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T. Bulduk[1], A. U. Yalcin[2], O. M. Akay[3], S. G. Ozkurt[2], H. U. Teke[3], G. Sahin[2], G. Temiz[2], G. Demirel[3]

Does erythropoietin therapy affect circulating endothelial cells in hemodialysis patients?

[1] Eskisehir Osmangazi University Medical School, Department of Internal Medicine, Eskisehir, Turkey
[2] Eskisehir Osmangazi University Medical School, Department of Nephrology, Eskisehir, Turkey
[3]Eskisehir Osmangazi University Medical School, Department of Hematology, Eskisehir, Turkey

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Abstract. *Anemia is a common complication of chronic kidney disease (CKD). The most common cause of anemia in CKD is erythropoietin deficiency; and the most important cause of mortality in CKD patients is atherosclerotic vascular complications which are associated with endothelial damage. One of the methods evaluating vascular integrity is the cytometric measurement of circulating endothelial cells and endothelial progenitor cells in peripheral blood. The study aimed to investigate the effects of erythropoietin therapy on endothelial dysfunction by evaluating circulating endothelial cells and endothelial progenitor cells in peripheral blood using the technique of flow cytometry.*

Methods. A total of 55 hemodialysis patients were evaluated in three groups; those having erythropoietin therapy for at least last 3 months (n = 20) / not having erythropoietin for at least the last 3 months (n = 20) and the patients who started erythropoietin treatment during the study (n = 5). The control group consisted of 20 people. Blood values of the 3rd Group were investigated three times as baseline, 2nd week and 8th week CD34 +, CD105 + cells were evaluated as activated circulating endothelial cells; CD133 +, CD146 + cells were evaluated as activated endothelial progenitor cells.

Results. There was no difference between the patients and healthy individuals in terms of circulating endothelial cells and endothelial progenitor cells. In the third group, no differences were observed in circulating endothelial cells / endothelial progenitor cell levels at baseline / 2nd and 8th weeks. There was no correlation between erythropoietin and circulating endothelial cells / endothelial progenitor cells.

Conclusion. A correlation is not available between the therapeutic doses of erythropoietin used in hemodialysis patients and circulating endothelial cells / endothelial progenitor cell levels; supratherapeutic doses could change the results.

Key words: *renal insufficiency, hemodialysis, erythropoietin, circulating endothelial cell.*

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Correspondence should be addressed to Tuba Bulduk: tuba.kiraz@hotmail.com

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Т. Булдук[1], А.У. Ялцин[2], О.М. Окай[3], С.Г. Озкурт[2], Х.У. Теке[3], Г. Сахін[2], Г. Теміз[2], Г. Демірель[3]

Чи впливає терапія еритропоетином на циркулюючі ендотеліальні клітини у хворих, які лікуються методом гемодіалізу?

[1]Медична школа Університету Ескішехір Османгазі, кафедра внутрішніх хвороб, Ескішехір, Туреччина
[2]Медична школа Університету Ескішехір Османгазі, кафедра нефрології, Ескішехір, Туреччина
[3]Медична школа університету Ескішехір Османгазі, відділення гематології, Ескішехір, Туреччина

Резюме. *Анемія є частим ускладненням хронічної хвороби нирок (ХХН). Найбільш частою причиною анемії у хворих на ХХН є дефіцит еритропоетину; тоді як основною причиною смертності є атеросклеротичні судинні ускладнення, асоційовані з ураженням ендотелію. Одним із методів оцінки цілісності судин є цитометричне вимірювання циркулюючих клітин ендотелію та клітин-попередників ендотелію в периферичній крові. Метою роботи було дослідити вплив терапії еритропоетином на дисфункцію ендотелію шляхом оцінки циркулюючих клітин ендотелію та клітин-попередників ендотелію в периферичній крові за допомогою проточної цитометрії.*

Методи. 55 пацієнтів з ХХН V ст, які лікуються гемодіалізом (ГД) були розподілені на 3 групи: ГД пацієнти, які отримували терапію еритропоетином принаймні протягом останніх 3 місяців (n = 20), ГД пацієнти, яким не призначався еритропоетин принаймні протягом останніх 3 місяців (n = 20) та пацієнти, які розпочали лікування еритропоетином під час дослідження (n = 5). Контрольна група складалася з 20 осіб. Значення крові 3-ї групи досліджували тричі як вихідні, 2-й тиждень та 8-й тиждень CD34 +, CD105 + клітини оцінювали як активовані циркулюючі ендотеліальні клітини; Клітини CD133 +, CD146 + оцінювали як активовані ендотеліальні клітини-попередники.

