Neutrophil more than platelet activation associates with thrombotic complications in COVID-19 patients.

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Summary: Platelets and neutrophils are activated in COVID-19 patients. Increased neutrophil extracellular trap (NET) biomarkers, more than platelet activation, associated with severe evolution and thrombotic events. NET biomarkers may help to predict clinical worsening and VTE, and to guide LMWH-treatment intensity.

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Abstract

Background: SARS-CoV-2 infection is associated with hypercoagulability which predisposes to venous thromboembolism (VTE).

We analyzed platelet and neutrophil activation in COVID-19 patients and their association with VTE.

Methods: Hospitalized COVID-19 patients and age- and sex-matched healthy controls were studied. Platelet and leukocyte activation, neutrophil extracellular traps (NETs), and matrix metalloproteinase-9 (MMP-9), a neutrophil-released enzyme, were measured. Four patients were re-studied after recovery. The activating effect of COVID-19 plasma on control platelets and leukocytes and the inhibiting activity of common antithrombotic agents on it were studied.

Results: 36 COVID-19 patients and 31 healthy controls were studied; 8/36 COVID-19 patients (22.2%) developed VTE. Platelets and neutrophils were activated in COVID-19 patients. NET, but not platelet activation, biomarkers correlated with disease severity and were associated with thrombosis. Plasmatic MMP-9 was significantly increased in COVID-19 patients.

Platelet and neutrophil activation markers, but less so NETs, normalized after recovery.

In vitro, plasma from COVID-19 patients triggered platelet and neutrophil activation and NET formation, the latter blocked by therapeutic dose low-molecular weight heparin, but not by aspirin or dypiridamole.

Conclusions: Platelet and neutrophil activation are key features of COVID-19 patients. NET biomarkers may help to predict clinical worsening and VTE, and may guide LMWH-treatment intensity.

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Keywords

Low-molecular weight heparin

Neutrophil extracellular traps (NETs)

Matrix metalloproteinase-9 (MMP-9)

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Background

COVID-19 is a rapidly spreading viral pneumonia caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) declared pandemic by the World Health Organization on March 2020 [1].

Besides respiratory system involvement, SARS-CoV-2 infection induces severe blood clotting alterations with an unbalance towards hypercoagulability [2,3] predisposing to venous thromboembolism (VTE), arterial and microvascular thrombosis as a result of the strong systemic inflammatory reaction [4]. Indeed, abnormal coagulation parameters and high concentrations of proinflammatory cytokines are associated with disease severity, poor prognosis and with the incidence of VTE in COVID-19 patients [2,5,6]. Thrombocytopenia and elevated blood neutrophil counts have also been associated with disease severity and mortality in hospitalized COVID-19 patients, suggesting that these blood cells are deeply involved in the evolution of the infection [7,8].

While several studies have assessed coagulation and platelet count alterations in SARS-CoV-2 infection, only few focused on platelet and leukocyte activation, and on their relationship with VTE incidence [9–14].

Viral infections initiate a systemic inflammatory response stimulating the blood clotting system, a process called thromboinflammation in which platelet activation and platelet–neutrophil interactions play a crucial role [15]. Indeed, platelets act as blood sentinels quickly detecting invading pathogens through pattern recognition receptors (e.g., TLRs), get activated and form platelet-neutrophil complexes [15,16]. The interaction with activated platelets triggers the release by neutrophils of neutrophil-extracellular traps (NETs), 3-dimensional extracellular lattices composed by DNA, citrullinated histones, granule-derived enzymes and cytoplasmic proteins generated to limit infections but which may, when deranged, propagate inflammation and thrombosis [17].

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Activated neutrophils release also microparticles (PMN-MPs) which have been linked to thrombosis in vasculitis, inflammatory bowel disease and acute coronary syndromes [18,19]. PMN-MPs complex with NETs during sepsis enhancing thrombin generation, further highlighting the role of neutrophils in thrombosis [18]. Circulating PMN-MPs have not been studied so far in COVID-19 patients. NET formation in plasma and sera of COVID-19 patients, as well as their presence in autoptic lung specimens, have instead recently been reported, but no data on their correlation with VTE were provided [9,11,20].

Neutrophils contain in their tertiary granules and release upon activation matrix metalloproteinase-9 (MMP-9) which binds to NETs and induces endothelial dysfunction [21]. MMP-9 is also involved in neutrophil migration in lungs in response to viral infections [22]. Given that a strong neutrophil infiltration has been shown in the pulmonary microthrombi and in the bronchial tissue of COVID-19 patients [23], it is conceivable that MMP-9 may contribute to lung damage and/or thrombosis in COVID-19 patients. MMP-9 exists as a heterodimer with neutrophil gelatinase-associated lipocalin (MMP-9/NGAL) which prevents its inactivation, increasing MMP-9 proteolytic activity [24]. Increased plasmatic levels of MMP-9/NGAL have been reported in patients with pulmonary embolism, especially in the more severe cases [25]. Based on these considerations and on the pivotal role of MMPs in acute respiratory distress syndrome/acute lung injury, these enzymes have been recently postulated as a possible therapeutic target to prevent severe lung injury in COVID-19 [26], but MMP-9 levels in COVID-19 patients have not been measured so far.

Here we analyzed platelet and neutrophil activation and MMP-9 and MMP-9/NGAL levels in COVID-19 patients, focusing on their role in thrombosis. Moreover, we evaluated the effect of common antithrombotic agents in preventing COVID-19 patient plasma-triggered platelet and neutrophil activation and NET formation.

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Methods

Patients. Hospitalized COVID-19 patients were enrolled in a multicenter study carried out in the Umbria region approved by the local Ethics Committees (CEAS Umbria n. 3656/20 and University of Perugia Bioethics Committee n. 2020-36346) and each study participants or their legally authorized representative gave written informed consent in accordance with the Declaration of Helsinki. Patients were studied on average 3.4±0.6 days (95%CI 2.1-4.8) after the last positive nasopharyngeal swab. Thirty-one age- and sex-matched healthy volunteers who had not taken agents influencing platelet or leukocyte function in the previous seven days were also enrolled. Exclusion criteria for healthy controls were history of systemic autoimmune disease, active infection, allergic disorders and pregnancy. Four patients were restudied 2-3 months after hospital discharge made after 2 negative nasopharyngeal swabs (mean 72.7 days, 95% CI 32.8-112.6). Demographic, hemodynamic and prognostic variables, including PaO_2/FiO_2 ratio and sequential organ failure assessment (SOFA) score, were recorded.

Platelet and neutrophil activation markers. Citrated venous blood was collected and platelet P-selectin expression, platelet-leukocyte complexes, platelet- and neutrophil-derived microparticles were assessed by flow-cytometry (CytoFLEX, Beckman Coulter, Miami, Florida, USA); NET biomarkers (myeloperoxidase-DNA complexes and citrullinated Histone H3), soluble P-selectin, MMP-9, MMP-9/NGAL and TIMP-1 levels were measured in plasma by ELISA. See supplementary data for full details.

Effects of COVID-19 plasma on platelet/leukocyte activation from healthy donors in the presence of antithrombotic agents. Anticoagulated blood from healthy donors was centrifuged, plasma removed and replaced by an equal amount of plasma from either COVID-19 patients or healthy controls. See supplementary data for full details.

