



Pro-inflammatory cytokine (IL-6) and total count lymphocyte profiles in COVID-19 patients with different severity levels

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ABSTRACT

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COVID-19 is caused by the SARS-CoV-2 infection that attacked the human respiratory system. In severe conditions, it causes pneumonia, kidney failure, acute respiratory distress syndrome (ARDS), and even death. The SARS-CoV-2 infection triggers the immune cells to secrete an excess of pro-inflammatory cytokines lead to cytokine storm. It is believed to become one of the mechanisms that cause the ARDS condition. The level of pro-inflammatory cytokines will differ with each case severity. This study aimed to evaluate the profile of pro-inflammatory cytokines in COVID-19 patients with different severity. Therefore, it could be used as therapeutic approach for cytokine storm conditions. It was a cross sectional study using plasma samples of COVID-19 patients from Jakarta Islamic Hospital, Pondok Kopi and Dr. M. Goenawan Partowidigdo Hospital, Cisarua, Indonesia. The COVID-19 patients with severe (n=20) and mild to moderate (n=25) severity were involved in this study. As negative control plasma sample from healthy subjects (n=13) was used. Plasma IL-6 levels were measured using the ELISA technique and plasma lymphocyte levels were measured using a hematology analyzer. The results showed that no significant difference between severity and gender was observed (p=0.256). Meanwhile, there is a significant difference in IL-6 level between negative control, mild-moderate, and severe categories (p=0.015). The average IL-6 level in severe categories was higher than mild-moderate and negative control categories, with values 105.375, 59.75, and 64.577 pg/mL, respectively. This result becomes supporting evidence that there is a cytokine storm condition in severe COVID-19 patients. Furthermore, the lymphocyte level in the severe group is significantly lower than the mild to moderate group. This result may indicate lymphocytopenia in the severe group.

ABSTRAK

COVID-19 disebabkan oleh virus SARS-CoV-2 yang menyerang sistem pernapasan manusia. Pada kasus berat, COVID-19 menyebabkan pneumonia, gagal ginjal, *acute respiratory distress syndrome* (ARDS), bahkan kematian. Infeksi virus SARS-CoV-2 memicu sel imun mengsekresikan sitokin pro-inflamasi dalam jumlah berlebihan yang disebut sebagai badai sitokin. Hal ini dipercaya menjadi salah satu mekanisme penyebab terjadinya ARDS. Kadar sitokin pro-inflamasi akan berbeda pada setiap tingkat keparahan. Penelitian ini bertujuan untuk menentukan profil sitokin pro-inflamasi pada pasien COVID-19 dengan tingkat keparahan yang berbeda-beda, sehingga hasil tersebut dapat digunakan sebagai pendekatan terapi untuk mengatasi kondisi badai sitokin. Penelitian ini adalah penelitian potong lintang menggunakan sampel plasma pasien COVID-19 dari RSIJ Pondok Kopi dan RS Paru Dr. Goenawan Partowidigdo, Cisarua, Indonesia. Pasien COVID-19 dengan kelompok keparahan berat (n=20) dan ringan hingga sedang (n=25) dilibatkan dalam penelitian. Sebagai kontrol negatif digunakan subjek sehat sebanyak 13 sampel. Pengukuran kadar IL-6 plasma menggunakan teknik ELISA dan kadar limfosit plasma menggunakan

Keywords:
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hematologi analyzer. Hasil penelitian menunjukkan tidak ada perbedaan signifikan antara tingkat keparahan dan jenis kelamin ($p = 0,256$). Namun demikian, terdapat perbedaan signifikan kadar IL-6 antara kelompok kontrol negatif, ringan hingga sedang, dan berat ($p=0,015$). Rerata kadar IL-6 kelompok berat lebih tinggi dibandingkan kelompok kontrol negatif dan ringan hingga sedang, dengan nilai masing-masing sebesar 105,375, 59,750, dan 64,577 pg/mL. Hasil penelitian dapat mendukung adanya badai sitokin pada kelompok berat kasus COVID-19. Selain itu, kadar limfosit pada kelompok berat secara signifikan lebih rendah dibandingkan kelompok ringan hingga sedang. Hasil tersebut mungkin menunjukkan adanya limfositopenia pada kelompok berat.