Результати. Не було різниці між пацієнтами та здоровими особами щодо циркулюючих ендотеліальних клітин та клітин-попередників ендотелію. У третій групі не спостерігали відмінностей у рівні циркулюючих ендотеліальних клітин / рівнів клітин-попередників ендотелію на початковому рівні / 2-му та 8-му тижнях. Кореляції між еритропоетином та циркулюючими ендотеліальними клітинами / клітинами-попередниками ендотелію не визначено.

Висновок. Не існує взаємозв'язку між терапевтичними дозами еритропоетину, що застосовуються для лікування анемії у ГД пацієнтів та циркулюючими ендотеліальними клітинами / клітинами-попередниками ендотелію; надтерапевтичні дози еритропоетину можуть вплинути на результат.

Ключові слова: *гемодіаліз, еритропоетин, циркулююча ендотеліальна клітина.*

Introduction. Anemia is an early and frequent complication of Chronic Kidney Disease (CKD) and is one of the most important reasons that adversely affect the quality of life. Approximately 50% of the pre-dialysis patients and 80% of the dialysis patients are anemic [1]. The most common cause of anemia in renal failure is decreased erythropoiesis due to erythropoietin (EPO) deficiency [2]. The EPO which has been used for about 25 years, which is a development that can be considered a revolution in the treatment of CKD anemia, has increased the quality of life in patients with CKD.

Despite the beneficial effects of EPO, such as a decrease in angina symptoms, decrease in myocardial ischemia, decrease in left ventricular hypertrophy, increase in mental and cognitive functions, improvement in physical activity and quality of life, decrease in blood transfusion requirement, significant side effects, such as aggravation of hypertension, and thrombosis tendency are also present [3, 4].

Tuba Bulduk
tuba.kiraz@hotmail.com

The most important cause of mortality in CKD is atherosclerotic vascular complications [5]. In dialysis patients over 45 years of age, 87% have cardiovascular disease reported at the time of end-stage renal disease (ESRD) onset, and approximately 50% of deaths are attributed to cardiovascular disease [6]. The cause of this increase in mortality is not clear but is associated with endothelial damage. Clinically endothelial dysfunction concludes with vasospasm, thrombosis, hypertension, and atherosclerosis.

For measurement of endothelial function, angiography, acetylcholine (Ach) infusion response, venous occlusion plethysmography, arterial stiffness measurement, vascular ultrasonography, levels of endothelium-derived molecules are used. One of the frequently used methods for evaluating vascular integrity is Circulating Endothelial Cell (CEC) and Endothelial Progenitor Cell (EPC) measurements [7]. CEC and EPC are cells involved in the vascular injury and repair process. Circulating endothelial cells were first obtained from leukocyte concentrates of patients with tumors in the

1960s. During the following 25 years, several reports have been published showing the increase in CEC in various pathological conditions [8, 9].

CECs are identified by specific endothelial markers, such as CD144 or CD146. Since CECs are markers of severe endothelial damage, they are thought to play a role in the pathophysiology of cardiovascular and inflammatory diseases. The basic method of determining EPC is flow cytometry and culture. The common idea of the literature is that the EPCs originate from the bone marrow and regulate endothelialisation [10, 11]. Literature has shown that EPCs play a role in maintaining vascular integrity and repairing damaged tissues. CEC can be used as a marker of vascular damage and cardiovascular outcomes. Researches have shown that the increase in CEC counts correlates with the severity of endothelial damage [7].

The purpose of the study was to evaluate the relation between the use of EPO and endothelial dysfunction through circulating endothelial cells in patients with ESRD undergoing hemodialysis therapy.

Material and Methods. The study was initiated prospectively in 2012 and was performed in the Nephrology Clinic of Eskisehir Osmangazi University Medical Faculty Hospital Internal Medicine Department. All patients were informed about the study, and written consents were obtained from the patients stating that they agreed to participate in the study. Approval for the study was obtained from the local ethics committee by the decision dated October 31, 2011 and numbered 2011/37.