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Results

Patient characteristics

Clinical and demographic characteristics of enrolled COVID-19 patients and healthy subjects are reported in **Table 1**. Patient's age was 70.6±2.8 years (36.3-96.7 min-max), 55.5 % were males, the platelet count was 218.5±14.3x10^3/μL, neutrophil count 4.3±0.8x10^3/μL and neutrophil to lymphocyte ratio (NLR) 6.0±1.2. No significant differences between patients and healthy subjects were observed for demographics, platelet and neutrophil count, while NLR, D-dimer and VWF (Ag and RCo) were significantly different (**Table 1**). Enrolled patients were in general not severe [absence of leukocytosis, thrombocytopenia or hyperfibrinogenemia, only six admitted into ICU (16.6%), median SOFA score of non ICU patients 5] [27], although fourteen required mechanical ventilation, nine of which with endotracheal *intubation.* ICU patients as well as patients requiring mechanical ventilation had a significantly higher SOFA score compared to non ICU patients (**Supplementary Figure 1A**) or patients not requiring ventilation (**Supplementary Figure 1B**).

COVID-19 patients showed laboratory parameters compatible with hypercoagulability (**Table 1**). SOFA score positively and significantly correlated with D-dimer (r= 0.35; n= 33; p= 0.04) and NLR (r= 0.54; n= 23; p= 0.001), inversely and significantly correlated with PO_2/FiO_2 (r= -0.53; n= 17; p= 0.02).

Eight COVID-19 patients (22.2%) developed nine thrombotic events (one patient had two events) during or immediately after hospitalization (six pulmonary embolism, two deep vein thrombosis, one vein cava thrombosis): of these, six were under prophylactic LMWH (n=3 standard dose, n=3 intermediate-dose) and two under therapeutic dose LMWH treatment (one for atrial fibrillation and one for a previous pulmonary embolism). A significantly higher SOFA score was observed in patients who developed a thrombotic

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event compared to those who did not [without VTE 5.5±0.3 (n= 27), with VTE 8.2±1.2 (n= 8), p<0.05).

Platelets are activated in COVID-19 patients

We evaluated as platelet activation markers platelet P-selectin, soluble P-selectin and circulating PMPs [28]. A significant increase in platelet surface P-selectin expression was found in COVID-19 patients compared to healthy controls (**Figure 1A**). Soluble P-selectin (s-Psel) and PMPs were also significantly enhanced (**Figure 1B-C**). Circulating platelet-leukocyte, platelet-neutrophil and platelet-monocyte complexes, sensible markers of in vivo platelet activation [28], were also strikingly and significantly higher in COVID-19 patients compared to healthy controls (**Supplementary Figure 2A**, **Figure 1D** and **Supplementary Figure 2B** respectively). Plasma s-Psel inversely correlated with PO_2/FiO_2 (r= -0.52; n= 17; p= 0.032), and positively with NLR (r= 0.36; n= 33; p= 0.047), indicating that the degree of platelet activation is related to COVID-19 severity, confirming previous results [12].

For all measured platelet activation parameters, no significant differences were found between COVID-19 patients admitted into ICU and non-ICU COVID-19 wards, COVID-19 patients requiring mechanical ventilation and not, and COVID-19 patients who developed thrombotic events and those who did not (**Supplementary Figure 3A-F**). Moreover, no differences were observed between COVID-19 patients treated with LMWH at prophylactic dose (n=25), and COVID-19 patients who did not receive LMWH (n=8) (data not shown). Female COVID-19 patients showed greater in vivo platelet activation, as shown by higher platelet surface P-selectin expression and PMPs, compared to male COVID-19 patients (**Supplementary Figure 4A,B**), despite similar increase in D-dimer, VWF:Ag (**Supplementary Figure 4C,D**) and VWF:RCo (data not shown).

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Neutrophils are activated in COVID-19 patients

Circulating neutrophil derived microparticles (PMN-MPs), a marker of neutrophil activation [18,19,29], were significantly increased in COVID-19 patients compared to healthy controls (**Figure 2A**). It is known that neutrophil activation leads to the release of NETs which play a crucial role in thromboinflammation [17], we thus measured two highly specific markers of NET formation in plasma. Myeloperoxidase (MPO)-DNA complexes (**Figure 2B**) and citrullinated histone H3 (citH3) (**Figure 2C**) were significantly higher in plasma of COVID-19 patients compared to healthy controls. A statistically significant positive correlation between MPO-DNA complexes and citH3 was evident (**Figure 2D**). Interestingly, MPO-DNA complexes positively and significantly correlated with plasma levels of s-Psel (r= 0.39; n= 33; p= 0.027) and with NLR (r= 0.55; n= 33; p= 0.001). Moreover, NET biomarkers correlated with disease severity, in fact MPO-DNA complexes correlated with the SOFA score (r= 0.63; n= 29; p= $4.8X10^{-4}$) and were significantly higher in COVID-19 patients admitted into ICU (**Supplementary Figure 5A,B**), and in those requiring mechanical ventilation compared to milder COVID-19 patients (**Supplementary Figure 5C,D**), indicating that the degree of neutrophil activation is related to COVID-19 severity. No differences were observed for any of the assessed neutrophil activation parameters between COVID-19 patients treated with LMWH at prophylactic dose (n=25), and COVID-19 patients who did not receive LMWH (n=8) (data not shown). Interestingly, COVID-19 patients who developed thrombotic events had significantly higher MPO-DNA complexes and citH3 compared to COVID-19 patients who did not (**Figure 3A,B**). ROC curve analysis revealed that MPO-DNA complexes and cit-H3 showed moderate accuracy in discriminating patients developing VTE from those not (AUC= 0.769, p<0.001 and AUC=0.791, p<0.001, respectively) with a best cut-off of >0.12 OD for MPO-DNA complexes and of > 3.9 ng/ml for cit-H3 (**Supplementary Figure 6A,B**). Interestingly, male COVID-19 patients showed significantly greater in vivo NET

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formation, as shown by higher MPO-DNA complexes, compared to female COVID-19 patients (**Figure 3C**), despite similar increase in D-dimer, VWF:Ag (**Supplementary Figure 4C,D**) and VWF:RCo (data not shown).

MMP-9 and MMP-9/NGAL are increased in COVID-19 patients

MMP-9 and its MMP-9/NGAL heterodimer were significantly increased in plasma of COVID-19 patients compared to healthy controls (**Figure 4A, B**). Both parameters significantly correlated with circulating neutrophil number (MMP-9: r= 0.55; n= 24; p= 0.005, MMP-9/NGAL: r= 0.607; n=24; p= 0.003), and with the number of PMN-MPs in plasma (MMP-9: r= 0.44; n= 32; p= 0.014; MMP-9/NGAL: r= 0.39; n= 32; p= 0.03), in favour of their neutrophil origin. The physiological tissue inhibitor of metalloproteinase-1 (TIMP-1), a selective natural MMP-9 inhibitor binding to the MMP-9 active site in a 1:1 stoichiometric ratio, was also elevated in COVID-19 patients, but the ratio MMP-9/TIMP-1, which expresses residual MMP-9 proteolytic activity, was still significantly higher than in healthy controls (**Figure 4C, D**), showing that MMP-9 activity is enhanced in patient plasma.

No significant differences were found between COVID-19 patients admitted into ICU and non-ICU wards, between patients requiring or not mechanical ventilation and between patients who developed thrombotic events or not for any of the MMP-9-related biomarkers (data not shown).

