

# Level of Circulating Endothelial Cells and Expression of Nuclear Factor Kappa Beta of Human's Peripheral Blood Mononuclear Cells in Subjects with Certain Conditions

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## ABSTRAK

**Tujuan:** untuk mendeteksi kadar circulating endothelial cells (CEC) dan ekspresi NFκB pada 3 kelompok subjek dengan kondisi tertentu. **Metode:** penelitian ini merupakan penelitian eksploratif menggunakan sampel darah perifer manusia. Subyek penelitian terdiri 3 kelompok yaitu kelompok orang sehat sebanyak 23 orang, kelompok yang memiliki satu atau lebih faktor risiko penyakit vaskular sebanyak 35 orang dan kelompok penderita penyakit vaskular (penyakit jantung koroner, diabetes melitus, strok) sebanyak 15 orang. CEC diisolasi dari peripheral blood mononuclear cell (PBMC). Kadar CEC diidentifikasi melalui pengukuran CD45 dan CD146 dengan metode flowcytometry. Ekspresi NFκB diketahui dengan metoda ELISA (imgenex, USA). **Hasil:** nilai rerata tertinggi kadar CEC ditemukan pada kelompok sakit (28,6%). Nilai rerata tertinggi ekspresi NFκB (924,9495 pg/ml) ditemukan pada kelompok dengan faktor risiko. Adapun rerata terendah ekspresi NFκB dan CEC ditemukan pada kelompok sehat. Analisis ANOVA pada rentang kepercayaan 95% menunjukkan bahwa terdapat perbedaan yang signifikan ( $p=0,00$ ) kadar CEC dan ekspresi NFκB kelompok sehat dengan kelompok risiko dan kelompok sakit. Sedangkan ekspresi NFκB dan kadar CEC pada kelompok risiko tidak berbeda signifikan ( $p>0,05$ ) dengan kelompok sakit. **Kesimpulan:** peningkatan kadar CEC dan ekspresi NFκB memiliki hubungan yang kuat dengan penyakit vaskular dan faktor risikonya.

**Kata kunci:** circulating endothelial cells (CEC), nuclear factor kappa beta (NFκB), peripheral blood mononuclear cell (PBMC), penyakit vaskular.

## ABSTRACT

**Aim:** to detect the levels of CEC and expression of NFκB in the three groups of subjects with certain conditions. **Methods:** this study is an exploratory study using human peripheral blood samples. The study subjects comprised three groups, the group of 23 healthy people, a group of 35 people that has one or more risk factors for vascular disease and a group of 15 vascular disease patients (coronary heart disease, diabetes mellitus, stroke). CEC were isolated from peripheral blood mononuclear cells (PBMC). CEC level is identified through the measurement of CD45 and CD146 by flowcytometry method. NFκB expression is recognized by ELISA method (imgenex, USA). **Results:** the highest average levels of CEC were found in the sick group (28.6%). The highest average expression of NFκB (924.9495) is found in the group with risk factors. The lowest average expression of NFκB and CEC is found in the healthy group. Statistical analysis of ANOVA at the interval confidence of 95%

shows a significant difference ( $p=0.00$ ) levels of CEC and NFκB expression between the healthy group with the group with the risk of cardiovascular disease (CVD) and patients with known CVD. **Conclusion:** increase of level CEC and NFκB expression has a strong relationship with vascular disease and its risk factors.

**Key words:** circulating endothelial cells (CEC), nuclear factor kappa beta (NFκB), peripheral blood mononuclear cell (PBMC), vascular disease.

## INTRODUCTION

Atherosclerosis is a disorder in the blood vessel wall which is characterized by blockage (atheroma), so that blood flow becomes impaired.<sup>1</sup> The causes of atherosclerosis include dyslipidemia, free radicals, inflammation, and endothelial dysfunction.<sup>2</sup> Endothelial dysfunction plays an important role in the pathogenesis, progression, and prognosis of the vascular disease. Another form of vascular disease is diabetes mellitus (DM). Hyperglycemic environment occurred in DM increases ROS production which leads to the oxidative state.<sup>3</sup> ROS is a destructive substance against vascular and cause endothelial dysfunction afterward. According to Landim (2009) and Verma (2003), endothelial dysfunction initiates atherosclerosis.<sup>4</sup> One of the markers of endothelial dysfunction is the increased number of circulating endothelial cells (CECs).