INTRODUCTION

Corona virus disease-19 (COVID-19) is caused by the severe acute respiratory syndrome Corona virus – 2 (SARS-CoV-2) infection that attacks the human respiratory system. The SARS-CoV-2 has been initially transmitted from animals to humans. Then human-to-human transmission occurred through droplets, direct contact, or contaminated objects.¹ The first case of COVID-19 was reported on December 31, 2020, in Wuhan, China. Furthermore, the number of cases increased significantly and spread to various countries in a short time, including Indonesia. In March 2020, WHO declared the COVID-19 outbreak as a pandemic. As of March 2021, it has been one year after the first cases in Indonesia declared, the total number of confirmed cases in Indonesia was 1.379.662, with 37.266 total deaths and 1.194.656 total of recovered patients.² The rapid increase in COVID-19 cases may contribute to the possibility of transmission by the asymptomatic carrier.

Clinical manifestations of COVID-19 usually appear within two days to 14 days after exposure to the virus. Common signs and symptoms in mild cases include acute respiratory symptoms such as fever, cough, and shortness of breath. Severe cases can cause pneumonia, acute respiratory distress syndrome (ARDS), kidney failure, and even death.³ There were four classifications of COVID-19

severity, such as asymptomatic (without symptoms), mild, moderate, and severe. The patient that experienced the asymptomatic phase does not feel any symptoms and sign physically. When the acute respiratory symptoms and systemic malaise appear, the viral load will increase significantly, thus classified as a mild phase. Furthermore, in the moderate phase, the disease progresses from the upper respiratory system to the lower respiratory system and becomes a pneumonia infection.^{4,5} The clinical symptoms in this phase include shortness of breath, use of nasal oxygen, and pneumonia thorax profile.

In the mild and moderate phases of COVID-19, an adaptive immune response is triggered to eliminate the virus and prevent progression to a severe clinical stage. Most of the patients experienced gradual recovery in their health conditions. However, if the response is maladaptive, it will progress to the severe condition causing damage to the tissue that expresses ACE2. The cellular mechanism internalization of SARS-CoV-2 into the lung is by binding to the ACE2 receptor in the surface of lung epithelial cells, subsequently, the virus replicates rapidly. This condition caused massive death of endothelial and epithelial cells, also triggered the massive production of pro-inflammatory cytokine.⁶ In the severe phase, the innate immune response could not resist the viral load. Therefore, some patients experience a

cytokine storm with increased levels of pro-inflammatory cytokines and other markers of inflammation, lymphopenia, and progressive respiratory failure.⁷ This condition can progress to a severe phase and occur in ARDS and multi-organ failure. Some studies have found that the ARDS condition is closely related to pro-inflammatory cytokine (IL-6, TNF- α , IL-1) and genetic susceptibility).⁴⁻⁶ Pro-inflammatory cytokines are signaling molecules secreted by immune cells (T cells and macrophages) and other cells that promote inflammation and immunity.

Profiling IL-6 in COVID-19 patients was our early study to know the level of pro-inflammatory cytokine among other cytokines related to different severity of COVID-19. Further study related to another pro-inflammatory cytokine level should be conducted to determine which cytokine will increase significantly during the infection of the SARS-CoV-2. Some studies also reported that cytokine storm-related to decreased lymphocyte level in COVID-19 patients. Another study needs to be performed to profiling the other pro-and anti-inflammatory cytokines in COVID-19 patients with different severity to be used as a therapeutic approach for drug development.

