 2019 (COVID-19). The disease presents with flu-like symptoms and viral pneumonia, which may progress to acute respiratory distress syndrome (ARDS) and occasionally to multiple organ dysfunction syndrome (MODS) [1]. Arterial and venous thrombosis contribute to ARDS, MODS, and fatality in COVID-19 [2-4]. Before the availability of SARS-CoV-2 vaccines, specific anti-viral drugs and therapeutic neutralizing monoclonal antibodies, 14.5% of COVID-19 patients developed severe systemic inflammation with subsequent organ failure despite virus clearance [1]. Based on postmortem analysis, microvascular complications are a main driver of the pathophysiology of organ dysfunction in COVID-19 [5,6]. This is illustrated by the finding that SARS-CoV-2 may directly invade endothelial cells, with subsequent lysis, and induce microthrombosis, especially in the lungs [7-9]. Other studies have linked systemic complement activation in COVID-19 patients to respiratory failure [10] and described this as a distinct feature of COVID-19 [11]. Furthermore, uncontrolled complement activation at the level of C3 has been shown to correlate with disease severity and mortality [12,13]. Indeed, postmortem analyses of lung specimens of patients who died from COVID-19 demonstrate deposition of MASP-2, C4d, C3d, and C5b9 in the intra-alveolar septa and in the microvasculature, consistent with the activation of the lectin pathway and alternative pathway, respectively [14]. Interestingly, these patients' biopsy specimens of purpuric lesions showed thrombogenic vasculopathy with deposition of C4d and C5b9 [14].

High neutrophil:lymphocyte ratio and D-dimer levels has been suggested to predict death in patients with severe COVID-19 [15]. In addition, SARS-CoV-2 infection induces neutrophil influx in the nasopharyngeal epithelium and the lungs and increases their count in peripheral blood [16-18]. These data suggest a prominent role for neutrophils in the pathogenesis of microvascular complication with subsequent organ dysfunction. Indeed, neutrophil activation in the form of neutrophil extracellular traps (NETs) plays an important role in the pathogenesis of microvascular complications, since NETs are a main driver of immunothrombosis [19,20]. NETs are present in obstructed vessels in lungs from autopsies of COVID-19 patients studied by immunohistochemistry [21]. In addition, elevated markers of NETs can be detected in the plasma of COVID-19 patients, and these markers significantly correlate with D-dimers, pointing to a role for NETosis in the pathogenesis of ARDS and thrombosis [22,23]. Markers for NETs in COVID-19 patients increase with disease severity [24] and are involved in several affected organs [25]. Interestingly, C3-deficient mice infected with SARS-CoV have less respiratory distress, with a significant attenuation of the inflammatory response and less neutrophil infiltration, emphasizing not only the role of complement and neutrophils in COVID-19, but also linking complement activation to neutrophil activation [26]. NET degradation by deoxyribonuclease I (DNase 1) is an essential step in maintaining microvascular patency during inflammation [27-29]. Compared with healthy controls, DNase 1 activity was found to be significantly increased in COVID-19 patients; nevertheless, COVID-19 patients with sepsis had decreased DNase 1 activity compared with healthy volunteers. In the same study, COVID-19 patients' plasma cell-free DNA levels and NET formation were reduced by adding DNase 1 [30]. Besides the fact that elevated cfDNA in combination with markers for neutrophil activation points to NET formation, cfDNA in the form of nucleosomes as a surrogate marker for systemic inflammation, e.g. predicting severity and fatality in sepsis [31, 32]. CfDNA in the form of nucleosomes is the product of chromatin degradation by endonucleases and consists of an octamer of two copies of each of the four core histones (H2A, H2B, H3, and H4) wrapped by 145–147 base pairs of helical DNA [33]. CfDNA in the form of mtDNA, is released by mitochondria upon cell death and/or cell stress has been demonstrated to be increased in sepsis patients being highest in non-survivors [34].

The strength of our current study is that we comprehensively investigate the role of complement, neutrophil and coagulation activation in the pathogenesis of systemic inflammation in a unique cohort of COVID-19 patients included during the first two waves of COVID-19 before any vaccination was available. In detail, we studied the main drivers of the innate immune response involved in the development of microvascular complication in longitudinal samples with these markers: neutrophil activation in the form of NETs, DNase 1 activity, cell-free DNA (e.g. nucleosome and mitochondrial DNA forms), complement activation and D-dimer levels.

Our group identified cfDNA in the form of nucleosomes as a surrogate marker for systemic inflammation, predicting severity and fatality in sepsis [31, 32]. Analogously, nucleosome levels increased with disease severity in COVID-19 patients. CfDNA in the form of nucleosomes is the product of chromatin degradation by endonucleases and consists of an octamer of two copies of each of the four core histones (H2A, H2B, H3, and H4) wrapped by 145–147 base pairs of helical DNA. Our findings are in line with other studies demonstrating that cfDNA is a potentially useful marker to monitor COVID-19 progression and severity [35-38].

METHODS

Study Design and Participants

This study was a subproject of a larger COVID-19 cohort study (NCT04510012) aimed to characterize the immune response of SARS-CoV-2 infected individuals. Symptomatic patients with PCR-confirmed SARS-CoV-2 infection in the two first waves of COVID-19 were prospectively included from March 5, 2020, to December 15, 2020, at the Inselspital in Bern, Switzerland. Healthy controls were enrolled at the blood donation center SRK in Bern (projects P357). The blood bank physician assessed their health status. Individuals not feeling well or being in close contact with SARS-CoV-2 infected persons within five days before the intended blood donation were neither accepted as blood donors nor as healthy controls. The same was true for individuals who had confirmed COVID-19 or symptoms compatible with a SARS-CoV-2 infection within the previous two weeks. For legal reasons, we were not allowed to record demographic information of healthy blood donors.

COVID-19 disease severity was categorized according to the *COVID-19 WHO Ordinal Scale for Clinical Improvement* (WHO COVID-19 synopsis 2020) [39]. Patients with a score of 1–2 (outpatients) were defined as having mild disease, whereas patients with scores of 3–4 (admitted patients with or without oxygen by mask of nasal prongs) and 5–8 (non-invasive ventilation, high-flow oxygen, mechanical ventilation, extracorporeal membrane oxygenation, death) were defined as having moderate or severe disease, respectively. The time of COVID-19 symptom onset was self-reported and details about symptoms were collected using a standardized case report form. Some data of the COVID-19 project (NCT04510012) regarding other aspects of immune functionality and neuro-axonal damage were previously published [40,41]. The study was approved by the Ethics Committee of the Canton of Bern, Bern, Switzerland, Nr. 2020-00877 and registered at clinicaltrials.gov (NCT04510012). Patients were included after provision of informed consent. In case of lack of capacity and/or inability to provide consent, enrollment followed the procedures for research projects in emergency situations according to Swiss law.