Moreover, no differences were observed between COVID-19 patients treated with prophylactic dose LMWH (n=25), and COVID-19 patients who did not receive it (n=8) (data not shown).

Platelet and neutrophil activation in patients recovered from COVID-19

In patients who had recovered from COVID-19 a normalization of platelet P-selectin expression, PMPs, platelet-leukocyte and platelet-neutrophil complexes and MMP-9 was

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observed (**Supplementary Figure 7A-E**), showing that in vivo platelet and neutrophil activation are strictly linked to the ongoing COVID-19 infection. However, NET biomarkers seemed not to decrease (during infection: 0.13±0.03, after recovery: 0.15±0.03 O.D. 405 nm) (**Supplementary Figure 7F**), suggesting that some degree of thromboinflammation may persist.

COVID-19 plasma induces platelet and neutrophil activation

In order to unravel whether the inflammatory mediators present in plasma of COVID-19 patients, or the direct interaction of SARS-CoV-2 with platelets and neutrophils, are the main responsible of the observed systemic platelet and neutrophil activation, we incubated blood from healthy donors with plasma from either healthy subjects or COVID-19 patients. Inflammatory markers in COVID-19 plasma samples used for the co-incubation experiments were strikingly enhanced (**Supplementary Table 1**). Plasma from COVID-19 patients induced a significant and robust increase in platelet P-selectin surface expression, platelet-leukocyte and platelet-neutrophil complex formation (**Figure 5A, Supplementary Figure 8A** and **Figure 5B** respectively) and a strong increase in PMN-MPs, MPO-DNA complexes and MMP-9 (**Supplementary Figure 8B** and **Figure 5C,D** respectively) compared to plasma from healthy donors. In order to unravel if common antithrombotic agents could inhibit COVID-19 plasma-induced platelet and neutrophil activation, we pre-incubated venous blood from healthy donors with aspirin, dipyridamole and LMWH. Aspirin and dipyridamole showed no or a non significant effect on COVID plasma-induced NET formation (**Figure 6A,B**), while LMWH reduced it concentration-dependently, with a significant suppression with the highest dose (1.5U/ml) (**Figure 6C**).

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Discussion

COVID-19 is frequently associated with VTE, arterial and microvascular thrombosis which are considered a result of the strong systemic inflammatory reaction and the associated hypercoagulability [2–4]. Platelets play a connecting role between haemostasis and immunity getting activated by invading pathogens, crosstalking with neutrophils and contributing to thromboinflammation [15] which triggers VTE and microvascular thrombosis [30,31]. Activated neutrophils release NETs and microparticles (PMN-MPs), which induce endothelial damage and platelet activation [32,33], playing an important role in VTE [17].

Here we confirm that circulating platelets and neutrophils are strongly activated in COVID-19 patients [9,10], but we also show for the first time that NET formation, more than platelet activation, is associated with thrombotic events. We also report for the first time that COVID-19 patients have significantly increased plasma levels and activity of MMP-9. Finally, we show that plasma from COVID-19 patients induces platelet and neutrophil activation and NET formation which are prevented by high but not low concentrations of LMWH.

Differently from previous studies [10,12], our COVID-19 patients were mainly mild but still we found strong platelet and neutrophil activation, showing that the SARS-CoV-2 infection itself rather than the multiorgan dysfunction associated with severe forms triggers these blood cell function modifications [34]. On the other hand, platelet and neutrophil activation markers correlated with COVID-19 severity, suggesting that the respiratory tract infection triggers a vicious circle which starting from an uncontrolled systemic release of pro-inflammatory cytokines activates platelets and neutrophils, reinforcing lung damage on one hand and involving blood vessels on the other, with

consequent multiorgan dysfunction which in turn further boosts platelet and neutrophil activation [35].

We show significantly increased circulating levels of s-Psel and PMN-MPs in COVID-19 patients, suggesting that they may represent simple platelet and neutrophil activation biomarkers in SARS-CoV-2 infection.

On the other hand, we show for the first time that NET formation more than platelet activation is associated with thrombosis in COVID-19 patients. In fact, while for all platelet activation parameters no significant difference was found between patients with thrombotic events and those without, NET biomarkers were associated with thrombosis, showing moderate accuracy in discriminating patients developing VTE from those not. Interestingly, 7 of the 8 COVID-19 patients with thrombotic events were males, confirming the higher VTE risk in males [36], and male COVID-19 patients had significant higher NET, but not platelet activation, biomarkers compared to females further supporting the conclusion that neutrophils associate with thrombotic complications. Although platelets play a role in NET formation [15,16], several other pathways are also involved [37,38,39], including neutrophil activation by pro-inflammatory cytokines which are greatly increased in SARS-CoV-2 infection [35], and this may explain the stronger association of NETs respect to platelet activation with VTE. This central pathogenic role of NETs in COVID-19-associated VTE is in agreement with findings in cancer-associated thrombosis, another thromboinflammatory condition [15,40], and in animal models of viral infections [41]. No significant differences were found in PMN-MPs between COVID-19 patients who developed thrombosis or not, suggesting that it is specifically NETosis rather than simply neutrophil activation to be implicated in COVID-19-associated thrombosis.

We also show for the first time that MMP-9 and the MMP-9/NGAL heterodimer as well as the MMP-9/TIMP1 ratio, an index of MMP-9 proteolytic activity, are significantly

increased in plasma of COVID-19 patients. MMP-9 and MMP-9/NGAL correlated with the NLR, which is associated with severe outcome in COVID-19 [42] and which in turn correlated with the SOFA score (r= 0.54; n= 33; p= 0.01), but not with thrombotic events suggesting a pathogenic role of MMP-9 in the systemic rather than in the vascular complications of SARS-CoV-2 infection. Increased MMP-9, which participates in leukocyte migration in inflamed lungs, was previously found in plasma of patients with H1N1 and seasonal influenza A (IAV) infections, and lung injury triggered by IAV in mice was attenuated by MMP-9-gene deletion [43]. Thus, MMP-9 may be one trigger of the described neutrophil infiltration in lungs of COVID-19 patients [9] and thus represent a new potential therapeutic target for the pulmonary complications of SARS-CoV-2 infection [43].

Platelet and neutrophil activation normalized in patients recovered from COVID-19, suggesting that in vivo platelet and leukocyte activation are strictly linked to the ongoing infection. However, NET biomarkers did not seem to decrease to normal control levels, suggesting that some degree of neutrophil activation might persist after recovery, possibly contributing to the continued inflammatory reaction and long term sequelae which start to emerge in these patients [44,45]. The small number of COVID-19 patients studied during infection and after recovery, however, does not allow to draw definitive conclusions and further studies are warranted.

In order to unravel if the in vivo platelet and neutrophil activation observed in our COVID-19 patients was the results of direct SARS-CoV-2 interaction with platelets and neutrophils or of the cytokine storm triggered by SARS-CoV-2, we evaluated the effects of COVID-19 plasma on platelets and neutrophils from healthy subjects. Plasma from COVID-19 patients induced a strong activation of control platelets and neutrophils and NET formation, suggesting that inflammatory mediators, most probably cytokines,

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released during the SARS-CoV-2 infection [1] are responsible of the in vivo platelet and neutrophil activation observed in COVID-19 patients.