MATERIALS AND METHODS

Study design and subjects

This was a cross-sectional study using blood samples from COVID-19 patients collected from COVID-19 patients in Jakarta Islamic Hospital, Pondok Kopi, Jakarta and DR. M. Goenawan Partowidigdo Hospital, Cisarua, Bogor with different severity. The severity level of COVID-19 patients were categorized as mild to moderate and severe. The COVID-19 patients were considered mild if the patient confirmed COVID-19 with upper respiratory infection symptoms such as cough, nausea, fatigue, and fever,

with or without anosmia. The COVID-19 patients were considered moderate if the patient confirmed COVID-19 with upper respiratory infection symptoms, shortness of breath, used nasal oxygen as breathing support, and pneumonia thorax profile. The COVID-19 patients were considered severe if the patient confirmed COVID-19 with lower respiratory infection, shortness of breath, ventilator as breathing support, and pneumonia thorax profile. The people confirmed COVID-19 who not hospitalized were excluded from this study. The healthy subjects were used as negative. The protocol of this study was approved by the Research Ethic Committee of University of Muhammadiyah Prof. DR. HAMKA, Jakarta. There was no conflict of interest in this study.

Assessment of plasma IL-6 level

Blood samples were taken, centrifuged at 1372 x g for 10 min and the plasma was separated. Plasma samples were then stored at -80°C until analysis. the measurement of IL-6. Plasma IL-6 levels were measured with the ELISA technique. This technique was chosen based on literature that uses the ELISA technique in measuring the anti and/or pro-inflammatory cytokines in plasma.⁸⁻¹⁰ The measurement procedure was carried out by the ELISA Kit (Elabscience). Measurements using the ELISA technique are based on specific reactions between antigens and antibodies with high sensitivity and specificity, using enzymes as indicators to generate a detectable signal. The specific antibody will bind to the antigen, and then the enzyme causes a color change of the solution through an enzymatic reaction. The results were obtained quantitatively by observed optical density (OD) value from the color intensity of the solution measured by an ELISA reader and read as absorbance value. The standard curve was used to

calculate the concentration of samples by inputting the absorbance sample into the linear regression equation of standard curve lines.

Total count lymphocyte in plasma

The data of lymphocyte level was collected from medical record data COVID-19 patients in Jakarta Islamic Hospital, Pondok Kopi, Jakarta and DR. M. Goenawan Partowidigdo Hospital, Cisarua. The measurement of lymphocytes was carried out using a hematology analyzer.

Statistical analysis

The plasma IL-6 levels were compared among different severity and analyzed statistically by IBM SPSS-12 software using the chi-square test. The statistical significance was shown as $p < 0.05$. The total count lymphocyte profile was analyzed statistically by t-test methods. The correlation study between gender and severity, age and severity, IL-6 levels and severity, lymphocyte level, and severity were analyzed using Pearson correlation analysis.

RESULTS

Characteristics of patients

Thirteen healthy subjects and forty-five COVID-19 patients consisted of mild to moderate cases (25 patients) and severe cases (20 patients) were involved in this study. The general characteristics of patients are shown in TABLE 1. The age range of the subject in this study is approximately 19 – 73 y.o. The gender ratio between males and females was 3:2. The average age of subjects in the negative control, mild to moderate, and severe groups were 29, 43, and 50 y.o., respectively. Normality tests by Kolmogorov Smirnov methods showed that data gender was not normally distributed ($p < 0.05$). Comparative analysis between studied groups was conducted by chi-square methods. There was no significant difference between severity and gender ($p = 0.256$). Meanwhile, another result related to the correlation between age and severity showed a positive correlation based on the Pearson correlation analysis ($r_{\text{count}} > r_{\text{table}}$) as shown in TABLE 2.