Blood Collection from patients with COVID-19

According to the initial study protocol, serum samples (S-Monovette® serum tubes 9 mL, Sarstedt, Germany) were collected at the time of enrollment (baseline) and at day 28 ± 7 (D28). During the ongoing study, the protocol was amended, and we additionally collected EDTA (S-monovette® 2.7 mL tubes, Sarstedt, Germany) and citrate plasma samples (S-monovette® 3 mL tubes, Sarstedt, Germany) at baseline, day 11 ± 3 (D11) and at D28 using a butterfly needle.

Serum samples were left at room temperature for 30 min for complete coagulation. The tubes were then centrifuged at 2000g for 10 min at room temperature to collect the serum. EDTA tubes were centrifuged once and citrate tubes were centrifuged twice for 15 minutes at 2500g. Serum and EDTA / citrate plasma was stored in aliquots at -70°C.

Blood Collection from healthy donors

Serum of 29 healthy donors for measurement of DNase I activity was collected in S-Monovette® serum tubes (9 mL, Sarstedt, Germany) or vacuette® tubes (9 mL tubes, Greiner Bio-One, Switzerland) and left at room temperature for 30 min for complete coagulation. The tubes were then centrifuged at 2000g for 10 min at room temperature to collect the serum. EDTA plasma of another 24 blood donors was drawn using K3 EDTA vacuette® tubes (4 mL tubes, Greiner Bio-One, Switzerland) and used to measure nucleosome, elastase–α1-antitrypsin complexes and mitochondrial DNA levels. A subset of 15 EDTA plasma donors also donated citrate plasma, which was drawn using vacuette® citrate tubes (3.5 mL tubes, citrate 3.2%, Greiner Bio-One, Switzerland).

The preferred blood draw system at the blood donation center, where healthy donors were recruited, are vacutainers (vacuette® Greiner Bio-One, Switzerland). Since blood collection using vacutainer systems may activate complement (suppl. Fig. 1), additional 14 blood donors were recruited to donate EDTA blood drawn in a S-monovette® tube (2.7 mL tubes, Sarstedt, Germany) using a butterfly needle. These samples were used to measure complement activation products C3b/c and C4b/c.

Enzyme-linked immunosorbent assay for activated C3 and C4

Activated C3 and C4 was determined by detection of complement activation products C3b/c and C4b/c as described previously, using a sandwich ELISA with EDTA samples [42,43].

Quantification of mitochondrial DNA

Mitochondrial DNA (mtDNA) was purified from EDTA plasma using a QIAamp DSP Virus kit (Qiagen). Patient DNA samples were subsequently diluted (1:5) in DNase-free water. A digital droplet PCR (ddPCR) was performed according to the manufacturer's instructions: the ddPCR system included an automated droplet generator and

reader from Bio-Rad, (QX200 Droplet Digital PCR, Bio-Rad, Hercules, California, USA) and a T100 thermal cycler (Bio-Rad). For mtDNA quantification, primers and probes targeting the mitochondrial DNA encoded NADH dehydrogenase 1 (MT-ND1), NADH dehydrogenase 1 (ND1), Human (FAM) (Bio-Rad, unique assay ID dHsaCNS669425578) were used. Results were analyzed using QuantaSoft software (Bio-Rad), and absolute values of mtDNA (ND1) (copies/µL) were calculated for each DNA sample.

Nucleosome measurement

Nucleosome levels were determined using EDTA plasma with an ELISA as previously described [44]. Briefly, ELISA plates were coated with monoclonal anti–histone H3 antibody (CLB/ANA-60) and the samples were added and incubated for 1 hour at room temperature. After washing, biotin-labelled F(ab')2 fragments of monoclonal anti-nucleosome antibody (CLB/ANA-58) were added and incubated for another hour at room temperature. Binding of biotin-labelled antibodies was detected with streptavidin–horseradish peroxidase using tetramethylbenzidine as a substrate. The reaction was stopped with 2 M H_2SO_4 and the absorbance was measured at 450 nm.

Neutrophil activation

Elastase–α_1-antitrypsin complexes were measured in EDTA plasma by ELISA as described [44].

DNase activity

DNase I activity was quantified in serum with a Quant-iT PicoGreen® dsDNA Assay Kit (Invitrogen). Serum samples were diluted 1:10 and incubated with TE buffer (10 mM Tris-HCL in UltraPure DNase/RNase-free distilled water) without or with 10 µL of double-stranded calf thymus DNA solution (Invitrogen, ref 15633-019) at 5 µg/mL final concentration in a 96-well plate. The wells with plasma samples without added DNA provided the background signal. For the standard curve, different DNase I (#EN0521, Thermo Scientific) concentrations were used and added to wells containing only TE buffer and dsDNA. The total volume was adjusted to 100 µL before incubation. The plates were incubated for 15 min at 37°C. Samples were stained with PicoGreen according to the manufacturer's instructions. After 2 to 5 minutes, the reduction in PicoGreen staining by fluorescence was measured using TECAN. Before using samples from our cohort, we assessed our assay of DNase I activity (suppl. Fig. 2).

D-dimer measurement

D-dimer levels were measured in citrate plasma using immunoturbidimetry with INNOVANCE® (Siemens) Sysmex CS-S100 (Sysmex Europe GmbH, Germany). Polystyrene particles covalently was coated with a monoclonal antibody (8D3) then samples were added. The cross-linking region of the D-dimers has a symmetrical structure, which means that the epitope for the monoclonal antibody is present twice. Therefore, a single antibody is sufficient to trigger an aggregation reaction, which is detected by an increase in turbidity. The lower limit of detection of the assay used is 155 µg/L. For statistical analysis, D-dimer values below the limit of detection were assigned to 155 µg/L. Values below 500 µg/L allow acute venous thromboembolism to be ruled out with high specificity [45].

Statistical Analysis

All statistical analyses and figures were computed with GraphPad Prism software version 9.3 (GraphPad Software, La Jolla, CA, USA). Results are indicated as median ± interquartile range. For the same patients at different time points, statistical significance was determined by using the Wilcoxon test. For all other experiments, statistical significance was determined by using the non-parametric Mann-Whitney test to compare two groups, or the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data are presented as median with interquartile range. Correlation has been calculated using Spearman rank testing. Statistical significance was defined as $p < 0.05$.

