Analysis of Interleukin-17 Levels in Patients with Thrombocytopenia

Lidwina Septie Christyawardani*, Mansyur Arief, and Uleng Bahrun

1Department of Biomedical Science, Clinical Chemistry, Post-graduate Program of Universitas Hasanuddin
Jl. Perintis Kemerdekaan KM.10, Tamalanrea Indah, Kec. Tamalanrea, Kota Makassar, Sulawesi Selatan 90245, Indonesia

2Department of Clinical Pathology, Faculty of Medicine, Universitas Hasanuddin
Jl. Perintis Kemerdekaan KM.10, Tamalanrea Indah, Kec. Tamalanrea, Kota Makassar, Sulawesi Selatan 90245, Indonesia

3Department of Clinical Pathology, Faculty of Medicine, Universitas Hasanuddin
Jl. Perintis Kemerdekaan KM.10, Tamalanrea Indah, Kec. Tamalanrea, Kota Makassar, Sulawesi Selatan 90245, Indonesia

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Thrombocytopenia or platelet deficiency is a condition, in which platelet level in the blood circulation is below normal, which is less than 150,000 cells/µl. Thrombocytopenia is classified into some conditions, including decreased platelet production, increased need for platelets, and other thrombocytopenia. The need for increased platelets can be subdivided into primary immune thrombocytopenia, secondary immune thrombocytopenia, non-primary ITP, and thrombocytopenia that are not immune-mediated. Several cytokines play a role in the process of thrombocytopenia, one of which is Interleukin-17 (IL-17) that will be further discussed in this study. A previous study reported that IL-17 production increased in ITP and cITP patients. The objective of this study was to analyze the IL-17 levels and figure out the differences in IL-17 levels in the sera of patients with primary ITP and non-primary ITP. The samples were taken from Wahidin Sudirohusodo Hospital and the specimens were examined in the Research Unit Laboratory of the Faculty of Medicine, Universitas Hasanuddin/Hospital of Universitas Hasanuddin. The comparative test resulted in p-value = 0.005, where p < α = 0.05; and therefore, there was a significant difference between IL-17 levels in ITP and non-primary ITP.

*Corresponding author:
Lidwina Septie Christyawardani
Department of Biomedical Science, Clinical Chemistry, Post-graduate Program of Universitas Hasanuddin,
Makassar, Indonesia
Email: lidwinaseptiech@pasca.unhas.ac.id
INTRODUCTION
Platelets are also called blood chips, megakaryocyte cytoplasmic fragments that do not have a nucleus and are formed by bone marrow (Kosasih, 2008). The normal platelet level ranges between 150,000 - 400,000 cells/µl of blood (Lefever Joyce, 2008). Thrombocytopenia or platelet deficiency is a condition where the platelets in the circulatory system are below normal (Guyton and Hall, 2014).

Thrombocytopenia is classified into decreased platelet production, increased platelet consumption, and other thrombocytopenia. Decreased platelet production is caused by damage to the bone marrow, myelofibrosis, myelodysplastic syndrome, and iron deficiency. Meanwhile, the increased platelets can be further divided into primary immune thrombocytopenia, secondary immune thrombocytopenia, non-ITP, and thrombocytopenia not mediated by immunity (Matzdorff et al., 2018). Primary immune thrombocytopenia or Immune Thrombocytopenic Purpura (ITP) is an autoimmune disorder in the body without other secondary causes resulting in a platelet count <100,000 cells / µl (Makis et al., 2017), while secondary immune thrombocytopenia is a condition of thrombocytopenia attributed to a primary disease, which among others are autoimmune diseases, particularly the syndromes of anti-phospholipid antibody, Systemic Lupus Erythematosus/SLE, Rheumatoid Arthritis/RA, Helicobacter pylori infection, viral infections (including Hepatitis C and Human Immunodeficiency Virus [HIV]), and certain drugs (Zufferey, Lime, & Semple, 2017).

