Neutrophil–Platelet and Monocyte–Platelet Aggregates in COVID-19 Patients

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TITLE : Neutrophil- and Monocytes- Platelets aggregates in COVID-19 patients.

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KEY POINTS

- Leukocytes-Platelets aggregates are increased and correlated with both inflammation and severity
- Anti-IL-6 therapies may reduce leukocytes-Platelets aggregate

ABSTRACT

High prevalence of arterial and venous thrombotic event as well as pulmonary microthrombi has been found in COVID-19 patients. Platelets play a critical role in inflammation and thrombosis and leucocytes-platelets aggregates have been implicated in thrombosis and acute lung injury. Herein, we aimed to explore neutrophil- and monocytes- platelets aggregates on patients with moderate or severe COVID-19 pneumopathy. Whole blood sample of twenty-seven patients (14 were male, median age was 71 years old) were collected and analysed by flow cytometry for neutrophils- and monocytes-platelets aggregates. Thirteen patients were classified as “moderate” and 14 as “severe” COVID-19. Neutrophil platelets (NPA) and Monocytes-Platelets aggregates (MPA) were higher in severe patients relative to moderate patients and healthy donors ($p<0.001$). NPA and MPA correlated positively with C-reactive protein and IL-6 levels. Patients treated with sarilumab (anti-IL6 receptor) showed a dramatic decrease of NPA and MPA ($p=0.002$ for both). In conclusion, leukocytes-platelets aggregates are increased and correlated with both inflammation and severity in COVID-19 infection. Leukocytes-platelets aggregates may be implicated in COVID-19 coagulopathy.

Coronavirus disease 2019 (COVID-19), a viral respiratory illness caused by the new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) rapidly spread around the world. Besides severe pneumonia with acute respiratory distress syndrome, it has been more recently highlighted that SARS-CoV-2 could predispose to thrombotic disease, both in venous and arterial circulations(1). Lung autopsy from severe COVID19 patients revealed high recruitment of innate immune cells including neutrophils and macrophages contributing to the cytokine storm as well as microthrombi(2). Given the central role of platelets in inflammation and thrombosis, and more specifically leucocytes-platelets aggregates that have been implicated in arterial and venous thrombosis, we aimed to explore neutrophil- and monocytes-platelets (NPA, MPA) aggregates in patients hospitalized in a medical ward for COVID-19 infection.

Data were collected from patients with Covid-19 participating in an open label, randomized, clinical trial testing sarilumab (400 mg) versus standard of care (SOC) conducted at the Pitié-Salpêtrière Hospital. Patients with moderate or severe pneumopathy according to the WHO Criteria of severity of COVID pneumopathy and positive to SARS-CoV-2 real-time reverse transcriptase–polymerase chain reaction assay from nasal swabs were included in this study. Moderate cases were classified as clinical symptoms associated with a maximum of 3L/min of Oxygen. Severe cases were defined as patients requiring more than 3L/min of Oxygen. The study received Paris VI ethical committee approval, conforming to the principles of the Declaration of Helsinki, and is registered as NCT04341870. All patients gave informed consent.

Whole blood sample were collected by venipuncture into EDTA vacutainer rapidly diluted in 3.2% sodium citrate. Cells were stained less than 3 hours after venipuncture using PerFix-nc (Beckman Coulter) according to the manufacturer's instructions and using the following antibodies: Fluorophore-labelled anti-CD45, anti-CD15, anti-CD16, anti-CD14 and anti-CD41b. Acquisition was performed using Navios Cytometer and analysed with Kaluza software (Beckman Coulter). Gating strategy is shown in Figure 1A.

Continuous variables are presented as median (interquartile range, IQR) or mean (+/- SEM) and compared using the Mann Whitney and Kruskal-Wallis tests with Dunn's correction for multiple comparisons or the Wilcoxon test when appropriate. Categorical variables are presented as counts (percent) and compared using Fisher's exact test. Analyses were computed GraphPad Prism (GraphPad Software, San Diego, USA).