CEC identified as a marker of vascular damage in some diseases. Increasing number of CEC is correlated with disease activity.<sup>5</sup> CEC is found in the bloodstream due to various factors such as mechanical injury, subendothelial adhesion molecule, matrix protein binding defect and risk factors for atherosclerosis.<sup>6</sup> In this case the risk factor mentioned is the impact of ROS that causes hyperglycaemia and also hyperlipidemia especially LDL. Oxidized LDL is also cytotoxic and served as a chemotactic factor for monocyte resulting in accumulation of inflammatory cells. Inflammation happens because ROS activates the transcription factor, Nuclear Factor Kappa Beta (NFκB). NFκB is a transcription factor which is susceptible to oxidation reaction.<sup>7</sup>

NFκB plays an important role in chronic inflammatory process that occurs in atherosclerosis. Inflammation happens because NFκB is unintentionally activated.<sup>8,9</sup> NFκB activation is caused by variety of stimuli such

as local factors, namely vascular injury<sup>10</sup>, modification of LDL<sup>11</sup>, metabolic factors<sup>12,13</sup>, product and microbial agents<sup>14,15</sup>, cytokines<sup>16</sup> and T lymphocytes.<sup>9</sup> Product activation of NFκB initiates the process of atherosclerosis with endothelial dysfunction, occurrence of platelet adhesion, migration and proliferation of smooth muscle cells.<sup>14</sup> Increased levels of various inflammatory mediators were clearly seen in populations that were at risk of cardiovascular disease.<sup>17</sup>

This study aimed to detect and compare the level of CEC and NFκB in humans among the healthy group, the group with CVD risk factors and those who suffer from CVD.

## METHODS

This study is an exploratory study using human peripheral blood samples. The subject of the study as a whole amounted to 73 people divided into three groups. Group I, is a group of 23 healthy people, drawn from the population of students at Brawijaya University with the criteria of 25-35 years of age, not smoking, normal weight (BMI/normal body mass index), normal lipid profile, normal blood pressure and not diabetic, not having hypertension and infection. Group II is a group that has one or more risk factors for vascular disease. Risk factors mentioned in this study were age over 50 years of age, smokers, obese; suffer from hypertension or diabetes, and lipid profile test results showed hyperlipidemia (total cholesterol, LDL and triglycerides and high density lipoprotein (HDL) below normal above normal). There are 35 people included in this group II. They were taken from the employees at the Faculty who present at the medical examination at the main building. Group III is a group of 15 patients with vascular disease, who were already in the stage of stroke rehabilitation. Blood sampling is performed as

many as 7 cc for lipid profile examination, CEC and NFκB. CEC were isolated from peripheral blood mononuclear cells (PBMC). CEC levels identified through the measurement of CD45 and CD146 by flowcytometry method. Expression of NFκB (imgenex, USA) is identified by ELISA.

#### **Isolation and Identification of Circulating Endothelial Cell (CEC)**

CEC refers to the isolation in Blann et al (2005).<sup>18</sup> Peripheral blood samples were taken using syringes. Peripheral blood mononuclear (PBMN) cell is separated from the blood using Histopaque 1077. PBMN cells amounted to 100 μL were then incubated with 10 μL normal mouse serum (Sigma, USA) for five minutes and then re-suspended with 50 μL of anti human CD146-PE (SantaCruz, USA) and 50 μL CD45-PE (SantaCruz, USA) by comparison 1:100. Cells were incubated with antibodies for 20 minutes at room temperature. Samples were diluted up to 1 μL and read with flowcytometry (FACS Callibur, BD).