The study samples were selected from patients receiving hemodialysis treatment for the patient group, and from healthy humans for the control group. Hemodialysis treatment with standard bicarbonate dialysate was applied 3 times per week for about 4 hours at a time. The study groups were determined as follows: Group-1 consisted of 20 ESRD patients with a Hb value above 10 g/dL who were treated with EPO for at least 3 months, Group-2 consisted of 20 ESRD patients with a Hb value above 10 g/dL who were not treated with EPO for at least 3 months, Group-3 included 15 ESRD patients who did not use EPO for at least 3 months but started EPO during the study due to the need for EPO, Group-4 consisted of 15 healthy people. The EPO treatment was EPO α and β, and the initial dose was 75-150 IU/kg/week and the ongoing dose was 25-75 IU/kg/week. They also received iron supplements in the direction of the guidelines' recommendation. People in the healthy group did not have any disease stories and did not use any medication. Blood was taken for CEC, EPC, CBC, ferritin, C-Reactive Protein (CRP) to evaluate baseline values (week 0) for all groups; and in the third group, the samples were taken again in the acute phase (week 2) and chronic phase (week 8). Patients who had the acute coronary or acute cerebrovascular event in the last 6 weeks, active infection, active malignancy or malignancy history, hepatitis B, C, HIV infection, decompensated liver disease, surgical intervention, burn or severe trauma within the past 1-month and those using angiotensin-converting enzyme inhibitor/angiotensin receptor blocker, statin, steroid, and non-steroidal anti-inflammatory drugs were excluded from the study.

Patients' hemodialysis treatment periods and smoking habits were noted. Patients were questioned for diabetes mellitus, hypertension, peripheral artery disease, coronary artery disease, heart failure, arrhythmia, pulmonary disease (chronic obstructive pulmonary disease, asthma, asbestosis, and occupational pulmonary disease), cerebrovascular disease and endocrinological disease. The medicines used by the patients were also recorded. Pre - and post-HD systolic-diastolic blood pressures of patients were recorded. Body Mass Indexes (BMI) was calculated by weight (kg) / height (m^2) formula. Blood panel, biochemical parameters (sodium (Na), potassium (K), calcium (Ca), phosphorus (P), glucose, blood urea nitrogen (BUN), creatinine (Cr), uric acid, lipid profile (Low-density lipoprotein cholesterol (LDL-C), High-density lipoprotein cholesterol (HDL-C)), albumin (alb), alanine aminotransferase (ALT), alkaline phosphatase (ALP), parathyroid hormone (PTH), iron (Fe), iron saturation, total iron-binding capacity (TDBC), ferritin, CRP, sodium bicarbonate (HCO3) were studied from all patients and Kt/V ratios were calculated.

Blood samples for laboratory examination were taken just before the midweek hemodialysis session. Blood samples that were taken from hemodialysis artery catheter were placed in tubes containing EDTA for Hemoglobin, CEC and EPC, tubes containing gel for biochemical parameters, and were processed without waiting. Mouse IgG2 FITC, Mouse IgG1 PE, CD105 FITC, CD34 PE, CD146 FITC, CD133 PE and CD45 Per Cp were used as circulating endothelial cell markers; and 2 cc blood of the patients were placed in K3 EDTA tubes (12x75 mm falcon polystyrene round-bottom tube), and 100'er μ / L of this blood was placed in 3 different antibody tubes.

Tube 1 contained MIgG1 FITC (fluorescein isothiocyanate), Mouse IgG1 PE (phycoerythrin), CD45 PerCp (peridinin chlorophyll protein); tube 2 contained CD105 FITC, CD34 PE, CD45 Per Cp; and tube 3 contained CD146 FITC, CD133 PE, and CD45 Per Cp. After 30 minutes of incubation in the dark at room temperature, 2 cc of diluted lysine solution was added to remove the erythrocytes from the medium by lysis and waited for 10 minutes. It was centrifuged at 1800 rpm for 5 minutes. The resulting supernatant was poured off and washed with 2 cc of PBS (phosphate-buffered saline). It was centrifuged again at 1800 rpm. The resulting supernatant was poured off and washed with 2 cc of PBS followed by centrifugation at 1800 rpm (total 2 washes). The remaining pellet was re-suspended in 500 μl PBS.

Bacton Dickinson Flow Cytometry (FACS Calibur) was analyzed by counting 50.000 cells in the CellQuest program. In the analysis: CD34 + 105- cells were considered as resting CEC; CD34 + 105 + cells as activated CEC; CD133 + cells as resting EPC; CD146 + 133 + cells as activated EPC cells.