RESULTS

Study population

We enrolled eighty-seven SARS-CoV-2 infected patients in the overall cohort at the Inselspital in Bern. Eighty-three patients, who had any type of required blood samples available, were included in the present study. Serum samples (for DNase I activity) were available for 72/83 patients; EDTA (for activated C3 and C4, nucleosome, Elastase–α1-antitrypsin complexes and mitochondrial DNA levels) and citrate plasma samples (D-dimer) were available for 37/83 patients. A subset of 26 individuals had a complete set of blood samples (serum, EDTA plasma and citrate plasma; suppl. Fig. 3).

The median age of patients with COVID-19 was 52 years (range 22 to 86 years), 55% of patients were older than 50 years, and 69% were males (Table 1). The most common symptoms at enrollment were cough (76%), fever (58%), and myalgia (57%). Thirty-five percent (n=29) of the patients suffered from mild, 27% (n=22) from moderate, and 39% (n=32) from severe disease. Among the patients suffering from severe disease, 78% of patients were more than 50 years old (median age 64 years). Among the patients suffering from severe disease who were older than 50 years old, 56% had at least two comorbidities, including mainly hypertension, cardiovascular disease, or diabetes. Ten patients died during the study.

Nucleosome levels, neutrophil activation, mtDNA, and DNase 1 activity in COVID-19 patients

In the subset of patients with available EDTA and citrated plasma (37/83), we longitudinally measured nucleosome levels, neutrophil activation and mtDNA at baseline (36/83; one patient with samples for D11 and D28 had a missing baseline sample), D11 (25/83) and D28 (29/83). Nucleosome levels in patients with COVID-19 were significantly higher than those in healthy controls (Fig. 1a). In patients with mild disease, the nucleosome levels were not significantly different from those in controls (Fig. 1b). In contrast, in patients with moderate and severe disease, nucleosome levels were significantly higher than in controls. In patients with moderate disease, nucleosome level significantly declined over time. In contrast, in patients with severe disease, nucleosome levels remained high after 28 days and were highest in non-survivors (suppl. Fig. 4).

Next, we measured neutrophil activation as evidenced by elastase–α1-antitrypsin complex (EA) levels in our study patients (Fig. 1c,d). Overall, patients with COVID-19 had significantly higher EA levels than controls. In patients with mild disease, EA levels were similar to healthy controls and remained unchanged over time. At baseline, in patients with moderate and severe disease, EA levels were significantly higher than they were in controls. Whereas EA levels significantly decreased in patients with moderate disease, in patients with severe disease the levels remained high over time. There was a strong and significant correlation between nucleosome and EA levels (Fig. 1e).

Using a specific ddPCR, mtDNA was measured in 34/83 COVID-19 patients and in 20 controls (Fig. 2a,b). For three COVID-19 patients and four controls for whom EDTA plasma samples were drawn, there was not sufficient plasma for DNA extraction. Interestingly, mtDNA was significantly lower in patients with mild disease as compared with controls. In severe disease, mtDNA was significantly higher at the baseline and after 11 days than it was in the controls. We found a strong and significant correlation between neutrophil activation and the concentration of mtDNA (Fig. 1f).

Finally, DNase I activity was assessed longitudinally in 72/83 COVID-19 patients with available serum samples and in 29 healthy controls. DNase I activity was significantly higher in patients with COVID-19 as compared with the controls (Fig. 3a). DNase I activity decreased over time in patients with COVID-19 (Fig. 3b) and patients with severe disease had higher DNase I activity in comparison with the controls (baseline vs D28; Fig. 3c). In COVID-19 patients, DNase I activity was correlated with nucleosome level (Fig. 3d).

Levels for nucleosomes, elastase–α1-antitrypsin complexes, mtDNA and DNase I in non-survivors and survivors are shown in supplementary figure 5. Longitudinal levels for nucleosomes, elastase–α1-antitrypsin complexes, mtDNA and DNase I activity of ventilated vs. non-ventilated COVID-19 patients and for patients with vs. without corticosteroid therapy are provided in supplementary Figures 6 and 7.

Complement activation in COVID-19 patients

We longitudinally measured complement activation products of C3 and C4 in 37/83 COVID-19 patients with available EDTA plasma and in 14 healthy controls. In one COVID-19 patient, blood samples were not collected at the baseline, but at D11 and D28, respectively. Overall, C3b/c and C4b/c levels were significantly higher in COVID-19 patients than controls (Fig. 4a, c). At baseline, C3b/c and C4b/c concentrations were increased in all patients. However, in mild and moderate diseases, C3b/c levels decreased over time, whereas in severe disease the levels remained high (Fig. 4b). In C4b/c levels, such a decrease was observed exclusively in patients with mild disease

(baseline vs D11). Interestingly, in moderate and severe cases, C4b/c levels increased over time (baseline vs D11). Then in moderate disease, C4b/c levels had a tendency to decrease between D11 and D28 but remained higher than healthy controls (baseline vs D28). Whereas, in severe cases the C4b/c concentration remained high at D28 (Fig. 4d).
Complement activation in non-survivors was as compared to survivors (suppl. Fig. 5). Longitudinal complement activation products of ventilated vs. non-ventilated COVID-19 patients and for patients with vs. without corticosteroid therapy are provided in supplementary Figures 6 and 7.

D-dimer production in COVID-19 patients

In the subset of patients with available citrate plasma samples (37/83) we measured D-dimer levels at baseline (36/83; one patient with samples for D11 and D28 had a missing baseline sample), D11 (25/83) and D28 (27/83; Fig. 5). D-dimer levels in patients with COVID-19 were significantly higher as compared with controls (Fig. 5a). In most patients with mild disease, D-dimer levels were below the detection limit (<155 µg/L). Nevertheless, in both moderate and severe disease, D-dimer levels were significantly higher than controls. In moderate disease, the D-dimer level increased until D11, and by D28 we observed a downward trend. In severe disease, D-dimer levels increased significantly between baseline and D11, and remained high at D28.
D-Dimer levels were significantly higher in non-survivors as compared to survivors on admission and D11, respectively (suppl. Fig. 5). Longitudinal D-dimer levels of ventilated vs. non-ventilated COVID-19 patients and for patients with vs. without corticosteroid therapy are provided in supplementary Figures 6 and 7.

DISCUSSION

COVID-19 is a disease characterized by systemic inflammation, as evidenced by complement activation, neutrophil activation, and the release of cfDNA. Markers for complement and neutrophil activation, as well as cfDNA, increase with disease severity in systemic inflammation, and have been proven to be reliable markers for disease severity in sepsis [31, 32, 46]. In this comprehensive study, we measured cfDNA (in the form of nucleosomes and mtDNA), neutrophil activation (in the form of EA), DNase I activity, complement activation (C3bc, C4bc), and D-dimers in longitudinal samples of COVID-19 patients having different disease severities (ranging from mild to severe). We showed that neutrophil activation, complement activation, cfDNA, and D-dimer levels increased with disease severity. Moreover, we have provided evidence that in severe disease there is ongoing neutrophil and nucleosome release as well as complement activation, and D-dimer formation, whereas in mild and moderate disease all these variables of immunothrombosis decrease over time.