#### **Translocation Measurement of NFκB with ELISA Method**

Total amount of PBMC were 106 cells per ml, diluted with PBS containing MgCl, and resuspended 4 x vol with lyses buffer (10 mM KCl, 10 mM Tris Cl, pH 7.9 containing 1 mM PMSF DDT and 60 μm) in 4°C. Afterwards, it is incubated on ice for 20 minutes, and then homogenized with homogenizer. Centrifugation at 3000xg lasts for five minutes. Then, discard the supernatant. The pellet is washed two times with lysis buffer, 2 x vol. Resuspension of pellets uses extraction buffer 4 x vol, (50 mM Tris Cl pH 7.9, 0.42 M KCl, 5 mm MgCl, 0.1 mM EDTA, 20% glycerol, 10% sucrose, 2 mm DDT, 60 μm PMSF) which is stirred for 30 minutes and then centrifuged at 17,000 x g. Add (NH<sub>2</sub>)<sub>2</sub>SO<sub>4</sub> in the supernatant (0.33 g/mL), stir for 30 minutes and centrifuge for 10 min at 15,000 x g. Then, pellet is resuspended with TM 1M, 0.5 x vol, (50 mM Tris Cl pH 7.9, 0.1 M KCl, 12.5 mM MgCl, 0.1 mM EDTA, 20% glycerol, 1 mM DDT, 60μm PMSF). Two times dialysis on the 1M TM buffer and centrifugation at 15 x g last for 10 minutes. Take the supernatant containing the cell nucleus protein extracts.

In p50/p65 of NFκB translocation test, the first step is to determine the amount used in the micro titer well. To manufacture the standard curve, 100 μL assay buffer is pipetted into the wells (as well blank), 100 μL is pipetted of p50/p65 NFκB standards 1-7 into the mentioned well. For sample treatment, the thing to do is to pipette 100 μL of each sample treatment and collect it into the well. Micro titer is incubated at 37°C for two hours. Each well is washed with wash buffer 3x400 μL respectively for five minutes. Each well is filled with 100 μL of antibody, except the blank. Micro titer is incubated at 37°C for 1 hour. Each well was washed with wash buffer 3x 400 μL. Each well is filled 100 μL of conjugate, except in blank. Micro titer is incubated at 37°C for 30 minutes. Each well was washed with wash buffer 3x400 μL. TMB substrate as much as 100 μL is pipetted and added to each well, then incubated for 30 minutes at temperature room. Stop solution (HCl) as much as 100 μL is pipetted and added to each well for five minutes. Reading assessment of absorbency is done on the OD 492 nm.

The study was conducted after reviewed and approved by the research ethics committee of health, Faculty of Medicine of Brawijaya University number 069/EC/KEPK-JK/03/2011. All the study subjects were given information regarding the research which will be conducted (information sheet attached). After understanding the purpose of this study, the subjects were offered for willingness to follow, if they were willing then a consent form (informed consent attached) is given for signature. All recipients who were willing to follow the research were given the sheet to be filled based on interviews and direct measurement by the researchers, which include initials, age, gender, weight, height, blood pressure, history of disease (diabetes mellitus, hypertension, infection/TB, CVA ) and lifestyle (smoking, diet, exercise patterns).

The result of measurement of CEC and NFκB in each group differences were analyzed using ANOVA statistical test.

## RESULTS

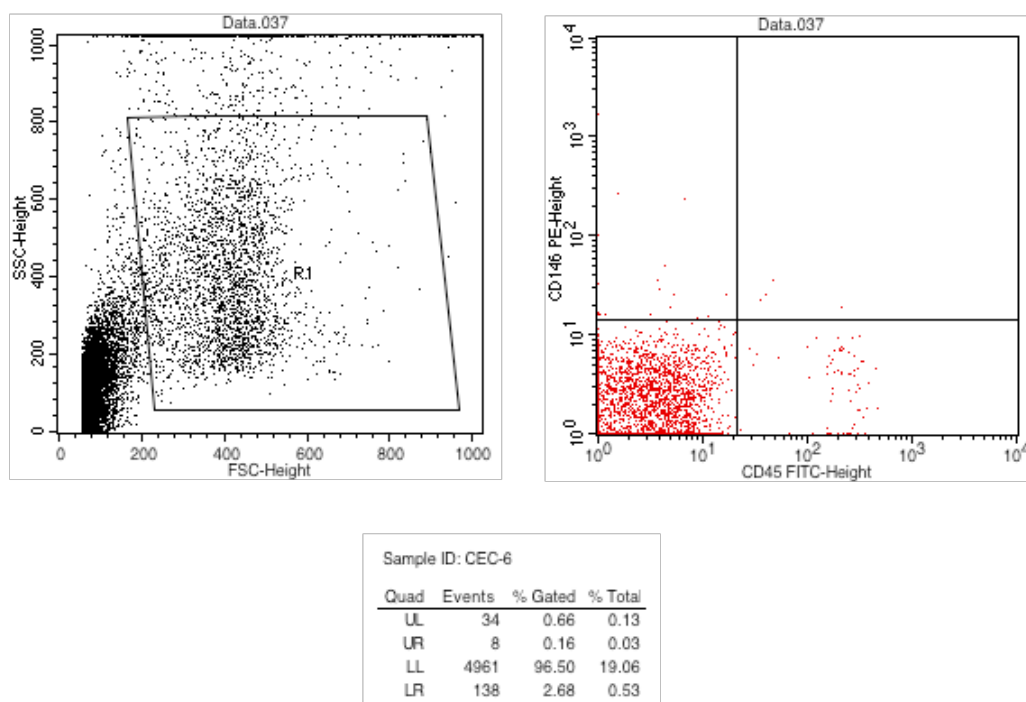
The highest average levels of CEC were seen in the sick group (28.6%) (**Table 1**), while the highest average of NFκB expression (644.80 pg/ml) was found in the group with risk factor (**Table 1**). The lowest average level of CEC and expression of NFκB was found in the healthy group. Statistical analysis ANOVA at the confidence interval 95% indicates that there is a significant difference ( $p=0.00$ ) CEC levels and expression of NFκB of the healthy group and the group with the risk group. While the CEC levels and expression of NFκB of the group with risk did not differ significantly ( $p>0.05$ ) with the sick group.