Statistical analysis. All data analyzes were performed with SPSS 20 and SigmaStat 3.5 package programs. Continuous quantitative data were expressed as mean and standard deviation, and qualitative data were expressed as n, median, 25th and 75th percentiles. Continuous data consisting of independent measurements and showing normal distribution were analyzed with One Way Anova test (Multiple comparisons of this test were performed using Student Newman Keuls Method and Tukey tests). Data consisting of score variables with non-normal distribution were analyzed by Kruskal Wallis (Multiple comparisons of this test were performed using Dunn's Method and Student Newman Keuls Method) and Mann-Whitney U test according to group numbers. Spearman Correlation tests were used to show the relationship between the variables according to the normality test results. Chi-square tests were applied to categorical datasets.

Results. 10 female and 10 male patients with a mean age of 50.2 ± 6.2 years were included in Group-1; 10 female and 10 male patients with a mean age of 56.2 ± 9.6 years were included in Group-2; 5 female and 10 male patients with a mean age of 58.3 ± 7.1 years were included in Group-3; 10 female and 10 male healthy people with a mean age of 42.1 ± 6.0 were included in Group-4 for control. Age distribution by groups was statistically significant (Kruskal Wallis = 35,285; $p < 0.001$). In the sub-analysis, it was found that this significant difference was between the 2nd and 4th Groups ($p = 0.0001$), between the 3rd and 4th Groups ($p = 0.0001$), between the 1st and 3rd Groups ($p = 0.024$). Age differences in sub-analyzes of other groups were not statistically significant. According to the groups, height, weight, BMI and gender distributions were not statistically significant (Table 1).

Table 1

Demographic characteristics of the study participants

	Group 1	Group 2	Group 3	Group 4	p
Age (years)	50.2 ± 6.2	56.0 ± 9.3	58.3 ± 7.1	42.1 ± 6.0	0.001
Height (cm)	165.1 ± 7.5	165.5 ± 4.6	168.0 ± 6.5	166.0 ± 8.2	0.625
Weight (kg)	66.3 ± 12.0	68.1 ± 9.7	73.5 ±11.0	75.3 ± 14.0	0.066
BMI	24.4 ± 3.7	25.2 ± 4.0	26.2 ± 3.8	27.1 ± 3.5	0.126
F/M	10/10	10/10	5/10	10/10	0.720

The data is presented as M±SD and compared using the Kruskal Wallis test.

*Sub-analysis results are detailed in the text

SD: standard deviation, cm: centimeter kg: kilogram BMI: body-mass index, F: female, M: male

There were 20 Diabetes Mellitus (DM) patients (36.3%), and 28 hypertension (HT) patients in the study. There was no difference in DM and HT frequency between patient groups ($p = 0.397$, $p = 0.501$, respectively). Hyperlipidemia, cerebrovascular accident and deep vein thrombosis were not seen in any case. The distribution of cigarette smoking, alcohol use, coronary artery disease, pulmonary thromboembolism rates according to the groups was not statistically significant ($p > 0.05$).

There was no difference in hemodialysis administration times between patient groups in terms of URR and Kt/V ($p = 0.817$, $p = 0.796$ and $p = 0.476$, respectively).

When the patient groups were compared in terms of laboratory parameters including Fe, iron saturation, ferritin, HCO3, HDL, LDL, triglyceride (TG), BUN and Cr (before dialysis), Cr (after dialysis), Ca, P, Alb, CRP, PTH, ALT, uric acid, glucose, biochemistry values; it was seen that there was no difference ($p > 0.05$)

When Hb levels were evaluated, it was seen that there was a significant difference between the groups. Hb levels of Group-2 were higher than Group-1 and 3, while Hb level in Group-3 was the lowest ($p < 0.0001$) (Table 2).