The incidence of secondary immune thrombocytopenia in adults accounts for 18% of the incidence of ITP patients that require health care. A total of 5.9% patients have malignant lymphoid disorders, 2.5% systemic lupus erythematosus, 2.3% myelodysplastic syndrome, 1.7% immune deficiency (excluding HIV infection), 0.9% HIV infection, 0.6% sarcoidosis, 0.3%, antiphospholipid syndrome, and 0.2% HCV infection. Forty-seven adult patients (1.63%) and nine children patients (1.1%) suffer from Evans syndrome. Secondary immune thrombocytopenia occurs in patients at a more mature age than ITP patients and is more common to happen in women. Only 2.4% of cases of secondary immune thrombocytopenia occur in children. The main causes are primary immune deficiency, systemic lupus erythematosus, blood cancer, and HIV infection (Moulis et al., 2014).

ITP disease is a health problem caused by immune dysregulation that triggers the loss of tolerance of the immune system to self-antigens on the surface of platelets and megakaryocytes. T cells are activated due to the introduction of platelet-specific antigens to APC (antigen-presenting cells), which then induce antigen-specific expansion in B cells. B cells then produce glycoprotein-specific autoantibodies that are expressed on platelets and megakaryocytes. The circulating platelets are bound by platelet autoantibodies and then adhere to the spleen macrophage FC receptor, which results in platelet destruction. In addition, anti-megakaryocyte autoantibodies are also formed and these reduce the ability of megakaryocytes to produce platelets (Sari, 2018). The research conducted by Li et al. (2015) reported that the levels of IL-17A in cITP (chronic Immune Thrombocytopenic Purpura) patients were significantly higher than in the controls. Further, the study by Ye (2015) found that ITP patients showed increased levels of mRNA expression in IL-23p19, IL-12p40, IL-23R, IL-12Rβ1, IL-17A, IL-17F, and RORC. Moreover, there were increases in Th17 cells and IL-17 levels in plasma.

The results of previous studies highlighted an increase in the number of patients with autoimmune thrombocytopenia. As science develops, it turns out that IL-17 cytokines play a particular role in ITP; however, further analysis of IL-17 in secondary immune thrombocytopenia has not been carried out. Therefore, the researchers were triggered to analyze IL-17 levels in the sera of ITP and non-primary ITP patients, and compare the results of IL-17 levels in both types of sera.
MATERIALS AND METHODS

This research belongs to an analytical study with a cross-sectional approach. This study was carried out in August – December 2019. The data used in this study were primary. The samples were taken from Wahidin Sudirohusodo Hospital and specimens were examined in the Research Unit Laboratory of the Faculty of Medicine, Universitas Hasanuddin/Hospital of Universitas Hasanuddin. The samples were all patients diagnosed with autoimmune thrombocytopenia at dr. Wahidin Sudirohusodo Hospital and the networks during the research period. A total of 60 samples were used, which were taken using a purposive sampling technique by considering the inclusion and exclusion criteria. The criteria of inclusion were: (1) the residual samples or patient serums resulted from routine blood tests showed a platelet count <100,000 μl, (2) the data of medical records of patients with ITP without any other blood cell abnormalities indicated that the hemoglobin and leukocyte levels were normal, and (3) the medical record data showed a platelet count <100,000 cells/μl accompanied by a diagnosis of other diseases causing thrombocytopenia, but it was not primary ITP. The exclusion criteria were: (1) the specimen volume was insufficient and (2) the specimens were hemolyzed, lipemic, and icteric and the sampling could not be repeated.

The IL-17 level examination was carried out using the ELISA method. 40 μl of samples were put into the sample holes and 10 μl of anti-IL-17 antibody was added. After that, 50 μl of streptavidin HRP was added into each sample hole and standard hole until homogenized. The plate was covered with a lid and incubated at 37 °C for 60 minutes. The lid was removed and the plate was washed five times with Wash Buffer. 50 μl of substrate solution A was added into each well and then 50 μl of substrate solution B was put into each well. The plate was later covered with a lid and incubated at 37 °C for 10 minutes. 50 μl of Stop solution was added into each well and homogenized. The color would turn yellow immediately. The absorbance was read at 450 nm in the microplate reader. Furthermore, the data obtained were processed using statistical software.