## DISCUSSION

CEC is a circulating endothelial cells participating along with blood flow or often defined as the expression of the membrane glycoprotein CD 146 and are rarely found in the blood of healthy individuals, but increased in patients with various conditions, such as inflammatory diseases, infections, metabolic disease and cardiovascular disease. The number of CEC was calculated by the method of flow cytometry (**Figure 1-3**). CEC Isolation refers to Blann et al (2005).<sup>18</sup> CEC are rarely or its number is very few in healthy people so that flow cytometry was chosen because of its high levels of sensitivity. To identify the CEC, another

**Table 1.** The comparison of circulating endothelial cells (%) level and nuclear factor Kappa beta expression (pg/mL) among population groups

Population group	CEC (%)		NFκB (pg/ml)	
	Mean ± SD	95% CI for Mean	Mean ± SD	95% CI for mean
Healthy Group	5.65 ± 3.95 <sup>a</sup>	3.94 - 7.35	166.64 ± 5.18 <sup>a</sup>	144.22 - 189.06
Risk Group	23.27 ± 9.42 <sup>bc</sup>	20.26 - 26.29	644.80 ± 2.53 <sup>bc</sup>	504.51 - 785.20
Sick Group	28.60 ± 6.60 <sup>cb</sup>	24.95 - 32.25	524.44 ± 3.52 <sup>cb</sup>	411.93 - 636.95