TABLE 1. General characteristics of COVID-19 patients

Characteristics	Control (n=13)	Mild to moderate (n=25)	Severe (n=20)
Age (median y.o.)	29 (28)	43 (42)	50 (49)
Sex (n)			
• Male	7	13	14
• Female	6	12	6

TABLE 2. Correlation between age and severity

Parameters	Mean	SD	n	r count	r table	p
Age	42.214	14.509	56	0.534	0.263	0.00
Group	2.11	0.755	56	0.534	0.263	0.00

IL-6 and lymphocyte levels

FIGURE 1 shows the distribution of plasma IL-6 levels in the three groups. The plasma IL-6 levels among the three groups were not normally distributed. The median plasma IL-6 levels in the

severe group (96.5 pg/mL) were higher than the negative control (61.5 pg/mL) and mild to moderate group (59.0 pg/mL). The analysis result showed a statistical difference between plasma IL-6 levels in mild to moderate group compared to the severe group ($p < 0.05$).

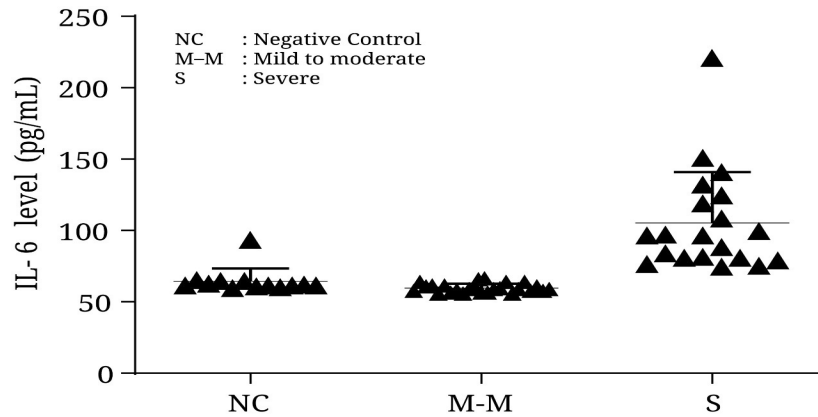


FIGURE 1. Distribution of plasma IL-6 level (pg/mL) in the negative control, mild to moderate, and severe categories

The lymphocyte level results showed a normal distribution ($p > 0.05$) and conducted a t-test analysis. The statistical analysis result showed a significant difference in lymphocyte level between the severe and mild to moderate groups ($p = 0.015$). Average lymphocyte levels in the severe group were lower than

the mild to moderate group (FIGURE 2) with 16.40% and 24.70%. The Pearson correlation was conducted among the two groups and showed no correlation between IL-6 and lymphocyte level. Meanwhile, IL-6 level and lymphocyte level may correlate to the severity of the disease.

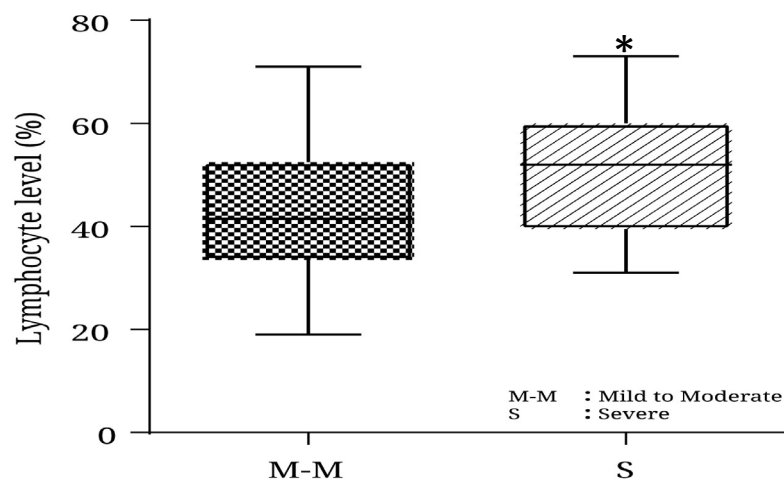


FIGURE 2. Level of lymphocytes in the mild to moderate and severe group. Value levels are shown as mean. Significant differences were performed by $*p < 0.05$.