Activation of the complement system is a well-recognized process of the innate immune response during sepsis [47-49]. C3a and C4a levels are increased in patients with sepsis and septic shock, and plasma concentrations of C3a correlate with mortality rate [50]. In this context it is important to acknowledge that increased C3 antigen levels in young COVID-19 patients is associated with poor outcome [51]. The circulating levels of C3b/c and C4b/c were significantly elevated in patients of our cohort with COVID-19 and were associated with disease severity. These findings are in line with the publication of Jarlhelt et al, who reported classical complement pathway activation in COVID-19 patients [52]. These results are coherent with a recent study that reported significantly increased levels of soluble C5b9 in patients with moderate and severe COVID-19 as compared with healthy controls [53]. Another study reported an association between mortality and the level of overactivation of C3, as evidenced by the C3a:C3 ratio, pointing to a possible central role of C3 [13]. In contrast, autopsy specimens of lung tissue from deceased COVID-19 patients showed strong deposition of MASP-2 but only weak C1q deposition, suggesting a prominent role of the lectin pathway in COVID-19 [54].

Our group identified cfDNA in the form of nucleosomes as a surrogate marker for systemic inflammation, predicting severity and fatality in sepsis [31, 32]. Analogously, nucleosome levels increased with disease severity in COVID-19 patients. CfDNA in the form of nucleosomes is the product of chromatin degradation by endonucleases and consists of an octamer of two copies of each of the four core histones (H2A, H2B, H3, and H4) wrapped by 145–147 base pairs of helical DNA. Our findings are in line with other studies demonstrating that cfDNA is a potentially useful marker to monitor COVID-19 progression and severity [35-38].

In our cohort of COVID-19 patients, neutrophil activation increased with disease severity and decreased during disease resolution. This is in analogy to sepsis, where neutrophil activation is a reliable surrogate marker of severity and fatality [2]. Neutrophil infiltration in the inflamed lung is a hallmark of ARDS [3]. Indeed, studies have reported increased numbers of neutrophils in patients with severe COVID-19 and in COVID-19 patients who died [55]. In addition, high neutrophil infiltration in pulmonary capillaries and extravasation into the alveolar space characterized by neutrophil mucositis of the trachea and fibrin deposition were observed in autopsy specimen of the lungs of COVID-19 patients [17].

Microvascular complications in systemic inflammation are a result of immunothrombosis. Neutrophil activation in the form of NETs has a central role in the pathogenesis of immunothrombosis [19, 20]. In our patients, we demonstrated that neutrophil activation, nucleosome levels, and mtDNA increase with disease severity. The strong correlation between neutrophil activation and cfDNA in the form of nucleosomes and mtDNA may suggest the presence of neutrophil activation in the form of NETs. Several studies report a role of NETs in the pathogenesis of COVID-19 and have demonstrated high levels of NETs as evidenced by circulating markers for NETs to be associated with thrombotic complications [37, 56-59]. One has to keep in mind that measurement of citrullinated histone 3–DNA or elastase–DNA complexes might be troublesome, since in vitro formation of these complexes after sampling cannot be excluded. To circumvent this issue, we assessed neutrophil activation using an elastase-complex assay as well as an assay for nucleosomes. Recent data show that the release of cfDNA early in inflammation occurs from hematopoietic cells and that only in later stages do parenchymal cells also release cfDNA [60]. Therefore, we also cannot rule out that the release of nucleosomes is caused by cell damage or by activated neutrophils.

NET degradation by deoxyribonuclease I (DNase1) is essential in maintaining microvascular patency [28]. Interestingly, we found increased DNase I activity in all COVID-19 patients, independent of disease severity, as compared with healthy controls. In contrast, a previous study reported increased DNase activity in COVID-19 patients, but only in severe forms of COVID-19 [30]. In a recent study, despite high levels of DNase activity, the

impaired DNase was not enough efficient in NET degradation contributing to disease severity, and was more associated to the elderly male patients [61]. The results of our study imply that even though DNase I activity is increased in COVID-19 patients, the endogenous DNase I activity might still be relatively too low in individuals with severe disease. One may speculate that in mild disease, increased DNase I activity ensures microvascular patency, but that DNase activity reaches a maximum in moderate to severe disease, with a relative DNase I insufficiency resulting in microvascular thrombosis. Therefore, inhaled DNase in severe COVID-19 might be an attractive therapeutic option for investigation in clinical trials [62, 63]. Unfortunately, we could not follow the dynamics of DNase activity as no serum from day 11 was available.

Elevated concentrations of D-dimers are associated with inflammation, organ injury, and poor outcome in SARS-CoV-2 infection [64, 65]. Our findings corroborate the results of other groups, who have reported that D-dimer levels are associated with disease severity in COVID-19 [66]. We observed a similar pattern of increasing levels of D-dimer, neutrophil activation, and complement activation, which are soluble markers of immunothrombosis, correlating with disease severity. Altogether, these results point to immunothrombosis as a main driver of microvascular complication in COVID-19.

To the best of our knowledge, this is the first study that measured the dynamic in timeline of all biomarkers of the crosstalk between neutrophil activation, complement system and thrombosis in patients with mild, moderate or severe disease of COVID-19. However, we also have to point out the limitations of our study. The patients were included in the first and second COVID-19-waves, before large parts of the population had either vaccine- or infection-elicited adaptive immunity to SARS-CoV-2. Even though pre-existing adaptive humoral and cell-mediated immunity to SARS-CoV-2 may not uniformly protect from re-infection, it is likely that it affects the extent of the innate immune response, such as complement activation, NETosis and immunothrombosis. Therefore, our findings may not directly translate to a pre-immunized population. As evidenced in table 1 the median age in patients with mild disease is lower as compared to the age of patients with moderate and severe disease, respectively. The same holds for the comorbidities being present in lower proportion in the mild group as compared to the moderate and severe group. This points to the findings that age as well as comorbidities and male sex, at least in the period before vaccination was available, went hand in hand with worse prognosis [67-69]. Given the multicollinearity of independent variables (age, sex, comorbidities) we did not perform a regression model to predict age-, sex-, and comorbidity adjusted values of the assessed biomarkers. In addition, the number of individuals in the groups of different disease severity as well as the healthy controls are rather small and neither age and gender in the control group. We did neither systematically screen for immunothrombotic pathologies in our cohort nor did we perform autopsies. Therefore, we cannot present data on microthrombotic or macrothrombotic pathologies. Finally, we exclusively focused on the innate immune axis. We acknowledge that the adaptive immune response may also play a role in pathogenesis of thromboembolic events in COVID-19. Another shortcoming of our study is the relatively low number of samples in the subgroups which may hamper proper statistical analysis. Since vacuum-based blood collection may induce complement activation we used aspiration-based blood collection for complement measurements in order to rule out collection bias. Finally, we only have anonymous healthy blood donors as control, and we miss sex and aged matched controls.