We then tested whether some common antithrombotic agents (aspirin, dipyridamole and LMWH), prevent platelet and neutrophil activation induced by COVID-19 plasma. In one observational study in severe SARS-CoV-2 infection, dipyridamole-treated patients improved–platelet counts and D-dimer levels [11]. We show that while aspirin and dipyridamole did not attenuate the platelet- and neutrophil-triggering activity of COVID-19 plasma, LMWH prevented NET formation in a dose-dependent manner, being significantly effective at a concentration corresponding to the therapeutic dose. The fact that the patients who developed VTE in our case series were under LMWH does not contradict its effectiveness in preventing NET formation concentration-dependently. Indeed, previous studies have shown a high frequency of VTE in LMWH-treated patients [46] but the incidence in untreated patients is unknown as no randomized studies comparing standard or intensified prophylactic-dose LMWH with placebo have been performed so far. Moreover, it has been suggested that LMWH dose should be increased from prophylactic towards therapeutic in high risk patients, like those hospitalized in ICU or with high D-dimer [47,48]. The dose-dependent ability of heparin to dismantle the NET scaffold has previously been reported [49]. Therefore, our results support the importance of LMWH prophylaxis for COVID-19 patients [3], and suggest that NET biomarkers may guide the switching to potentiated prophylactic dose or, in high-risk patients, to therapeutic dose to prevent neutrophil contribution to VTE [48].

In conclusion, we show that a strong in vivo platelet and neutrophil activation occurs in hospitalized COVID-19 patients, including in not severe cases, and that NETs most probably significantly contribute to the development of thrombosis. Our findings corroborate the idea that NET formation represents an important therapeutic target to reduce the thrombotic complications of COVID-19 [9,23].

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We recognize that a limitation of our study is the small number of COVID-19 patients who developed thrombotic events: further studies in larger patient cohorts are warranted to validate the predictive value of NET biomarkers for COVID-19-related thrombosis. However, while our manuscript was under review, a paper associating NET levels with the risk of developing thrombotic events has been published, supporting our conclusions [50].

Moreover, our findings concur to the hypothesis that MMP-9 may contribute to neutrophil infiltration in lungs [9,23] and that it might represent a potential therapeutic target for the prevention of lung damage in COVID-19 patients [26].

In summary, our results suggest that plasmatic NET biomarkers may be used for prognostication of COVID-19 severity and VTE risk and encourage the investigation of novel NET-targeting approaches for the prevention of thrombotic complications [9,20,23].

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Author contributions

E. Petito, E. Falcinelli, E. Cesari, M. Sebastiano, M. Malvestiti and L. Bury performed experiments; E. Petito and E. Falcinelli analyzed and interpreted data; G. Guglielmini carried out statistical analyses; U. Paliani, G. Vaudo, V. Cerotto, F. Gori, C. Becattini, E. De Robertis and T. Lazzarini enrolled the patients for the study; F. Paciullo collected patient's clinical data; P. Gresele designed and supervised the study; E. Petito and P. Gresele wrote the manuscript; COVIR-Study Investigators contributed to patient enrollment.

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Disclosure of conflict of interest

The authors declare no conflict of interest, except for Dr. Becattini Cecilia that reports personal fees from Bayer HealthCare, from Daiichi Sankyo, from Bristol Myers Squibb, and from Pfizer, outside the submitted work.

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References

1. Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LFP. The trinity of COVID-19: immunity, inflammation and intervention. Nat Rev Immunol. 2020; 20:363–374.

2. Connors JM, Levy JH. COVID-19 and its implications for thrombosis and anticoagulation. Blood. 2020; 135:2033–2040.

3. Marietta M, Ageno W, Artoni A, et al. COVID-19 and haemostasis: a position paper from Italian Society on Thrombosis and Haemostasis (SISET). Blood Transfus. 2020; 18(3):167-169.

4. Al-Samkari H, Karp Leaf RS, Dzik WH, et al. COVID-19 and coagulation: bleeding and thrombotic manifestations of SARS-CoV-2 infection. Blood. 2020; 136(4):489-500.

5. Velavan TP, Meyer CG. Mild versus severe COVID-19: laboratory markers. Int J Infect Dis. 2020; 95:304–307.

6. Levi M, Thachil J, Iba T, Levy JH. Coagulation abnormalities and thrombosis in patients with COVID-19. Lancet Haematol. 2020; 7:e438–e440.

7. Lippi G, Plebani M, Henry BM. Thrombocytopenia is associated with severe coronavirus disease 2019 (COVID-19) infections: A meta-analysis. Clin Chim Acta. 2020; 506:145–148.

8. Zhang B, Zhou X, Zhu C, et al. Immune phenotyping based on the neutrophil-to-lymphocyte ratio and IgG level predicts disease severity and outcome for patients with COVID-19. Front Mol Biosci. 2020; 7:157.

Accepted Manuscript

9. Middleton EA, He XY, Denorme F, et al. Neutrophil extracellular traps contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome. Blood. 2020; 136(10):1169-1179.

10. Manne BK, Denorme F, Middleton EA, et al. Platelet gene expression and function in patients with COVID-19. Blood. 2020; 136(11):1317-1329.

11. Zuo Y, Yalavarthi S, Shi H, et al. Neutrophil extracellular traps in COVID-19. JCI Insight. 2020; 5(11):e138999.

12. Hottz ED, Azevedo-Quintanilha IG, Palhinha L, et al. Platelet activation and platelet-monocyte aggregate formation trigger tissue factor expression in patients with severe COVID-19. Blood. 2020; 136(11):1330-1341.

13. Zhang S, Liu Y, Wang X, et al. SARS-CoV-2 binds platelet ACE2 to enhance thrombosis in COVID-19. J Hematol Oncol. 2020; 13(1):120.

14. Zaid Y, Puhm F, Allaeys I, et al. Platelets can associate with SARS-Cov-2 RNA and are hyperactivated in COVID-19. Circ Res 2020; doi: 10.1161/CIRCRESAHA.120.317703.

15. Guo L, Rondina MT. The era of thromboinflammation: platelets are dynamic sensors and effector cells during infectious diseases. Front Immunol. 2019; 10:2204.

16. Menter DG, Kopetz S, Hawk E, et al. Platelet “first responders” in wound response, cancer, and metastasis. Cancer Metastasis Rev. 2017; 36:199–213.

17. Kimball AS, Obi AT, Diaz JA, Henke PK. The emerging role of NETs in venous thrombosis and immunothrombosis. Front Immunol. 2016; 7:236.

18. Kambas K, Chrysanthopoulou A, Vassilopoulos D, et al. Tissue factor expression in neutrophil extracellular traps and neutrophil derived microparticles in antineutrophil

Accepted Manuscript

cytoplasmic antibody associated vasculitis may promote thromboinflammation and the thrombophilic state associated with the disease. Ann Rheum Dis. 2014; 73:1854–1863.

19. He Z, Si Y, Jiang T, et al. Phosphotidylserine exposure and neutrophil extracellular traps enhance procoagulant activity in patients with inflammatory bowel disease. Thromb Haemost. 2016; 115:738–751.

20. Radermecker C, Detrembleur N, Guiot J, et al. Neutrophil extracellular traps infiltrate the lung airway, interstitial, and vascular compartments in severe COVID-19. J Exp Med. 2020; 217(12):e20201012.