Table 2

Comparison of laboratory parameters of patient groups

	Group 1	Group 2	Group 3	p
LDL (mg/dL)	83.9±34.1	87.9±26.8	88.2±31.1	0.892
TG (mg/dL)	225.4±137.9	170.3±86.1	172.5±72.3	0.389
BUN (before HD) (mg/dL)	55.6±12.2	60.3±13.2	52.3±11.5	0.133
Cr (before HD) (mg/dL)	7.9±2.4	7.8±2.4	7.2±1.9	0.606
Calcium (mg/dL)	9.4±0.7	8.8±0.9	9.1±0.7	0.079
Phosphorus (mg/dL)	5.3±1.1	4.7±1.7	4.1±1.2	0.039

Table 2 continuation

	Group 1	Group 2	Group 3	p
Albumin (g/dL)	4.0±0.4	3.9±0.3	3.8±0.4	0.267
PTH (pg/mL)	325.2±300.0	267.7±211.5	240.7±205.2	0.821
ALT (U/L)	17.5±12.9	12.1±5.2	13.6±4.7	0.0519
Uric acid (mg/dL)	5.9±1.1	5.7±1.2	6.0±0.9	0.740
Glucose (mg/dL)	117.6±49.7	154.2±83.3	156.5±88.8	0.329
Bicarbonate (mEq/L)	23.0±1.9	23.4±5.3	23.3±4.1	0.803
CRP (mg/dL)	9.3±7.2	7.6±5.1	10.2±13.6	0.088
Hemoglobin (gr/dl)	11.2±0.5	12.9±0.8	10.4±0.8	0.0001
Ferritin (ml/ng)	739.7±428.7	558.8±225.5	520.4±319.2	0.194
Iron saturation (%)	39.8±49.3	27.8±11.1	24.1±9.0	0.581

The data is presented as M±SD and compared using the Kruskal Wallis test.

SD: standard deviation, BUN: blood urea nitrogen, HD: hemodialysis, Cr: creatinine, LDL: Low-density lipoprotein, TG: triglyceride, PTH: Low-density lipoprotein, ALT: alanine aminotransferase, CRP: C-reactive protein.

When resting/activated CECs and resting EPCs levels were evaluated, there was no difference in both patient groups and between patient groups and control groups ($p > 0.05$) (Table 3). Activated EPCs levels were too low and were not included in statistical analysis.

Table 3

Comparison of baseline values of resting / activated CEC and EPC levels

	EPO + (1st group)	EPO – (2nd group)	EPO started (3rd group)	Control (4th group)	P
	n=20	n=20	n=15	n=20	
Resting CEC	0.55 (0.36-1.68)	1.26 (0.89-2.13)	1.36 (0.73-2.41)	1.32 (0.80-2.23)	0.108
Activated CEC	0.74 (0.41-1.82)	0.66 (0.36-1.72)	0.78 (0.49-1.93)	0.71 (0.47-1.86)	0.328
Resting EPC	1.94 (1.12-3.22)	2.53 (1.35-3.71)	2.92 (1.80-3.98)	3.10 (1.92-4.75)	0.395

EPO: erythropoietin, CEC: Circulating endothelium cell, EPC: Endothelial progenitor cell. Median was shown as (25% -75%) and the Kruskal Wallis test was used in the intergroup comparisons.

There was no difference in gender, age, smoking status, DM / HT presence or absence at resting/ activated CECs and resting EPCs levels ($p > 0.05$). Resting EPCs levels were higher in the presence of a history of Coronary Artery Disease (p values: 0.04) (Table 4). Activated EPCs levels were too low and were not included in statistical analysis.

Table 4

Comparison of resting / activated CEC and EPC levels with sex, age, smoking, diabetes mellitus, hypertension, coronary artery disease presence

		n	Rest-CEC	Activated -CEC	Rest-EPC
Gender	Female	35	1.12 (0.54-2.15)	0.13 (0.00-0.27)	2.53 (1.60-3.66)
	Male	40	1.32 (0.64-2.18)	0.25 (0.00-0.46)	2.79 (1.45-4.13)
P			0.754	0.516	0.610
Age	<45 years	19	1.30 (0.72-1.77)	0.25 (0.00-0.53)	3.24 (1.68-4.79)
	≥45 years	56	1.15 (0.55-2.20)	0.16 (0.00-0.29)	2.53(1.19-3.59)
P			0.932	0.483	0.182
Smoking	no	63	1.17 (0.56-2.15)	0.15 (0.00-0.36)	2.57 (1.41-3.66)
	yes	12	1.06 (0.56-2.16)	0.25 (0.03-0.51)	2.89 (1.65-4.13)
P			0.800	0.469	0.665

