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## THE CORRELATION BETWEEN THE MEAN PLATELET VOLUME VALUES WITH THROMBOCYTE AGGREGATION IN NEPHROPATHY DIABETIC PATIENTS

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### ABSTRACT

Diabetic nephropathy is the most important cause of end-stage renal failure. Chronic hyperglycemia will cause glomerular endothelial damage, and this damage will stimulate hemostasis activation including platelets so that platelet aggregation will increase. The increase of platelet aggregation will increase platelet consumption, which further stimulates thrombopoiesis which will lead to immature platelets of large size to be released into the circulation. This research aimed to determine the positive correlation between MPV with platelet aggregation in patients with diabetic nephropathy. This study was an analytic observational study with a cross-sectional study design. The research was conducted in the Dr. Hasan Sadikin Hospital Bandung from July 2016 to October 2017. A total of 52 subjects who met the inclusion criteria were included in the study. Mean platelet volume and platelet aggregation were performed with venous examination with EDTA and sodium citrate 3.2% anticoagulants. The result of platelet aggregation examination showing platelet hyper-aggregation was found in 44.2% of subjects, 50% normal-aggregation, 5.8% hypo-aggregation. While the median value of MPV in this study was 9.2 fL with the range of 8.00 – 11.80 fL. A positive correlation was found between MPV value with platelet aggregation with  $r= 0.067$ ,  $p= 0.634$ . The conclusion was that there was no correlation between MPV values with platelet aggregation in diabetic nephropathy patients. This small and insignificant  $r$ -value might be due to several factors that also affect platelet aggregation in diabetic nephropathy patients, requiring further investigation.

**Key words:** Platelet aggregation, diabetic nephropathy, MPV values

### INTRODUCTION

Inadequate management of Diabetes Mellitus (DM) can cause various long-term complications related to macrovascular disorders such as cardiovascular, cerebrovascular, and peripheral arteries; and microvascular disorders such as nephropathy, retinopathy, and neuropathy.<sup>1</sup> Clinical manifestations of DM are usually mild and asymptomatic, but the course of the disease can develop into progressive and cause various acute and chronic complications.<sup>2</sup>

World Health Organization (WHO) explains diabetic nephropathy occurs in 20-40% of people with diabetes mellitus and can become end-stage renal failure.<sup>3</sup> Diabetes mellitus is the leading cause of kidney failure by 44% of all new cases in the world in 2011 and of all diabetic nephropathy patients, with the amount of 228,924 people who can survive routine hemodialysis or kidney transplantation.<sup>4</sup> Microvascular abnormalities in DM also have an effect on the hemostasis system. High blood glucose levels that occur continuously will cause endothelial

damage including glomerular endothelium and, the endothelial damage will stimulate the activation of the hemostasis system including platelets, causing platelet activity to increase which results in increased platelet aggregation. Increased platelet aggregation will cause the number of platelets consumed to stimulate thrombopoiesis, eventually young platelet cells will be found in the peripheral circulation. Immature platelet cells have a higher volume so the Mean Platelet Volume (MPV) will increase. Mean Platelet Volume is an indicator of platelet activation and function, large metabolic and enzymatic platelets have higher thrombotic power.<sup>5</sup>

Several studies have shown that in patients with diabetes mellitus there is an increase in adhesion activity, aggregation, thromboxane synthesis, and plasma platelet factor.<sup>4</sup> Increased platelet volume is associated with increased platelet activity so that MPV can be used as a marker of thrombocyte activity.<sup>5-7</sup> In the event of vascular endothelial damage, endothelial cell membrane proteins such as von Willebrand Factor (vWF) and collagen will be exposed to the blood. Platelet adhesion in an open