In summary, we have shown that complement and neutrophil activation, D-dimers, and cfDNA increase with disease severity, decrease in patients with disease resolution, and remain high in non-survivors. Therefore, neutrophil activation, cfDNA, and complement activation have the potential to serve as reliable biomarkers for disease severity and fatality in COVID-19. They may also serve as suitable markers for monitoring the efficacy of therapeutic interventions in COVID-19. To demonstrate the reliability of these markers to predict severity and outcome in COVID-19, larger, prospective multicenter trials are needed.

Statement of Ethics

Human blood samples were collected from healthy donors with the blood donation center SRK in Bern (project P357) and the help of a physician in agreement with the local Ethical Board. The study was reviewed and approved by the Ethics Committee of the Canton of Bern, Bern, Switzerland [Nr. 2020-00877] and registered at clinicaltrials.gov [NCT04510012]. Patients were included after written informed consent. In case of lack of capacity and/or inability to provide consent, enrollment followed the procedures for research projects in emergency situations according to Swiss law. Our research comply with the guidelines for human studies and was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Conflict of Interest Statement

The authors declare no competing financial interests.

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Author Contributions
Contributions: Tiphaine Ruggeri performed the experiments, analyzed and interpreted the data, wrote, edited and reviewed the manuscript and figures; Yasmin De Wit performed the experiments, analyzed and interpreted the data, wrote, edited and reviewed the manuscript and figures; Noëlia Schärz performed the experiments and analyzed the data; Gerard van Mierlo assisted with the experiment and reviewed the manuscript; Anne Angelillo-Scherrer analyzed the data and reviewed the manuscript; Justine Brodard collected plasma samples from COVID-19 patients and was involved in data analysis; Joerg C. Schefold helped with patients, reviewed the manuscript; Cédric Hirzel wrote the ethical approval, established the cohort, collected clinical data from patients and reviewed the manuscript; Ilse Jongerius was involved in data analysis and reviewed the manuscript; Sacha Zeerleder designed and supervised the work.

Data Availability Statement
The data that support the findings of this study are not publicly available in order to protect the privacy of research participants but are available in an anonymized form from the corresponding author (SZ) upon reasonable request.

Author notes
* Tiphaine Ruggeri and Yasmin de Wit contributed equally to this study
# Sacha Zeerleder, Ilse Jongerius and Cédric Hirzel share senior authorship

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Figure Legends

Fig. 1. Nucleosome release and neutrophil activation in COVID-19 patients.
Nucleosome levels at baseline in COVID-19 patients (n=36) and in controls (n=24) (**a**), Nucleosome levels in patients with different severity levels of COVID-19 at different time points and in controls (**b**), Elastase–α_1-antitrypsin complex levels in patients with COVID-19 included at the baseline (n=36) and in controls (n=24) (**c**), Elastase–α_1-antitrypsin complex levels in patients with different levels of COVID-19 disease severity at different time points and in controls (**d**), Correlation between elastase–α_1-antitrypsin complex and nucleosome levels (**e**), Correlation between elastase–α1-antitrypsin complexes and cell-free mitochondrial DNA (mtDNA) (**f**). Plasma from healthy donors (controls) and from SARS-CoV-2–infected patients with different disease severities (mild, moderate, and severe) were analyzed at different time points: baseline corresponding to the enrollment day; 11 ± 3 days after enrollment (D11); and 28 ± 7 days after enrollment (D28). Squares represent patients who died. Data are presented as median ± interquartile range. Mann-Whitney-U test was used to test for differences between two groups (a, c). For the same patients at different time points, statistical significance was determined by using the Wilcoxon test. Kruskal-Wallis test following Dunn's multiple comparisons test was used to compare multiple groups (b, d). Spearman rank test was used to measure the ranked association between variables. The relation between Statistical significance was set at $p<0.05$.

Fig. 2. Cell-free mtDNA in COVID-19 patients
Mitochondrial DNA (mtDNA) amount in patients with COVID-19 included at the baseline (n=34) and in controls (n=20) (**a**), mtDNA amount in patients and in controls with different severity levels of COVID-19 at different time points (**b**). Plasma from healthy donors (controls) and from SARS-CoV-2–infected patients with different disease severities (mild, moderate, and severe) were analyzed at different time points: baseline corresponding to the enrollment day; 11± 3 days after enrollment (D11); and 28 ± 7 days after enrollment (D28). Squares represent patients who died. Data are presented as median ± interquartile range. Mann-Whitney-U test was used to test for differences between two groups (a). For the same patients at different time points, statistical significance was determined by using the Wilcoxon test (b). Kruskal-Wallis test following Dunn's multiple comparisons test was used to compare multiple groups (b). Statistical significance was set at $p<0.05$.

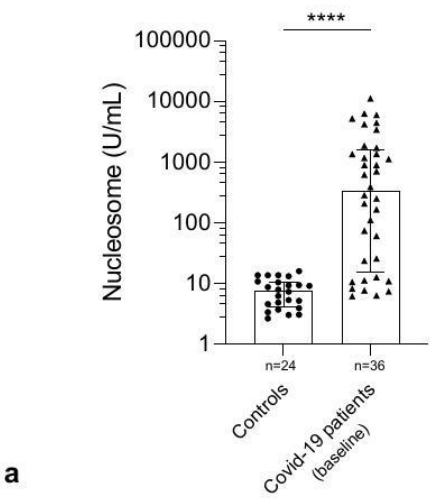
Fig. 3. DNase I activity in COVID-19 patients.
DNase I activity in patients with COVID-19 at baseline (n=72) and in controls (n=29) (**a**), DNase I activity in patients with COVID-19 at baseline and day 28 ± 7 (D28) (**b**), DNase I activity in patients with different COVID-19 disease severity at different time points and in controls (**c**), Correlation between DNase I activity and nucleosomes levels in all patients with COVID-19 at any time point (n=48 pairs) (**d**). Serum from healthy donors (controls) and from SARS-CoV-2–infected patients with different disease severities (mild, moderate, and severe) were analyzed at different time points: baseline corresponding to the enrollment day; and 28 ± 7 days after enrollment (D28). Squares represent patients who died. Data are presented as median ± interquartile range. Mann-Whitney-U test was used to test for differences between two groups (a). For the same patients at different time points, statistical significance was determined by using the Wilcoxon test (b, c), Kruskal-Wallis test following Dunn's multiple comparisons test was used to compare multiple groups (c). Spearman rank test was used to measure the ranked association between variables (d). Statistical significance was set at $p <0.05$.