RESULT AND DISCUSSION

The samples in this study were the serums of primary ITP and non-primary ITP patients, which were then tested for the interleukin-17 levels. A total of 60 samples, consisting of ITP samples (24 women and 6 men) and 30 non-primary ITP samples (12 women and 18 men), were used. The samples had a platelet count <100,000 cells/μl. The samples were taken from patients at Wahidin Sudirohusodo Hospital from August to October 2019. The general characteristics of the research subjects are presented in Table 1.

Results of Comparative Test of IL-17 Levels in the ITP and Non-Primary ITP Subjects

The test on the differences in IL-17 levels was performed using a statistical test, initiated by the data normality test. The data normality test with the Shapiro-Wilk test resulted in α = 0.05. The results of the normality test showed the value of p = 0.000, where the p-value was smaller than the value of α (p < α), denoting that the data were not normally distributed, so the Mann Whitney test was used. The Mann Whitney test was conducted to spot the differences in the median of the two groups. The results of the Mann Whitney test are demonstrated in Table 2.

Based on the results of the statistical test presented in Table 2, the median value of interleukin-17 levels in primary ITP was 13.50 and the median value of interleukin-17 levels in non-primary ITP was 19.50. The results of the statistical test using Mann Whitney also yielded the value of p = 0.005 and the value of α = 0.05, which indicate that the p-value was smaller than the value of α (p <α), exemplifying a significant difference between the levels of interleukin-17 in primary ITP and non-primary ITP.
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Table 1. General characteristics of research subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Primary ITP (n=30)</th>
<th>Non-Primary ITP (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Sex</td>
<td>Man</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Woman</td>
<td>24</td>
</tr>
<tr>
<td>Age classification (year)</td>
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<tr>
<td>0 – 17</td>
<td>4</td>
<td>13.3</td>
</tr>
<tr>
<td>18 – 65</td>
<td>22</td>
<td>73.3</td>
</tr>
<tr>
<td>66 – 79</td>
<td>4</td>
<td>13.3</td>
</tr>
<tr>
<td>Classification of causes of thrombocytopenia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. PLT destruction</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>1. Primary ITP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Secondary ITP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. PLT production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c. Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>thrombocytopenia</td>
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<td></td>
</tr>
</tbody>
</table>

Table 2. Comparison of IL-17 levels in primary ITP and non-primary ITP Subjects

<table>
<thead>
<tr>
<th>Subyek</th>
<th>Primary ITP (n=30)</th>
<th>Non-Primary ITP (n=30)</th>
<th>*p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukinin-17 (ng/L)</td>
<td>17.57</td>
<td>126.27</td>
<td>0.005</td>
</tr>
</tbody>
</table>

*p Mann Whitney test

The characteristics of the subjects based on gender showed that ITP occurred more in female subjects (80%) than in male subjects (20%). This ratio is in line with the finding in a previous study by Ayesh, Alawneh, Khassawneh, Khader, & Kasasbeh (2013), with women and men ratio of 2.5:1. The latest research conducted by Yun Liang from the University of Michigan reported that women were more likely to develop autoimmune diseases due to the differences in gene expression as many as 661 of all participants. The study by Liang & Gibson (2017) found that women had higher risk factors in autoimmune diseases.

The characteristics of subjects in this study were also classified based on age. Both types of thrombocytopenia occurred mostly in patients aged 18-75 years old, 73.3% of ITP subjects, and 70% of secondary immune thrombocytopenia subjects. The data are following the finding in a previous study by Sultan, Ahmed, Murad, & Irfan (2016) that ITP patients were predominantly found in the 30-year-old age group and occurred at the peak age of ≥ 60 years in developed countries.