matrix is the first step for thrombus formation. Selectin, especially P-selectin, mediates the movement of platelets (platelet rolling) above the endothelium. Mediator P-selectin is expressed on the endothelial surface in response to inflammatory stimulation. This inflammation causes P-selectin mediator translocation from the supply of granules in the endothelial membrane (Weibel Pallade Bodies) to the plasma endothelial membrane. GPIb $\alpha$  and PSGL-1 platelet surface receptors will interact with endothelial P-selectin causing thrombocyte movement on the endothelial surface.<sup>8</sup> Thrombocytes attach to vWF via adhesion receptors on glycoprotein Ib/IX/V membranes (GPIb/IX/V) and collagen via glycoproteins VI (GPVI). The attachment causes platelet activation and integrin  $\alpha$ IIb $\beta$ 3 receptor transformation (GPIIb/IIIa, fibrinogen receptor) and collagen receptors ( $\alpha$ 2 $\beta$ 1) so that platelets are firmly bound to the subendothelial.<sup>8</sup> The process of endothelial platelet interaction can be seen in Figure 1.

During adhesion, platelets become activated and secrete mitogen and inflammatory substances that play a role in aggregation, chemotaxis, and proteolytic of the endothelial membrane. These thrombocytes induce changes in endothelial cell shape to support chemotaxis, adhesion and monocyte migration to the inflammatory site. Platelets release adhesion proteins (fibrinogen, fibronectin, vWF, thrombospondin, vitronectin, P-selectin, GP IIb/IIIa), growth factors (PDGF, TGF- $\beta$ , EGF, bFGF), chemokine (RANTES, platelet factor 4

CXC chemokine ligand 4, neutrophil-activating protein 78 epithelial CXC chemokine ligand 5, cytokine-like factor (such as IL-1 $\beta$ , CD40 ligand,  $\beta$ -thromboglobulin) and coagulant factors (such as factor V, factor X). These proteins affect biological functions such as adhesion, aggregation, chemotaxis, proliferation, coagulation, and proteolysis which in turn will accelerate the inflammatory process and withdrawal of inflammatory cells to the site of damage to blood vessels. Interleukin 1 $\beta$  that interacts with thrombin activates platelets inducing endothelial cell membranes to secrete IL-8 and IL-6. The interaction of endothelial cells with thrombin stimulates platelets to secrete endothelial monocyte chemoattractant protein-1 (MCP-1). Besides, platelet interactions with IL-1 $\beta$  can increase the expression of endothelial adhesion molecules. Platelet interactions with IL-1 $\beta$  also strengthened expression of ICAM-1 and  $\alpha$ IIb $\beta$ 3 on endothelial cells. Platelet interactions with IL-1 $\beta$  will also increase neutrophil and monocyte adhesion to the endothelium. Interleukin-1 $\beta$  also expresses inflammatory genes for MCP-1 or ICAM-1 by activating the transcription factor NF- $\beta$ .<sup>9</sup>

Platelets increase inflammation with leukocyte chemoattractants through mediators such as platelet-activating factor and inflammatory macrophage protein-1 $\alpha$ . These two mediators can increase smooth muscle proliferation (TGF- $\beta$ , PDGF and serotonin) and also contribute to degrading the matrix by secreting MMP-2.<sup>8</sup> Platelet interactions