21. Carmona-Rivera C, Zhao W, Yalavarthi S, Kaplan MJ. Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2. Ann Rheum Dis. 2015; 74:1417–1424.

22. Bradley LM, Douglass MF, Chatterjee D, Akira S, Baaten BJG. Matrix metalloprotease 9 mediates neutrophil migration into the airways in response to influenza virus-induced toll-like receptor signaling. PLoS Pathog. 2012; 8:e1002641.

23. Barnes BJ, Adrover JM, Baxter-Stoltzfus A, et al. Targeting potential drivers of COVID-19: Neutrophil extracellular traps. J Exp Med. 2020; 217(6):e20200652.

24. Yan L, Borregaard N, Kjeldsen L, Moses MA. The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL. J Biol Chem. 2001; 276:37258–37265.

25. Busceti MT, Grande R, Amato B, et al. Pulmonary embolism, metalloproteinases and neutrophil gelatinase associated lipocalin. Acta Phlebol. 2013; 14:115-21.

ted Manuscript

26. Solun B, Shoenfeld Y. Inhibition of metalloproteinases in therapy for severe lung injury due to COVID-19. Med Drug Discov. 2020; 7:100052.

27. Lin J, Yan H, Chen H, et al. COVID-19 and coagulation dysfunction in adults: A systematic review and meta-analysis. J Med Virol. 2020; 10.1002/jmv.26346.

28. Gresele P, Bury L, Mezzasoma AM, Falcinelli E. Platelet function assays in diagnosis: an update. Expert Rev Hematol. 2019; 12:29–46.

29. Wang Y, Luo L, Braun OÖ, et al. Neutrophil extracellular trap-microparticle complexes enhance thrombin generation via the intrinsic pathway of coagulation in mice. Scientific Reports 2018; 8:4020.

30. Montoro-García S, Schindewolf M, Stanford S, Larsen OH, Thiele T. The role of platelets in venous thromboembolism. Semin Thromb Hemost. 2016; 42:242–251.

31. Momi S, Falcinelli E, Giannini S, et al. Loss of matrix metalloproteinase 2 in platelets reduces arterial thrombosis in vivo. J Exp Med. 2009; 206:2365–2379.

32. Pitanga TN, de Aragão França L, Rocha VCJ, et al. Neutrophil-derived microparticles induce myeloperoxidase-mediated damage of vascular endothelial cells. BMC Cell Biol. 2014; 15:21.

33. Pluskota E, Woody NM, Szpak D, et al. Expression, activation, and function of integrin αMβ2 (Mac-1) on neutrophil-derived microparticles. Blood. 2008; 112:2327–2335.

34. Wu T, Zuo Z, Kang S, et al. Multi-organ dysfunction in patients with COVID-19: a systematic review and meta-analysis. Aging Dis. 2020; 11:874–894.

Accepted Manuscript

35. Ragab D, Salah Eldin H, Taeimah M, Khattab R, Salem R. The COVID-19 cytokine storm; what we know so far. Front Immunol. 2020; 11:1446.

36. Nopp S, Moik F, Jilma B, Pabinger I, Ay C. Risk of venous thromboembolism in patients with COVID-19: A systematic review and meta-analysis. Res Pract Thromb Haemost. 2020; 4(7):1178–91.

37. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. Nat Rev Immunol. 2018; 18(2):134-147.

38. An Z, Li J, Yu J, et al. Neutrophil extracellular traps induced by IL-8 aggravate atherosclerosis via activation NF-κB signaling in macrophages. Cell Cycle. 2019; 18(21):2928-2938.

39. Keshari RS, Jyoti A, Dubey M, et al. Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. PLoS One. 2012; 7(10):e48111.

40. Thålin C, Hisada Y, Lundström S, Mackman N, Wallén H. Neutrophil extracellular traps. Arterioscler Thromb Vasc Biol. 2019; 39:1724–1738.

41. Beristain-Covarrubias N, Perez-Toledo M, Thomas MR, Henderson IR, Watson SP, Cunningham AF. Understanding infection-induced thrombosis: lessons learned from animal models. Front Immunol. 2019; 10:2569.

42. Kong M, Zhang H, Cao X, Mao X, Lu Z. Higher level of neutrophil-to-lymphocyte is associated with severe COVID-19. Epidemiol Infect. 2020; 148:e139.

43. Rojas-Quintero J, Wang X, Tipper J, et al. Matrix metalloproteinase-9 deficiency protects mice from severe influenza A viral infection. JCI Insight. 2018; 3(24):e99022.

Accepted Manuscript

44. Zhao G, Su Y, Sun X, et al. A comparative study of the laboratory features of COVID-19 and other viral pneumonias in the recovery stage. J Clin Lab Anal. 2020; e23483.

45. Carfì A, Bernabei R, Landi F, Gemelli Against COVID-19 Post-Acute Care Study Group. Persistent symptoms in patients after acute COVID-19. JAMA 2020; 324:603–605.

46. Middeldorp S, Coppens M, van Haaps TF, et al. Incidence of venous thromboembolism in hospitalized patients with COVID-19. J Thromb Haemost. 2020 Aug;18(8):1995-2002.

47. Nadkarni GN, Lala A, Bagiella E, et al. Anticoagulation, Bleeding, Mortality, and Pathology in Hospitalized Patients With COVID-19. J Am Coll Cardiol. 2020 Oct 20;76(16):1815-1826.

48. Gerotziafas GT, Catalano M, Colgan MP, et al. Scientific Reviewer Committee. Guidance for the Management of Patients with Vascular Disease or Cardiovascular Risk Factors and COVID-19: Position Paper from VAS-European Independent Foundation in Angiology/Vascular Medicine. Thromb Haemost. 2020 Sep 13. doi: 10.1055/s-0040-1715798. Epub ahead of print. PMID: 32920811.

49. Fuchs TA, Brill A, Duerschmied D, et al. Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A 2010; 107:15880–15885.

50. Zuo Y, Zuo M, Yalavarthi S, et al. Neutrophil extracellular traps and thrombosis in COVID-19. J Thromb Thrombolysis. 2020 Nov 5:1–8. doi: 10.1007/s11239-020-02324-z. Epub ahead of print.

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Table 1. Demographics and clinical characteristics of the study population.

	COVID-19 patients n= 36	Healthy subjects n= 31	p value
Age	70.6±2.8	64.0±3.4	ns
Sex (% M)	55.5%	37.9%	ns
Neutrophils (x 10^3/uL)	4.3±0.8	3.4±0.1	ns
Platelets (x 10^3/uL)	209.1±22.3	220.0±15.7	ns
NLR (neutrophil to lymphocyte ratio)	6.0±1.2	1.8±0.1	p=0.0001
D-dimer (ng/mL)	1634±325.3	161.6±27.7	p<0.0001
Fibrinogen (mg/dL)	371.6±30.4	323.2±26	ns
VWF Ag (%)	297.8±26.3	101.0±9.5	p<0.0001
VWF RCo (%)	319.2±27.8	100.9±8.3	p<0.0001
Procalcitonin (ng/ml)	1.5±1.2	N.A.	
CRP (mg/dL)	5.1±1.5	N.A.	
LDH (U/L)	251.2±28.3	N.A.	
PO_2/FiO_2	276.2±29.1	N.A.	
Days from positive swab	3.4±0.6	N.A.	
Thrombotic events (n)	9	N.A.	
SOFA score (total)	6.2±0.4	N.A.	
Comorbidities			
Hypertension (n)	15	3	p<0.01
Type 2 Diabetes Mellitus (n)	6	0	p<0.05
Obesity (n)	5	1	ns
Smoker (n)	3	2	ns
Atrial Fibrillation (n)	5	0	p=0.056
Cirrhosis (n)	1	0	ns
Kidney failure (n)	3	0	ns
Stroke (n)	2	0	ns
Peripheral artery disease (n)	2	0	ns
Drugs			
Antihypertensive agents (n)	7	1	ns
Statins (n)	4	0	ns
Antithrombotic treatments:			
Aspirin (n)	5	0	p=0.056
Anti P_2Y_{12} (n)	2	0	ns
LMWH (n)	28	0	p<0.0001
Apixaban (n)	3	0	ns
COVID-19 Treatments			
Hydroxycloroquine (n)	5	N.A.	
Darunavir/Cobicistat (n)	2	N.A.	
Tolicizumab (n)	1	N.A.	