Fig. 4. Complement activation in COVID-19 patients
C3b/c levels in patients with COVID-19 included at the baseline (n=36) and in control (n=20) (**a**), C3b/c level in patients with different severity levels of COVID-19 at different time points and in controls, with Wilcoxon test *p=0.0391 (**b**), C4b/c levels in patients with COVID-19 included at the baseline (n=36) and in controls (n=20) (**c**), C4b/c levels in patients with different severity levels of COVID-19 at different time points and in controls (**d**). Plasma from healthy donors (controls) and from SARS-CoV-2–infected patients with different disease severities (mild, moderate, and severe) were analyzed at different time points: baseline corresponding to the enrollment day; 11 ± 3 days after enrollment (D11); and 28 ± 7 days after enrollment (D28). Squares represent patients who died. Data are presented as median ± interquartile range. Mann-Whitney-U test was used to test for differences between two groups (a, c). For the same patients at different time points, statistical significance was determined by using the Wilcoxon test (b, d). Kruskal-Wallis test following Dunn's multiple comparisons test was used to

compare multiple groups (b, d). Statistical significance was set at $p<0.05$. #1 control dropout for C4b/c because of technical issue.

Fig. 5. D-dimer production in COVID-19 patients

D-dimer levels in COVID-19 patients at baseline (n=36) and in controls (n=15) (**a**), D-dimer levels in patients with different COVID-19 disease severities at different time points and in controls (**b**). Plasma from healthy donors (controls) and from SARS-CoV-2–infected patients with different disease severities (mild, moderate, and severe) were analyzed at different time points: baseline corresponding to the enrollment day; 11 ± 3 days after enrollment (D11); and 28 ± 7 days after enrollment (D28). Squares represent patients who died. The dotted line represents the detection limit (DL) of the D-dimer assay used (155 µg/L). Squares represent patients who died. Data are presented as median ± interquartile range. Mann-Whitney-U test was used to test for differences between two groups (a). For the same patients at different time points, statistical significance was determined by using the Wilcoxon test (b), Kruskal-Wallis test following Dunn's multiple comparisons test was used to compare multiple groups (b). Statistical significance was set at $p <0.05$.



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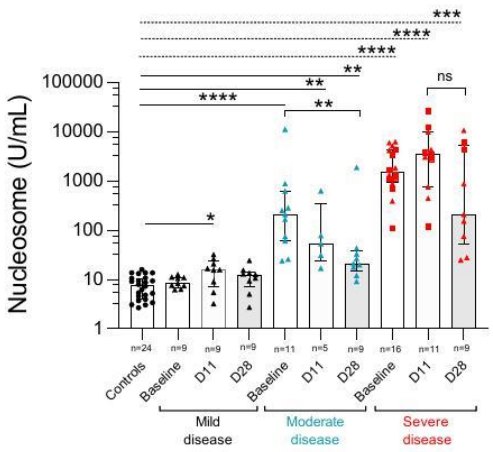

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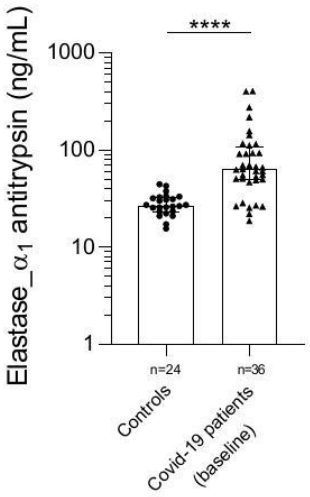

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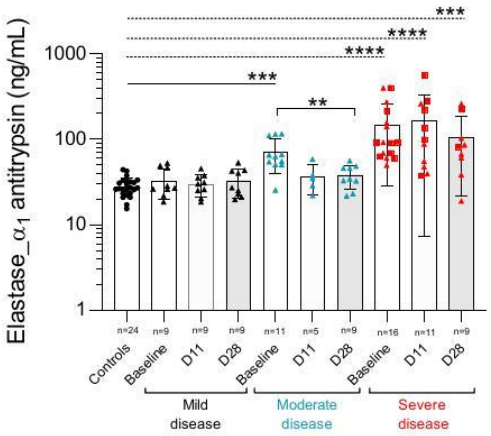

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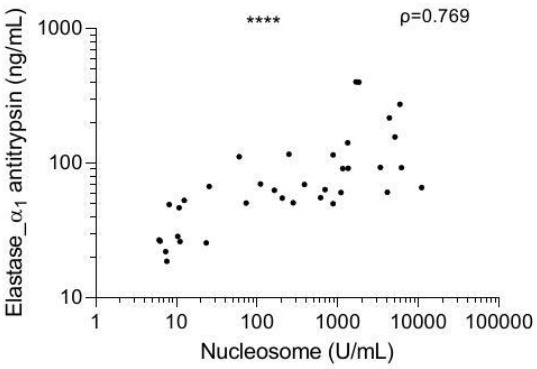

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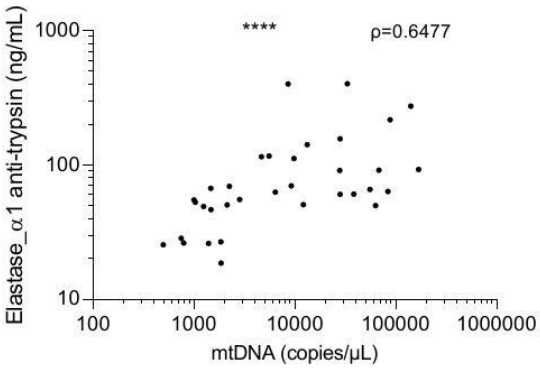

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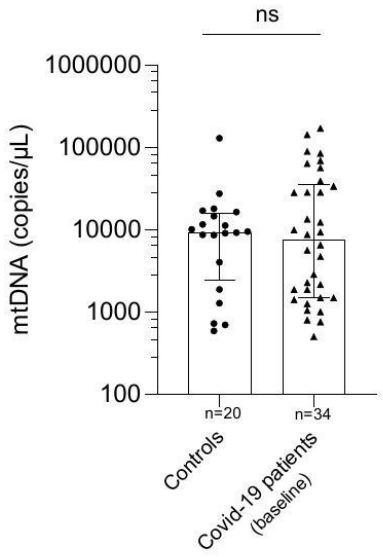