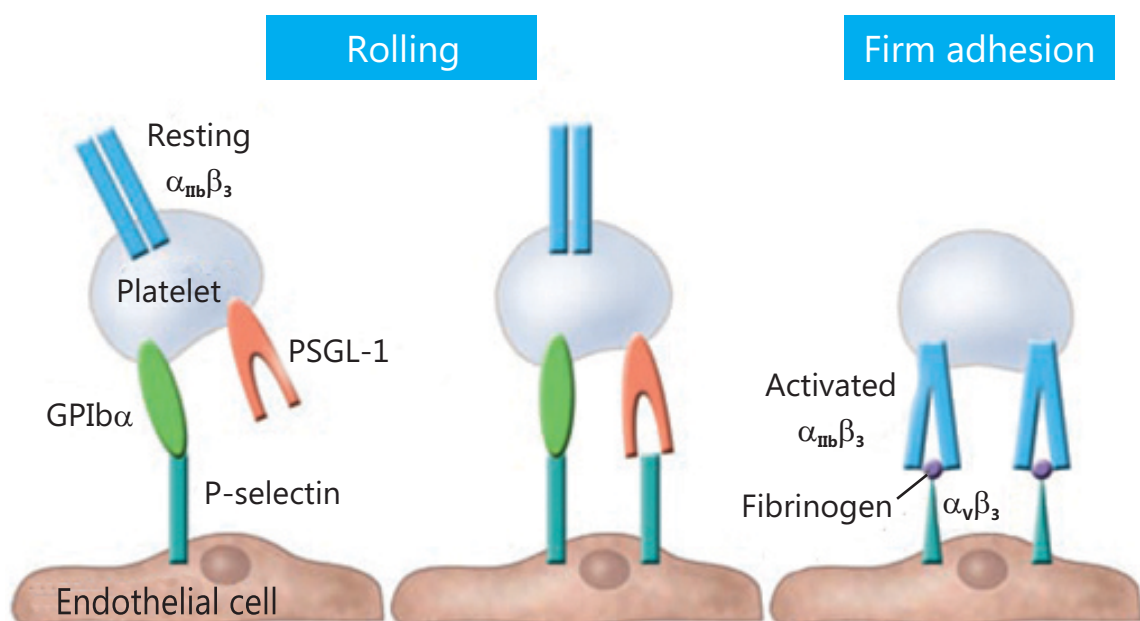


Figure 1. Platelet interactions (Quoted from Meinrad Gawaz)



with chemokines have implications for atherogenesis by increasing platelet aggregation and adhesion. Activated platelets can release chemokines and can also induce vascular cells to produce chemokines.

Hyperglycemia can increase the expression activity of Protein Kinase C (PKC), which plays a role in the regulation of platelet activity. Another consequence of hyperglycemia in platelets is the induction of mitochondrial dysfunction. One new mechanism that links hyperglycemia and mitochondrial dysfunction is the activation of aldose reductase and ROS production which results in PKC stimulation.<sup>10,11</sup>

Under normal circumstances, platelet adhesion to the vascular wall is inhibited by the antithrombotic properties of the endothelial cell surface and the release of anti-platelet factors by the endothelium.<sup>12</sup> In diabetic nephropathy occurs vascular endothelial disorders so that platelets can approach the subendothelial collagen-mediated by von Willebrand factor (vWF) and glycoprotein Ib/IX/V complex. This initial interaction is reinforced by the interaction of collagen with glycoprotein VI (GPVI) receptors and  $\alpha 2\beta 1$  integrins. This receptor bond will activate Src Family tyrosine kinase (SFKs) which causes phosphorylation and activation of phospholipase C $\gamma 2$  (PLC  $\gamma 2$ ), then hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) membrane to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP<sub>3</sub>) which activates protein kinase C (PKC) and stimulates the release of intracellular Ca<sup>2+</sup>. Protein kinase C binds and phosphorylates cytoplasm from the  $\beta 3$  complex subunit of the  $\alpha \text{IIb}\beta 3$  integrin, which then attracts talin and kindlin-3. Fibrinogen will be activated by adjacent platelet  $\alpha \text{IIb}\beta 3$  integrins, and initiate platelet aggregation.<sup>13</sup>

This study aims to see the correlation between MPV values and platelet aggregation, based on the explanation of the relationship between MPV values and platelet aggregation described above. The results of this study are expected to provide information to clinicians that more platelet hyper-aggregation can be related to an increase in MPV values. Thus the examination of MPV is expected to be a new modality for monitoring antiplatelet therapy in patients with diabetic nephropathy.