Results are reported as mean±SEM if not differently indicated. N.A. = not applicable; ns = not significant.

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Figure legends

Figure 1. Platelet activation. **(A)** Platelet surface P-selectin expression in whole blood of COVID-19 patients and healthy controls. Results are reported as percentage of positive cells (healthy controls n= 18, COVID-19 n= 31; *p<0.001). **(B)** Soluble platelet P-selectin in plasma of COVID-19 patients and healthy controls. Results are reported as ng/ml (healthy controls n= 20, COVID-19 n= 33; *p<0.0001). **(C)** Plasma levels of platelet-derived microparticles (PMPs) in COVID-19 patients and healthy controls. Results are reported as number/µl (healthy controls n= 21, COVID-19 n= 33; *p<0.0001). **(D)** Circulating platelet-neutrophil complexes $CD66b^{+}$-$CD41^{+}$ in COVID-19 patients and healthy controls. Results are reported as percentage of positive cells (healthy controls n= 17, COVID-19 n= 31; *p<0.0001).

Platelet surface P-selectin positively and significantly correlated with circulating platelet-leukocyte complexes (r= 0.36; n= 31; p= 0.046).

Figure 2. Neutrophil activation. **(A)** Plasma levels of neutrophil-derived microparticles in COVID-19 patients and healthy controls. Results are reported as number/µl (healthy controls n= 14, COVID-19 n= 32; *p<0.001). **(B)** Myeloperoxidase (MPO)-DNA complexes in plasma of COVID-19 patients and healthy controls. Results are reported as Optical Density (O.D.) at 405 nm (healthy controls n= 14, COVID-19 n= 34; *p<0.01). **(C)** Citrullinated histone H3 in plasma of COVID-19 patients and healthy controls. Results are reported as ng/ml (healthy controls n= 8, COVID-19 n= 31; *p<0.05). **(D)** Correlation between MPO-DNA complexes and cit H3 (Spearman r= 0.36; p= 0.03; n= 33).

PMN-MPs positively and significantly correlated with platelet count (r= 0.40; n= 32; p= 0.025) and NLR (r= 0.37; n= 32; p= 0.044).

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Figure 3. NET biomarkers in COVID-19 patients who developed thrombosis compared to those who did not and in COVID-19 patients grouped by sex. (**A**) MPO-DNA complexes in plasma of COVID-19 patients who developed thrombosis compared to COVID-19 patients who did not. Results are reported as Optical Density (O.D.) at 405 nm (n= 26 without VTE, n= 8 with VTE; *p<0.05). (**B**) Citrullinated histone H3 in plasma of COVID-19 patients of COVID-19 patients who developed thrombosis compared to COVID-19 patients who did not. Results are reported as ng/ml (n= 26 without VTE, n= 7 with VTE; *p<0.05). (**C**) Myeloperoxidase (MPO)-DNA complexes in plasma of COVID-19 patients grouped by sex. Results are reported as Optical Density (O.D.) at 405 nm (male COVID-19 n= 19, female COVID-19 n= 15; *p<0.05).

Figure 4. Plasma levels of MMP-9, pro-MMP-9/NGAL, TIMP-1 and MMP-9/TIMP-1 ratio. (**A**) MMP-9 in plasma of COVID-19 patients and healthy controls (healthy controls n= 14, COVID-19 patients n= 31; *p<0.01). In the inset a representative zymography. (**B**) pro-MMP-9/NGAL in plasma of COVID-19 patients and healthy controls (healthy controls n= 20, COVID-19 patients n= 30; *p<0.0001). In the inset a representative zymography. (**C**) TIMP-1 in plasma of COVID-19 patients and healthy controls (healthy controls n= 20, COVID-19 patients n= 32; *p<0.0001). (**D**) MMP-9/TIMP-1 ratio of COVID-19 patients and healthy controls (healthy controls n= 12, COVID-19 patients n= 30; *p<0.05).

A significant positive correlation between MMP-9 and MMP-9/NGAL in plasma of COVID-19 patients (r= 0.76; n= 33; p= $1.1x10^{-6}$) was found. Moreover, MMP-9 and MMP-9/NGAL positively correlated with the NLR (MMP-9: r= 0.37; n= 33; p= 0.04; MMP-9/NGAL: r= 0.46; n= 33; p= 0.007), and with the plasmatic levels of s-Psel (r= 0.37; n= 33; p= 0.04) (r= 0.35; n= 33; p= 0.05).

Figure 5. Effect of COVID-19 plasma on platelets and neutrophils from healthy controls. (**A**) Platelet surface P-selectin expression in whole blood of healthy controls mixed with

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plasma from healthy controls or from COVID-19 patients. Results are reported as percentage of positive cells. (n=6; *p<0.05). **(B)** $CD66b^{+}$-$CD41^{+}$ complexes in whole blood of healthy controls stimulated by plasma from healthy controls and from COVID-19 patients. Results are reported as percentage of positive cells. (n=9; *p<0.01). **(C)** MPO-DNA complex formation upon mixing with plasma from healthy controls or from COVID-19 patients (n=11 healthy controls, n=15 COVID-19 patients; *p<0.05). **(D)** Plasmatic levels of MMP-9 upon mixing with plasma from healthy controls or from COVID-19 patients (n= 12; *p<0.05).

Figure 6. Effect of aspirin, dipyridamole and LMWH on COVID-19 plasma-induced NET formation. **(A)** MPO-DNA complexes formation upon stimulation with plasma from COVID-19 patients, in the presence of aspirin 100 μM (n= 8; *p<0.05 vs healthy control plasma). **(B)** MPO-DNA complexes formation upon stimulation with plasma from COVID-19 patients, in the presence of dipyridamole 25 μM (n= 8; *p<0.05 vs healthy control plasma). **(C)** MPO-DNA complexes formation upon stimulation with plasma from COVID-19 patients, in the presence of LMWH at two concentrations (prophylactic dose: 0.5U/ml, and therapeutic dose: 1.5U/ml) (n= 8; *p<0.05 vs healthy control plasma, # p<0.05 vs vehicle). Results are reported as Optical Density (O.D.) at 405 nm.