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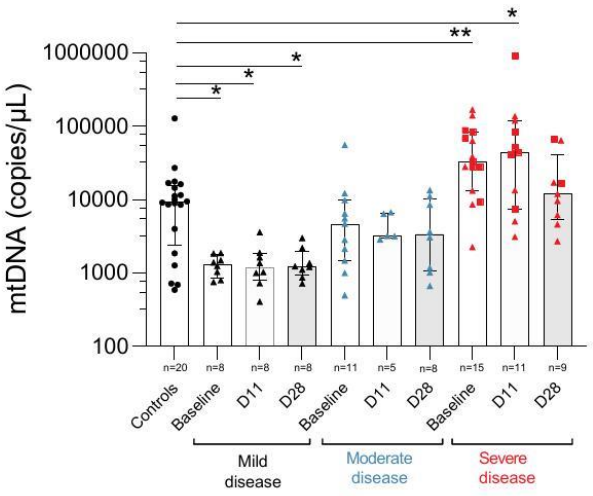

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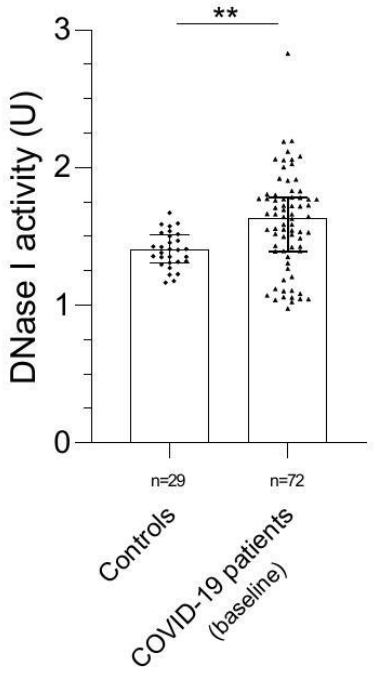

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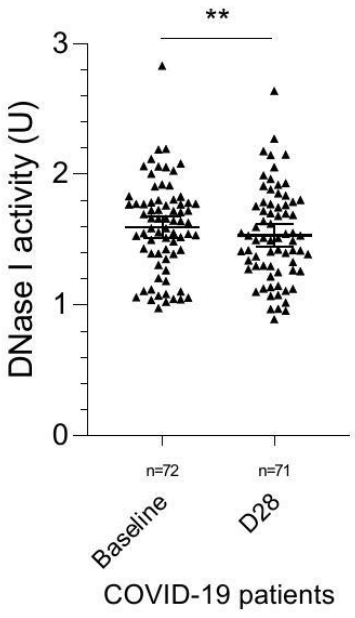

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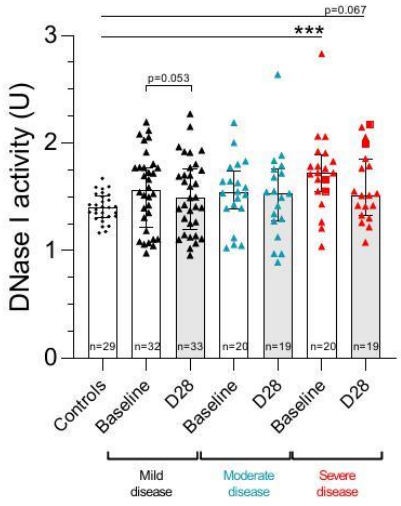

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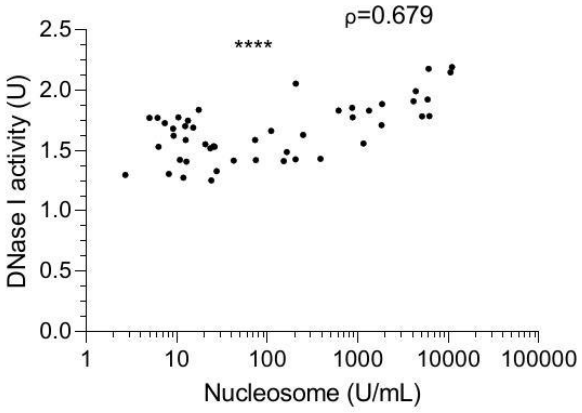

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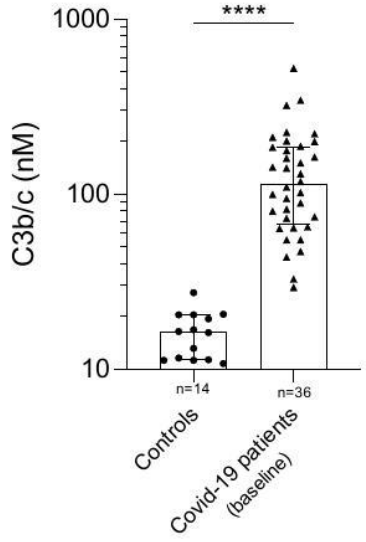

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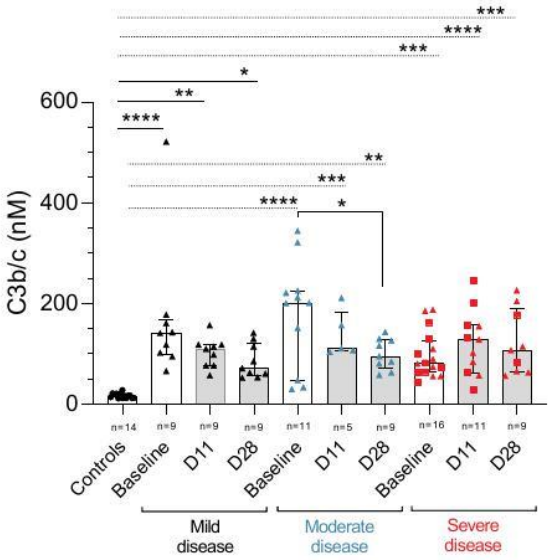

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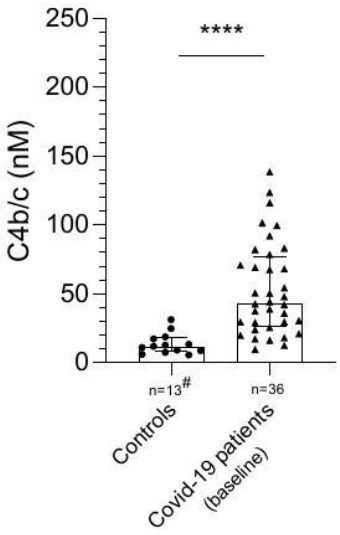