Twenty-seven patients were included, 14(52%) were male with a median age of 71 years old. Thirteen patients were classified with "moderate" and 14 with "severe" COVID-19 pneumopathy. Patients with severe disease were more frequently males compared to patients with moderate disease 10/14(71%) vs. 4/13(31%), p=0.02. Other demographic parameters were not statistically different. Levels of hemoglobin, C-reactive protein (CRP), fibrinogen and ferritin were higher in severe patients compared to moderate patients **(Supplementary Table 1)**.

At baseline, we found an increase proportion of both NPA and MPA in COVID-19 patients compared to HD (17.9% *vs.* 3.1%, p<0.001 and 20.1% *vs.* 4.5%, p<0.001, respectively). Furthermore, levels of NPA and MPA were significantly higher in severe patients relative to patients with moderate disease (25.2%[17.4-35] vs. 14.1% [10.8-18.2], p=0.001 and 33.6%[20.4-46.9] vs. 18.4%[13.8-20.1], p=0.001, respectively) **(Figure 1B)**. We report here the first evidence of platelets and leucocytes aggregates in COVID-19 suggesting that platelets are in a preactivated state and can contribute to the microthrombotic complication in severe patients. In agreement with our observation, Monocytes platelets aggregates were found associated with the development of ARDS in a study by Abdulnour et al. (3). Moreover, in a mouse model of acute lung injury, NPA were found to exert a critical role(4).

Next, we pointed out a positive correlation between levels of NPA and MPA with CRP (r=0.658, p=0.005 and r=0.563, p=0.002, respectively) and IL-6 (r=0.628, p=0.01 and r=0.694, p=0.003) **(Figure 1C)**. Yan et al. showed in a model of colitis that IL-6 was the key mediator of neutrophils platelets aggregates suggesting that IL-6 is a suitable target to manage thrombo-inflammatory diseases (YAN).

Lastly, we investigated the impact of sarilumab (anti-IL-6 receptor) in NPA and MPA. Leucocytes-platelets aggregates were analyzed at baseline and 7 days after a single infusion of sarilumab on top

of SOC (n=15) or SOC without sarilumab (n=5) for which samples were available. All but 3 patients treated with sarilumab showed a decrease in NPA and MPA levels (p=0.002 for both) **(Figure 1D)**. This reduction of MPA and NPA may have contributed to the better outcome observed under sarilumab or other anti IL-6 therapies that have been proposed to tip down the cytokine storm of COVID-19 pneumonia(5). Anti IL-6 therapies are known to diminished neutrophil, platelets and monocytes. Overall, our study suggests that targeting the cytokine storm may reduce platelet/leukocytes complexes which can alleviate the thrombotic/microthrombotic complications in severe COVID-19 patients.

In conclusion, neutrophils- and monocytes- platelets aggregates are increased and correlated with both inflammation and severity in COVID-19 patients. Leukocytes-platelets aggregates may be implicated in the pathophysiology of COVID-19. Targeting IL-6 pathway may result in a reduction of leukocytes-platelets aggregates.

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FIGURE LEGEND :

Figure 1 : Neutrophil and Monocytes Platelets aggregates in COVID-19 patients.

A. Gating strategy and representative Dot-pots of flow cytometry analysis **B.** Levels of NPA and MPA according do disease severity. **C.** Correlation of NPA and MPA with CRP and IL-6. **D.** Levels of NPA and MPA before and after anti IL-6 receptor or SOC.

Data are shown as means ±SEM. For statistical analyses, Kruskal-Wallis , Spearman and Wilcoxon tests were used; * $p<0.05$, ** $p<0.01$, *** $p<0.001$

NPA : Neutrophil-Platelets Aggregates; MPA : Monocytes-Platelets Aggregates; SOC : Standard of Care