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Figure 1

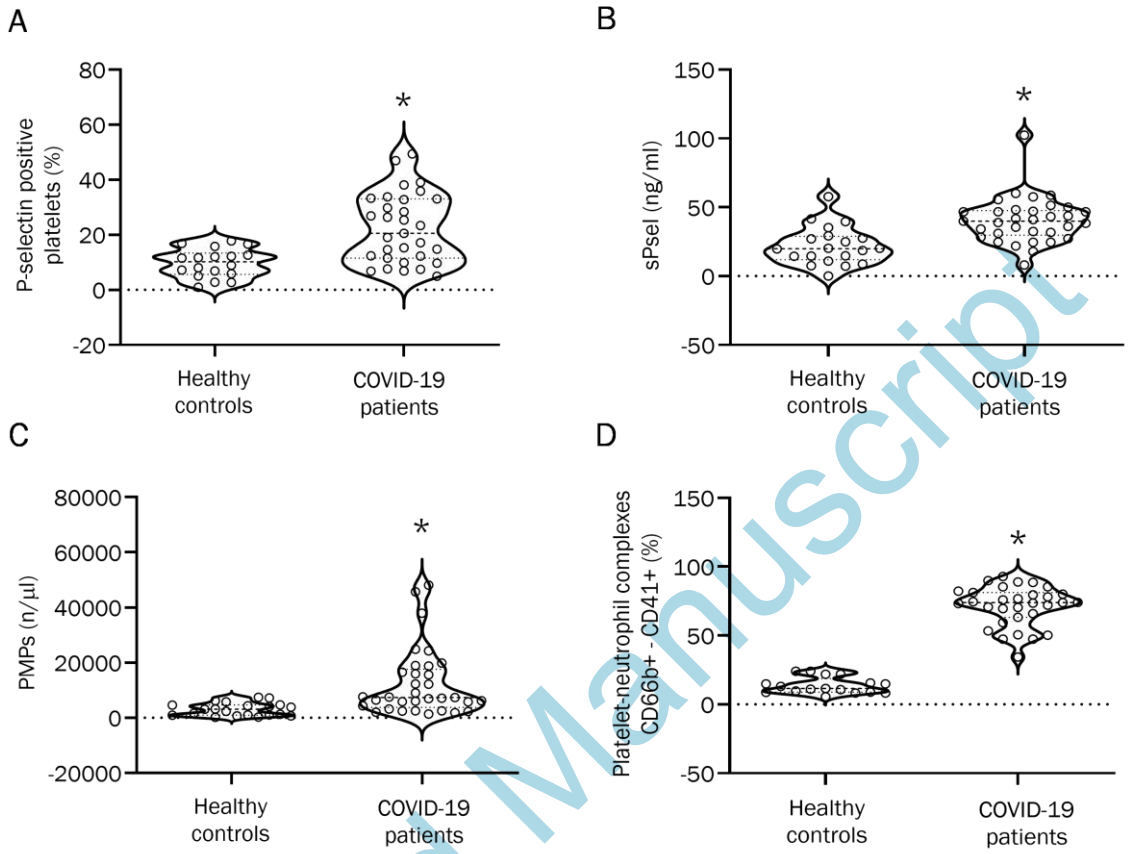

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Figure 2

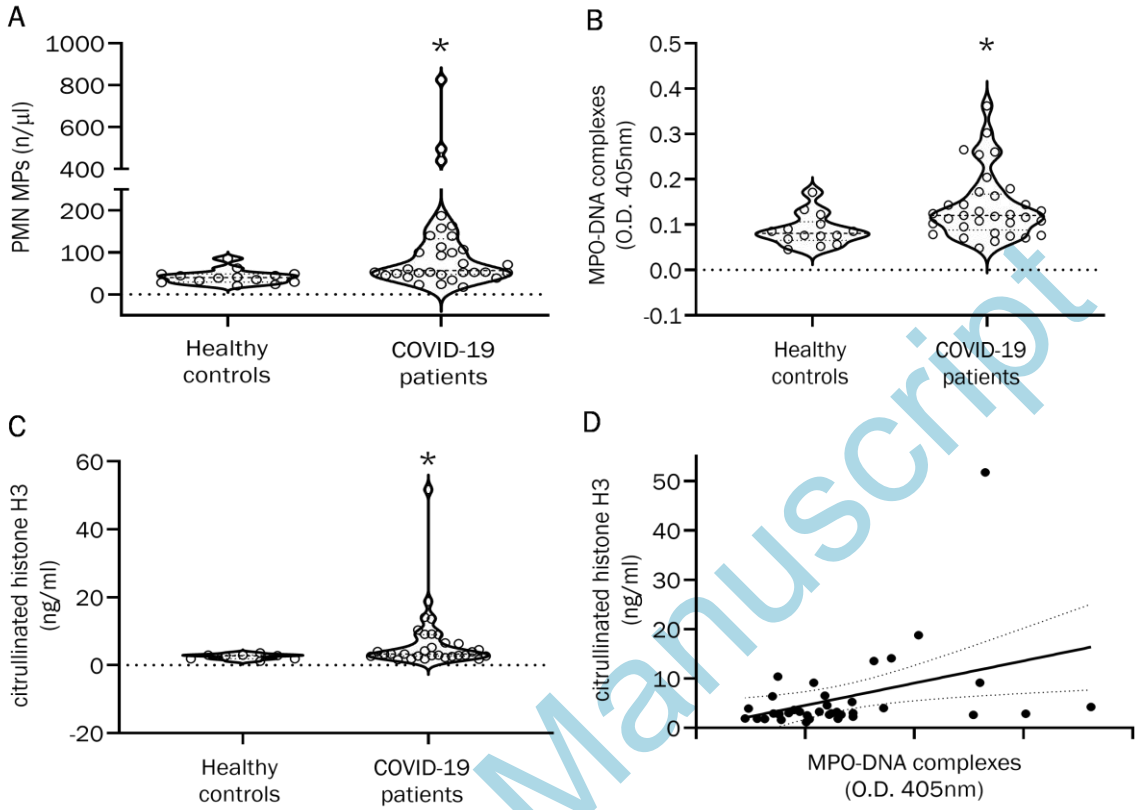

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Figure 3

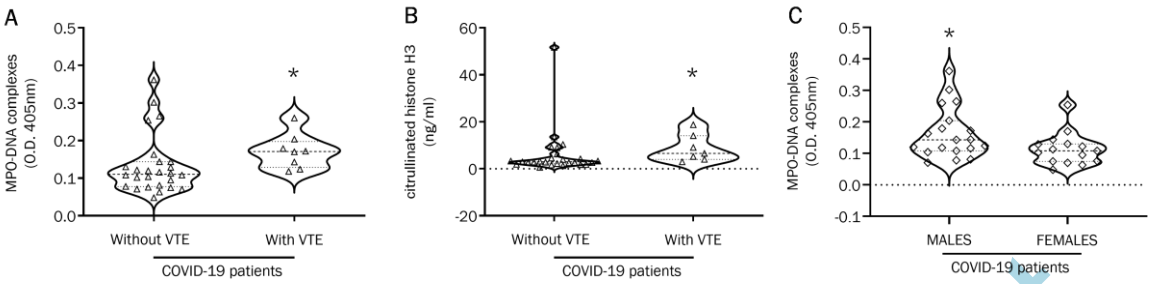