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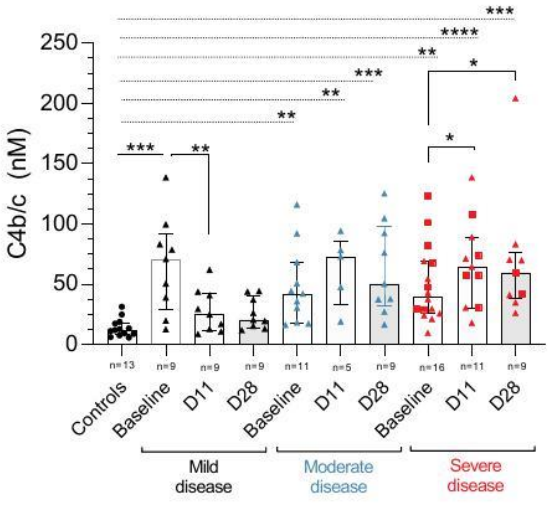

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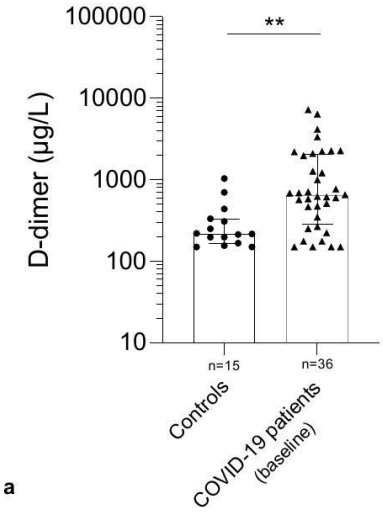

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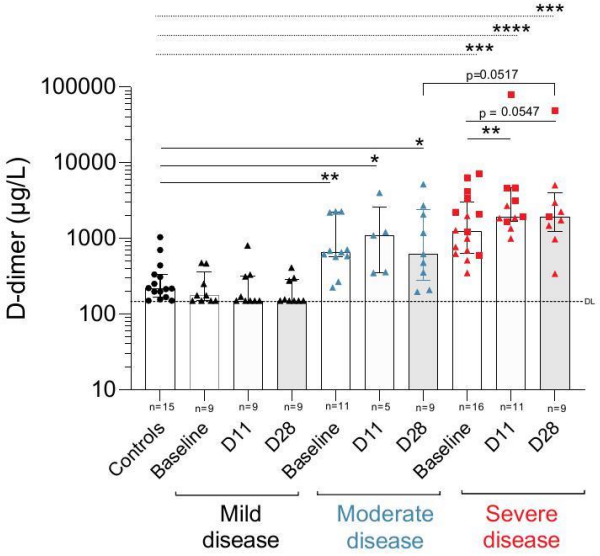

b

Table 1. Clinical features of patients with COVID-19

	All COVID-19 patients (n = 83)		**Mild disease** (n = 29)		**Moderate disease** (n = 22)		**Severe disease** (n = 32)	
	Total number	%	Total number	%	Total number	%	Total number	%
Sex								
Female	26/83	31	13/29	45	4/22	18	9/32	28
Male	57/83	69	16/29	55	18/22	82	23/32	72
Age group								
18-49 y	37/83	45	25/29	6	5/22	3	7/32	22
≥50 y	46/83	55	4/29	14	17/22	77	25/32	78
Median age [range] (y)	52 [22-86]		30 [22-71]		62 [22-84]		64 [25-86]	
Symptoms								
Fever	46/79	58	11/29	38	15/22	68	20/25	71
Rhinorrhea	30/69	43	20/29	69	4/22	18	6/18	33
Sore throat	24/70	34	17/29	59	3/22	14	4/19	21
Cough	58/76	76	19/29	66	18/22	82	21/25	84
Dyspnea	33/77	43	6/29	21	8/22	36	19/26	73
Myalgia	39/69	57	20/29	69	7/22	32	12/18	67
Nausea	4/69	6	1/29	3	1/22	5	2/18	11
Diarrhea	18/70	26	7/29	24	6/22	27	5/19	26
Anosmia	2/2	100	2/2	100	-	-	-	-
Days of enrollment after Symptoms onset								
Baseline median ± [IQR] (d)	-		4 ± [3 – 6]		8 ± [4 – 11]		7 ± [5 – 9]	
D11 median ± [IQR] (d)	-		15 ± [13 – 17]		20 ± [13 – 23]		17 ± [14 – 19]	
D28 median ± [IQR] (d)	-		33 ± [31 – 35]		36 ± [32 – 39]		32 ± [29 – 37]	
Comorbidities								
All comorbidities	37/83	45						
Diabetes	14/83	17	0/29	0	5/22	23	9/32	28
Cardiovascular	22/83	26	0/29	0	9/22	41	13/32	41
Hypertension	23/83	28	0/29	0	9/22	41	14/32	44
Pulmonary	10/83	12	5/29	17	3/22	14	2/32	6
Immune	4/83	5	0/29	0	1/22	5	3/32	9
Malignancy	8/83	10	1/29	3	2/22	9	5/32	16
Kidney	6/83	7	0/29	0	0/22	0	6/32	19
Other	17/83	20	0/29	0	9/22	41	8/32	25
At least 2 comorbidities	30/83	36	1/29	3	11/22	50	18/32	56
Other information								
Hospitalized	50/83	60	0/29	3	22/22	100	27/32	84
ICU at enrollment	25/83	30	0/29	0	1/22	4	24/32	75
ICU at any time	29/83	35	0/29	0	2/22	9	27/32	84
Death	8/83	10	0/29	0	0/22	0	8/32	25
Treatment								
No antiviral or anti-inflammatory therapy or anti-inflammatory	54/83	65	28/29	97	16/22	73	10/32	31
Antiviral therapy	5/83	6	0/29	0	1/22[#]	4	4/32[§]	13
Corticosteroid therapy	24/83	29	1/29	3	5/22	23	18/32	56

1 patient treated with lopinavir and ritonavir; § 4 patients treated respectively with: hydroxychloroquine, remdesivir and hydroxychloroquine, atazanavir, atazanavir and hydroxychloroquine. Abbreviations: d: days, Baseline: corresponding to the enrollment day, D11: 11 ± 3 days after enrollment, D28: 28 ± 7 days after enrollment, ICU: Intensive Care Unit, IQR: Intequartile Q1 (25%) and Q3 (75%), y: years