**Figure 1.** Flowcytometry of CEC using CD45-FITC marker and CD146-PE on a sample from healthy group

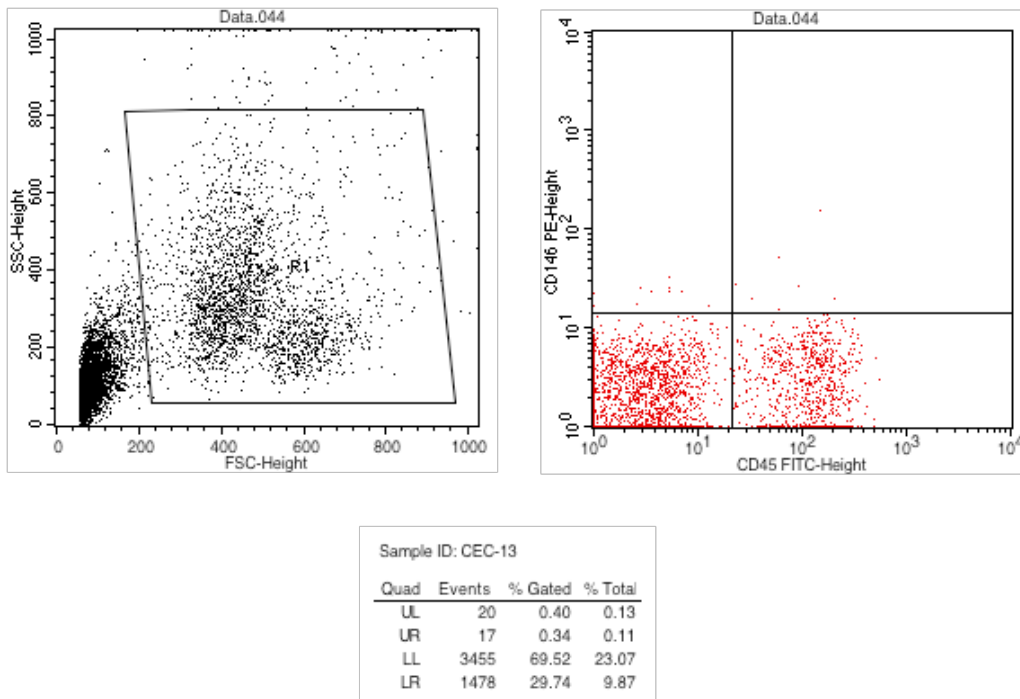


Figure 2. Flowcytometry of CEC using CD45-FITC marker and CD146-PE on a sample from risk factor group

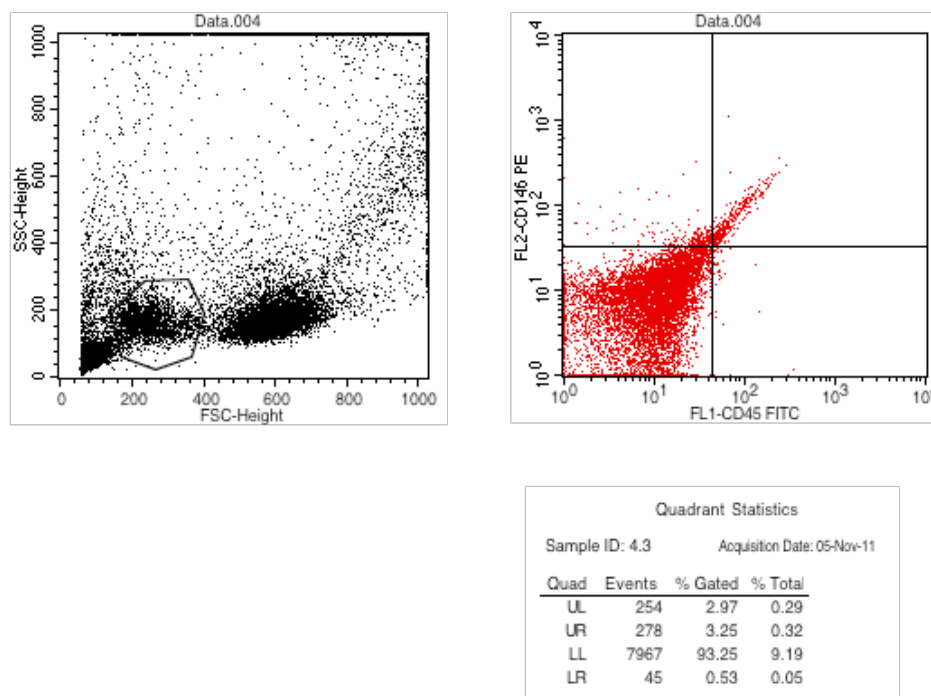


Figure 3. Flowcytometry of CEC using CD45-FITC marker and CD146-PE on a sample from sick group

marker is added to be more specific, namely the CD 45. CD 45 is a marker that exists in all white blood cells with varied intensity but not found in red blood cells. In relative relation, lymphocytes have a low SSC and high number of CD 45,

granulocytes have the high number of SSC and low CD 45 number, while monocytes' are among the granulocytes and lymphocytes. Based on the theory above, the part viewed from the results of flow cytometry is in the upper right or on the

lymphocyte area. This area is commonly called double positive because it shows both marker that are the number of CD 45 and CD 146 so that it is more representative of the number of CEC in the blood.<sup>19</sup>