## METHODS

This study was an analytic observational with cross-sectional design. A total of 52 research subjects were patients who had been diagnosed with

diabetic nephropathy by clinicians who came to the Clinical Laboratory Installation/Dr. Hasan Sadikin Hospital, Bandung from June 2017 to August 2017. The sampling technique used in this study was nonprobability sampling with consecutive sampling method. Samples that did not meet the requirements (quantity or quality) which were hemolysis, jaundice or lipemic, EDTA blood test frozen, the patient is taking antiplatelet drugs (Aspirin, Ticlopidine, Clopidogrel, Ticagrelor, Cilostazol, Dipyridamol, Lamifiban etc.), and platelet counts <200,000/uL were excluded in this study.

The examination material in this study is 3.2% Citrate Na whole blood for examination of platelet aggregation and whole blood EDTA whole blood for examination of MPV values. Inspections of MPV values using automatic hematology instruments with flow cytometry (Sysmex XN-1000) method, an analysis of platelet aggregation using turbidimetry method, all examinations were carried out at the Clinical Hematology Division of the RSHS Clinical Pathology Department, Bandung.

The collected data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20. The normality test was conducted using Kolmogorov-Smirnov's test. If the normality test showed that the data was normally distributed ( $p > 0.05$ ), the variable will be presented in the mean and standard intersections, then the statistical analysis used parametric tests. If the normality test showed that the data were not normally distributed ( $p \leq 0.05$ ), the variable would be presented in the median and range (minimum and maximum values), then the statistical analysis would use a non-parametric test.<sup>14,15</sup>

## RESULTS AND DISCUSSION

In this study, 52 patients were diagnosed with diabetic nephropathy that met the inclusion criteria and became the subject of the investigation. The age range of the study subjects was 36-89 years with the highest proportion in the age range of 50-59 years (32.7%). A description of the characteristics of the research subjects is presented in Table 1.

Mean platelet volume value data were not normally distributed so the correlation test between MPV value and platelet aggregation used Spearman's correlation test. The results of the correlation test between MPV values and platelet aggregation at the level of confidence (confidence interval) 95% are presented in Table 2.

Based on Table 2, the Spearman correlation

analysis showed that there was a positive correlation between MPV values and platelet aggregation; the strength of the correlation  $r = 0.067$  and the value of  $p = 0.634$ . These results indicate a very weak positive correlation that was not statistically significant. A positive correlation means that there was a unidirectional relationship between MPV values and

platelet aggregation, i.e. the higher the MPV value the more platelet hyperaggregation and vice versa.

Research on the correlation between MPV values and platelet aggregation in patients with T2DM had been carried out with various results. Based on a literature search, this study was the first time in

**Table 1.** Characteristics of research subjects (n= 52)

Variable	Mean ± SD	Median (Min-max)	n (%)	Reference range
<b>Age (years)</b>				
30-40			2 (3.8%)	
40-50			10 (19.2%)	
50-60			17 (32.7%)	
60-70			15 (28.8%)	
≤70			8 (15.4%)	
<b>Sex</b>				
Male			27 (51.9%)	
Female			25 (48.1%)	
<b>Duration of T2DM</b>				
≤ 5 years			19 (36.5%)	
6-10 years			11 (21.2%)	
>10 years			22 (42.3%)	
<b>Body Mass Index</b>				
Underweight and normal			21 (40.4%)	
Overweight and obese			31 (59.6%)	
<b>Thrombocyte aggregation results</b>				
Hypoaggregation			3 (5.8%)	
Normoaggregation			26 (50.0%)	
Hyper-aggregation			23 (44.2%)	
<b>Hb level (g/dL)</b>	11.93 ± 1.794			
<b>Thrombocyte count (/uL)</b>		280500 (203,000 - 550,000)		150,000 - 450,000
<b>MPV (fL)</b>		9.2 (8.00 - 11.80)		6.8 - 10.4
<b>PDW (fL)</b>		9.30 (6.90 - 13.60)		9-14
<b>PCT(%)</b>		0.23 (0.15 - 0.48)		0.19 - 0.34
<b>P-LCR(10 - 30 %)</b>		18.45 (9.60 - 38.30)		10-30
<b>IPF(%)</b>		2.95 (0.80-9.60)		1.1 - 6.1