Thrombocytopenia is categorized into decreased platelet production, increased platelet consumption, and other thrombocytopenia. Decreased platelet production is attributed to the impairment of the bone marrow, myelofibrosis, myelodysplastic syndrome, and iron deficiency. Table 1 demonstrates that 43.3% of non-primary ITP subjects had decreased platelet production. In this case, some patients suffered from aplastic anemia and myelofibrosis, both of which are diseases related to damage to the bone marrow in producing blood cells. In aplastic anemia, a decrease in the production of blood cells from the bone marrow occurs, triggering reticulocytopenia, anemia, granulocytopenia, monocytopenia, and thrombocytopenia. Meanwhile, myelofibrosis is cancer that occurs in the bone marrow, which instigates abnormal development and function of red blood cells that can lead to anemia, thrombocytopenia, and an enlarged liver.

The consumption of platelets would increase in this study, where ITP occurred in the total sufferers. As mentioned earlier, primary immune thrombocytopenia is an abnormality in the platelet count, in which the platelet count is under the normal value, <100,000 cells/μl, without any other secondary causes. This disease may be resulted from increased platelet destruction by
humoral or cellular immune mechanisms, as well as improper platelet production in the bone marrow. (Aboud, Depré, & Salama, 2017).

The increase in platelet consumption also occurred in secondary ITP, as much as 40% of the total of 30 samples. Secondary ITP was accompanied by diseases, such as HIV, HBV, and hepatic cirrhosis. The process of secondary immune thrombocytopenia can be explained like any other autoimmune process. The body's antigens, the HPA, react with the immune system in the body. Self-antigens can cross-react with microbial antigens and the activity is called molecular mimicry. Furthermore, microbes will activate specific T cells against self-antigens and then an immune response will occur (Abbas, Lichtman, & Pillai; 2015).

Thrombocytopenia not immune-mediated in this study were found in patients who experienced bleeding and sepsis. Bleeding is a condition where the blood comes out of damaged blood vessels, both inside and outside the body. When bleeding occurs, the body will try to stop the blood loss, but when it is enormously heavy, the production of platelets in the body is not enough to overcome the bleeding and the body will experience a decrease in the number of platelets. This is similar to sepsis; when the tissue is injured or infected, there will be a simultaneous release of pro-inflammatory and anti-inflammatory factors. The balance of these disparate signals will aid in tissue repair and healing. When the inflammatory process is not in balance, serious tissue damage will happen, and this mediator will produce adverse systemic effects on the body.

This study found patients suspected with community-acquired pneumonia, in which cytokines increased to control the spread of bacterial infections. The study conducted by Jin & Dong (2013) confirmed that cytokines IL-17A and IL-17F are important in cleaning bacteria, such as S. aureus, C. rodentium, and Klebsiella pneumoniae. These cytokines increase in bacterial control and produce proinflammatory chemokines.

Sepsis and renal failure were also diagnosed in the samples, where sepsis-related renal failure would increase IL-17 levels and neutrophils, which subsequently triggered apoptosis. The results of this study are along the lines with the findings in Das & Khader’s research (2017) on in vitro with PBMC from healthy donors and patients with severe sepsis that reported an increase in Th-17 cells in patients with sepsis compared to healthy donors.

The results of the comparative test using statistical tests on primary ITP and non-primary ITP showed a significant difference in the results of IL-17 levels between primary ITP and non-primary ITP. This difference occurs because patients with non-primary ITP also suffered from other diseases, such as sepsis, aplastic anemia, and myelofibrosis that could increase the IL-17 levels. Interleukin-17 is a strong proinflammatory cytokine capable of recruiting neutrophils and monocytes and inducing the activation of various cytokines. Interleukin-17 will increase when it is first detected and has never been treated, and IL-17 levels will increase when the body experiences sepsis.

CONCLUSION
This study concludes a significant difference between IL-17 levels in primary ITP and non-primary ITP.

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