DISCUSSION

This study showed the profile of plasma IL-6 as a cytokine pro-inflammatory and lymphocyte of COVID-19 patients with different severity. In general, the characteristics of patients showed no differences between severity and gender. The women and men patients have the same potency to undergo mild to moderate or severe in the COVID-19 cases. de Brito *et al.*¹¹ studies showed the same result regarding the differences between severity and gender in COVID-19 patients. The data also showed the varied ages in each group severity of COVID-19 and showed the positive correlation between age and severity. This finding considered that the older age has a higher risk of experiencing COVID-19 and progression of the disease. However, it is also important to consider other factors that could elevate the risk of COVID-19. Marlin *et al.*¹² also reported that older age is associated with mortality and severity of the disease. Many factors are related to the severity of COVID-19, including demographic, comorbidity, alcohol, and smoking. Therefore, another study with more comprehensible risk factors should be conducted.

The severity of COVID-19 is also related to immune response, including pro-and anti-inflammatory cytokine. An excessive secretion of cytokine proinflammatory is associated with severe conditions of COVID-19 patients and could trigger the ARDS condition and multi-organ failure.⁷ This study has shown elevated IL-6 levels in the severe group than the mild to moderate group. A sample in the severe group showed the highest level of IL-6 then the patient undergoes a worsening condition. These findings are considered the evidence for the presence of cytokine storms in severe conditions. de Brito *et al.*¹¹ and Zhang *et al.*¹³ also reported the elevated IL-6 levels in the severe group. The

presence of pro-inflammatory cytokines triggered another immune response to increased secretion of anti-inflammatory cytokines (IL-10, IL-4, IL-13) by T cells or macrophages. Therefore, further analysis regarding the ratio of pro-and anti-inflammatory cytokines needs to be performed to estimate the possibility of the ARDS condition.

Anti-inflammatory cytokines play a role in suppressing inflammatory and neoplastic processes by inhibiting IFN- γ production and activating specific antigen T cells. The balance of plasma level between pro-and anti-inflammatory cytokines can affect the degree of inflammation and, in severe conditions, may prevent the progression of the disease.¹⁴ Therefore, the elevated IL-6 levels should be followed by a high level of anti-inflammatory cytokines, as the body may be able to cope with the cytokine storm, thereby reduce the risk of worsening condition and ARDS. As one example from the severe group, there was a highest IL-6 level (220 pg/mL) among the others group and experienced progression of the disease and even death. However, other factors such as physiological conditions or comorbidity can also cause worsening of the patient's condition.

The occurrence of systemic inflammatory conditions or cytokine storms in the severe group is also related to lymphocytopenia.¹⁵ Jafarzadeh *et al.*¹⁶ studies showed the level of blood lymphocytes has manifested the most significant and reliable correlation with the disease progression and severity in COVID-19 patients and considered as an accurate indicator for the classification of COVID-19 patients in moderate, severe, and critical cases. This study showed decreased lymphocyte level in the severe group compared to the mild – moderate group. Chen *et al.*¹⁷ studies also showed a decrease in lymphocyte levels in the severe group compared to the mild-moderate group. Some

mechanisms may be underlying the decrease of lymphocytes in the severe group of COVID-19, such as apoptosis lymphocytes induced by SARS-CoV-2 infection, SARS-CoV-2 infection mediated thymus suppression, and the occurrence of cytokine storm that could induce the apoptosis of lymphocyte.¹⁶ Therefore, another study should be performed to understanding the mechanism of lymphocytopenia in COVID-19 cases.

CONCLUSION

There is no correlation between gender and severity of COVID-19, otherwise a significant difference between age and severity of COVID-19 is observed. The plasma IL-6 levels in the severe group are higher than the mild-moderate group. It can be concluded that the COVID-19 patients in the severe group potentially undergo the cytokine storm condition that could lead to the progression of the disease. Furthermore, the severity of COVID-19 related to low lymphocyte level and lymphocytopenia may cause serious effects in the severe group. Lymphocyte level in the severe group is significantly lower than the mild to moderate group. Further study related to lymphocytopenia in severe conditions should be performed to comprehend some possible mechanisms underlying this condition.

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