Layer of endothelial cells constitute the vascular structure construction attached to the basal membrane (basement membrane). Endothelial cells overlay the inner part of the lumen from all blood vessels and acts as a connector between blood circulation and plain muscle cells of blood vessels. Cell of endothelium plays an important role in the vascular system because it facilitates a variety of complex functions of plain muscle cell of blood vessels and cells in the blood compartment. Various studies have shown that endothelial cells play an important role in the process of homeostasis that occurs through the integration of various chemical mediators. This system has a good effect on the plain muscle cells of blood vessels and blood cells that can cause a variety of changes such as vasodilatation or vasoconstriction that can regulate the blood supply needs of all human organs, growth and or changes in panoptic characteristics of the plain muscle cells of blood vessels, changes in pro-inflammation or anti-inflammation, maintaining blood viscosity and prevent bleeding.<sup>20</sup> Recent studies identify the dysfunction of Endothelia by measuring circulating endothelial cells (CEC).<sup>6</sup> In healthy condition, endothelial cells will remain in the basal membrane and less likely to experience detachment in the bloodstream in the form of circulating endothelial cells (CEC). Like the case in this study, the average of the lowest level of CEC (5.65%) and the average of the lowest level of NFκB expression (166.64) was found in the healthy group. The healthy group in this study is represented by a population of Brawijaya University students with criteria of 25-35 years of age, not smoking, healthy weight (BMI/normal body mass index), normal lipid profile, normal blood pressure and not diabetic, not having hypertension and infection. The result of the analysis by using one way of ANOVA notes significant differences of both CEC and the expression levels of NFκB between the healthy group and the other groups (the group with risk

and the sick group). The lowness of CEC level and expression of NFκB in the healthy group population showed that a small possibility of the occurrence of a vascular inflammatory processes that trigger endothelial dysfunction.

CEC was found in the bloodstream due to various factors such as mechanical injury, adhesion molecule of subendothelium, defect matrix protein binding and risk factors for atherosclerosis.<sup>6</sup> One of risk factors of atherosclerosis is DM because oxidative stress occurred within leads to vascular.<sup>21</sup> Mechanism of endothelial cell release is complex and influenced by many factors, including the mechanisms of endothelial injury, atherosclerosis risk factors and endothelial adhesion damage and apoptotic cell with decreased function of the cytoskeleton. The highest average level of CEC in this study was found in the sick group with CVD (28.6%). Pathological conditions such as dyslipidemia, ischemic heart disease, coronary angioplasty, and heart disease and lung cancer, such as acute myocardial infarction, stroke and hypertension increase the number of CEC.<sup>5</sup> In addition, other factors that cause the release of endothelial cells is the presence of interference with attachment factors endothelial cells, proteases or cytokines that mediate the release of endothelial, or due to apoptosis.

The highest average expression of NFκB (644.80 pg/ml) was found in the risk group. NFκB activation is induced by ROS (reactive oxygen species) of a product, one of which is generated due to dyslipidemia, hiperglycemia and other risk factors for atherosclerosis. Data of the group with risk (524.44 pg/ml) in this group showed a tendency of atherosclerosis. Factor of age is more than 50 years of age, smokers, obese; suffer from hypertension or diabetes, and the test result of lipid profile showed hyperlipidemia (total cholesterol, LDL and triglycerides above normal and HDL below normal). These conditions will increase H<sub>2</sub>O<sub>2</sub>, which causes the increase of a phosphate cluster so that bond of NFκB-IKB gets loose. This causes NFκB to move into the nucleus of the cell automatically, because the P50 has a nuclear localization signal (NLS) is inhibited when bound to IKBNFKB function as a transcription

factor genes, especially genes involved in the immune response and Inflammation and apoptosis.<sup>22</sup> Since the beginning NFκB is involved in the pathogenesis of atherosclerosis by stimulating inflammatory cells and molecules that facilitate adhesion and apoptosis which cause atherosclerotic plaque buried and also cause rupture of the plaque. The high level of NFκB expression of the group with risk showed that the activation of NFκB precedes endothelial dysfunction so that CEC levels are still relatively low compared to the sick group. NFκB plays an important role in chronic inflammatory process that occurs in atherosclerosis.<sup>9</sup> NFκB activation stimulates the expression of pro-inflammatory genes that initiate the occurrence of endothelial dysfunction, the occurrence of platelet aggregation, migration and proliferation of smooth muscle cells.<sup>14</sup> Increased levels of various inflammatory mediators is clearly seen in populations with risk factors of cardiovascular disease.<sup>17</sup>

## CONCLUSION

CEC and NFκB were detected in human PBMC of all groups. There is a significant difference of CEC levels and expression of NFκB between the healthy group and the group with the risk of vascular diseases and group of patients with known CVD. While the CEC levels and expression of NFκB in the groups with risk did not different significantly with the sick group.

## ACKNOWLEDGMENTS

This research is funded from the Indonesia ministry of research and technology.

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