Note: SD = Standard Deviation; T2DM = Type 2Diabetes Melitus; MPV = Mean Platelet Volume; PDW = Platelet Distribution Width; PCT = Plateletcrit; P-LCR = Platelet-Large Cell Ratio; IPF = Immature Platelet Fraction

**Table 2.** The correlation between MPV values and thrombocyte aggregation

Research variable	$r_s$ (CI 95%)	p-value
MPV values and thrombocyte aggregation	0.067	0.634

Indonesia to analyze the correlation between MPV values and platelet aggregation in patients with diabetic nephropathy, because in the previous studies there were differences in both research subjects, research methods, examination instruments or data processing methods. The subjects of this study were aged 36-89 years with the highest proportion in the age range 50-59 years (32.7%). Various studies showed that the prevalence of T2DM increased with age and reached a peak in the 5<sup>th</sup> and 6<sup>th</sup> decades, as well as the complications of T2DM. These results were also similar to the results of the 2013 Riskesdas which received the highest DM prevalence at the age of 55-64 years. Aging was one of the risk factors for T2DM related to decreased insulin secretion by pancreatic  $\beta$  cells and the occurrence of insulin resistance.<sup>16,17</sup>

The duration of suffering from T2DM in this study was not limited, so it varied between 1 year to 36 years. Most of the study subjects (42.3%) suffered from T2DM over ten years, similar to the study of Ulutas *et al.* in 2014.<sup>18</sup> The duration of T2DM could be related to compliance, which was the patient's visit to the doctor. The study of El-khawaga *et al.* in 2015 in Egypt showed that research subjects with the length of T2DM  $\leq 5$  years had better compliance than the duration of T2DM over five years.<sup>19</sup> However, compliance is not only related to the adherence of controlling to the doctor but also to diet and exercise recommendations as recommended by PERKENI. Most of the research subjects (59.6%) had a BMI  $\geq 25$  kg/m<sup>2</sup> which included overweight and obese. This result was similar to the study of Shimodaira *et al.*<sup>7</sup> which obtained 63.4% of the study subjects had a BMI  $\geq 25$  kg/m<sup>2</sup>. Increased BMI indicated the presence of excess fat tissue, especially in visceral organs. Fat metabolism would produce free fatty acids, leptin, as well as various pro-inflammatory cytokines (adipokines), especially TNF- $\alpha$ , IL-6 which could trigger insulin resistance as the basis of T2DM pathogenesis. Thus obesity was a risk factor for T2DM. Based on hemoglobin examination results, the subjects of this study had a mean  $\pm$  SD of  $11.93 \pm 1.794$  g/dL and, the results were almost the same as that of Yenigün *et al.*<sup>5</sup> in 2014 which obtained a hemoglobin yield of  $12.13 \pm 1.12$  g/dL. Based on the results of platelet examination, the subject of this study had a median of 280,500/ $\mu$ L, the results were similar to the study by Yenigün *et al.*<sup>5</sup> in 2014 which received platelet results of 249,500/ $\mu$ L.