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Figure 4

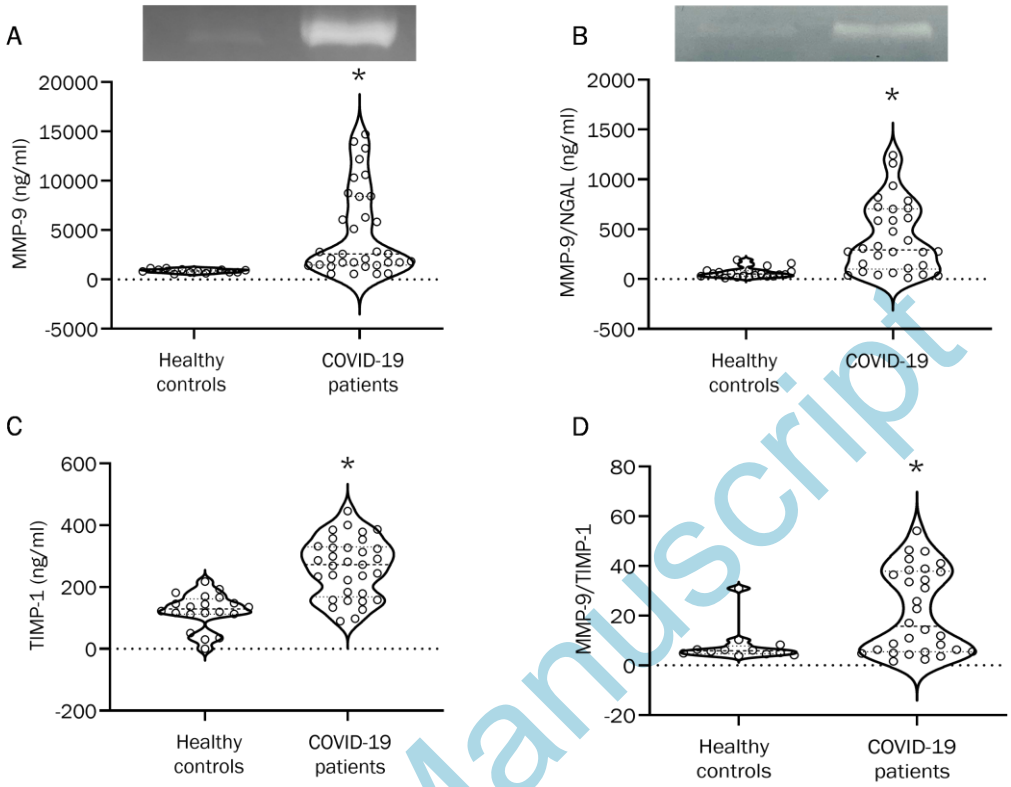

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Figure 5

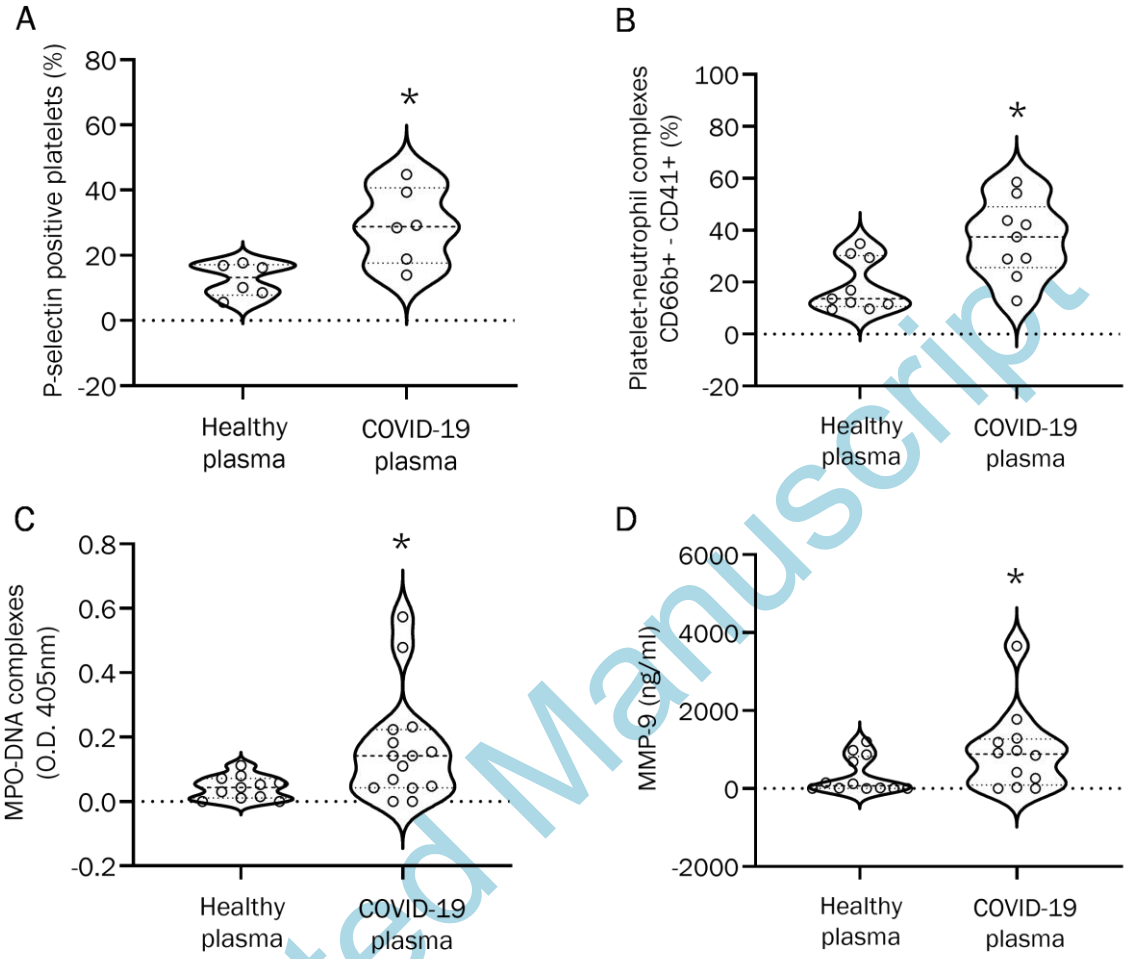

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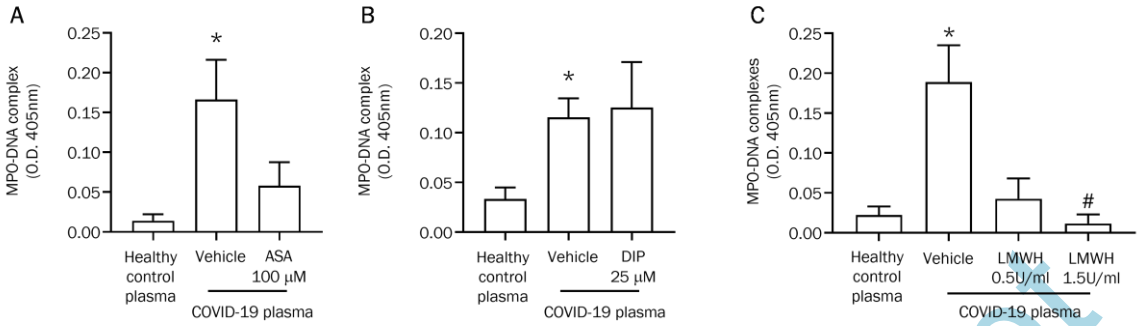
Figure 6



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