This study found 41 subjects (78.85%) suffering from anemia with hemoglobin level  $< 13.00$  g/dL, and one subject with severe anemia, with a Hb level of 7.1 g/dL.<sup>20</sup> Anemia in diabetic nephropathy occurs due

to erythropoietin deficiency, another thing that can play a role in the occurrence of anemia in diabetic nephropathy is iron deficiency, and inflammatory processes.<sup>21</sup> Astor *et al.* study in 2002, found a higher incidence of anemia (16-41%) patients of diabetic kidney disorders compared with patients with non-diabetic kidney disorders.<sup>22</sup> Horwich *et al.* in 2002, found that in patients with diabetic nephropathy, the incidence of anemia was associated with severe renal impairment.<sup>23</sup>

Based on platelet aggregation, the subjects of this study were grouped into platelet hypo-aggregation, platelet normal aggregation, and platelet hyper aggregation. In this study, the three groups had different proportions, 5.8% (3/52) platelet hypo aggregation, 50% (26/52) platelet normal aggregation, and 44.2% (23/52) platelet hyper aggregation. Based on literature searches, this study was the first time to classify patients with diabetic nephropathy into diabetic nephropathy with platelet hypo aggregation, platelet normal aggregation, and platelet hyper aggregation.

Subjects taking anti platelet drugs were excluded in this study because it could affect platelet aggregation. In this study information on anti platelet drugs was obtained based on interviews with research subjects, through leaflets in the form of pictures and trade names of the anti platelet drugs. However, the assessment was still subjective. Other factors could affect platelet aggregation that could not be excluded, such as fibrinogen levels, glycoprotein IIb-IIIa, and calcium ions, but in this study, no examination was carried out.<sup>24,25</sup> In the nephropathy group with platelet hyper aggregation had the smallest IPF value of 2.3% and the largest is 9.6%. This finding indicates that there was high thrombopoiesis activity in subjects with platelet hyper aggregation.

Several things could cause decreasing MPV values: platelet count, plateletcrit (PCT), disrupted fluorescent staining. Fluorescent staining was disrupted if in the blood of the study subject there was an antiplatelet monoclonal antibody.<sup>26</sup> In this study, confounding factors in fluorescent staining could not be removed. Platelet examination using flow cytometry method. The method used monoclonal antibodies such as PAC 128 which was an Ig-M monoclonal antibody that bound to fibrinogen receptors on platelets.<sup>27</sup> In this study flow cytometry method used two dyes, namely Polymethine dye to analyze reticulated platelets (immature platelets) and Oxazine dye. This dye will color platelet granules, RNA and platelet cell DNA. In the case of a platelet that was not intact, the dye would still color the platelets as a ghost cell.<sup>28,29</sup>

MPV values come from the comparison between plateletcrit (PCT) and platelet count. A decrease in MPV value would occur if plateletcrit (PCT) is low and platelet count is high. There are three conditions that caused an increase in platelet counts compare to the actual platelet count, such as fragmentocytes, microsporocytes, and non-human cell particles.<sup>28</sup> In this study, all of these things cannot be removed. In this study a median MPV value of 9.20 fL was obtained with a range of 8.00 fL - 11.80 fL, there were only seven subjects with MPV above normal. Of the seven subjects all platelet hyperaggregation. MPV value depended on thrombopoiesis, the more active thrombopoiesis, the higher the MPV value. Thrombopoiesis activity can be monitored through IPF parameters, if thrombopoiesis activity increased, there would be an increased IPF and vice versa. In this study, the IPF distribution was not normal with a range of 0.8% - 9.6%, with a mean value of 2.95%, and a mode of 3.25%. Based on the distribution of primary data, it showed that most subjects had a low IPF value, which meant that thrombopoiesis activity was not increased, there were not many immature platelets found, so the MPV value did not increase. Low thrombopoiesis activity in diabetic nephropathy can be caused by low thrombopoietin. Thrombopoietin is a hormone produced by the kidneys, which functions to stimulate platelet formation.<sup>29,30</sup>

## CONCLUSIONS AND SUGGESTIONS

There is no correlation between MPV values and platelet aggregation in patients with diabetic nephropathy. Mean platelet volume values cannot be used as a single parameter for the guidance of antiplatelet administration. Suggestions, Further research needs to be done to determine the factors that affect platelet aggregation in diabetic nephropathy, such as: thrombopoietin, fibrinogen levels, and calcium ions

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