Table 4 continuation

		n	Rest-CEC	Activated -CEC	Rest-EPC
Diabetes mellitus	no	55	1.21 (0.69-2.15)	0.18 (0.00-0.36)	2.80 (1.60-3.64)
	yes	20	0.99 (0.46-2.26)	0.10 (0.00-0.45)	2.12 (1.12-4.13)
P			0.323	0.619	0.952
Hypertension	no	47	1.34(0.53-2.25)	0.24 (0.00-0.36)	2.80 (1.87-3.64)
	yes	28	1.09 (0.64-1.71)	0.11 (0.00-0.41)	1.94 (0.96-5.35)
P			0.450	0.626	0.352
Coronary artery disease	no	69	1.17 (0.54-2.17)	1.13 (0.00-0.13)	2.53 (1.38-3.65)
	yes	6	1.14 (0.74-2.17)	0.38 (0.19-0.86)	4.09 (2.79-7.32)
P			0.792	0.089	0.040

There was no difference in the weekly comparison of activated / resting CEC and resting EPC values in group-3, in which EPO was started (Table 5). Activated EPCs levels were too low and were not included in statistical analysis.

Table 5

Weekly comparison of activated / resting CEC and EPC values in the EPO group (Group 3)

	N	Median
Resting CEC baseline	15	1.36
Resting CEC 2. week	15	0.84
Resting CEC 8. week	15	0.88
p	Friedman test = 1.2; p=0.549	
Activated CEC baseline	15	0.27
Activated CEC 2. week	15	0.05
Activated CEC 8. week	15	0.01
p	Friedman test = 4.76 ; p=0.092	
Resting EPC baseline	15	2.92
Resting EPC 2. week	15	2.85
Resting EPC 8. week	15	3.50
p	Friedman test =0.13; p=0.936	

CEC: Circulating Endothelial Cell; EPC: Endothelial Progenitor Cell

When baseline Hb on the 2nd week and 8th week Hb levels were evaluated in the group treated with EPO, there was a difference between weeks. However, since this difference was too low (p = 0.049), the Tukey Comparison Test was conducted to determine the groups with a difference. This test showed that this difference was between the baseline Hb value (median 10.5 gr/dl) and the 8th week Hb value (median 10.6 gr/dl) (p=0.022).

When the correlation analysis of baseline values in the patient groups was evaluated. significant negative correlations was found between resting/activated CECs and albumin (in resting CEC / Albumin r: -0.270. p = 0.046 and inactivated CEC / Albumin r = -0.312. p = 0.020). In addition, a significant relationship was found between baseline activated CEC and baseline hemoglobin level (r = -0.601, p = 0.018) but there was no correlation between hemoglobin levels at 2th and 8th weeks and activated CEC.

Discussion. There are a number of studies on chronic renal disease related to anemia. endothelial dysfunction. circulating endothelial cells and endothelial progenitor cells [**7, 12-14**] but only a few studies are investigating endothelial cell relation with erythropoietin. Therefore. we aimed to evaluate the relationship between the use of erythropoietin and endothelial dysfunction through circulating endothelial cells in patients with chronic renal failure who underwent HD treatment in our study.

There were no differences in the presence of diabetes mellitus, hypertension, coronary artery disease, pulmonary thromboembolism and chronic obstructive pulmonary disease in the HD patients. Furthermore, there were no differences in the distribution of the majority of examined blood markers and Kt/V.

Although Ayala et al. have demonstrated that CEC levels in CKD patients were higher than healthy controls [**15**], Eizawa et al and Choi et al have found that

EPC levels in HD patients were lower than in healthy controls [16, 17]. Another study has been conducted by Groot et al which consisted of 46 hemodialysis patients and 46 healthy control individuals of similar age and sex, supported the role of uremia and Kt/V in EPC physiology. In the same study, the increase in EPC level after renal transplantation with the improvement of urea level strengthens the relationship between urea and EPC [18]. It also shows us that the kidney is a complex and indispensable organ. not only with the filtering functions but also with the endocrinological functions at last. Unlike these studies. in a study conducted by Herbrig et al. EPC levels were significantly higher in hemodialysis patients compared to healthy controls. When migrator activity and adhesion properties were evaluated in the same study, the decrease in the migration feature was found to be incompatible with the increase in the number of EPCs [19]. We also found no difference in the levels of resting / activated CEC and resting EPC between the patients' group and the control group. In addition, there was no difference between the patient groups in our study. However, we found that resting EPC levels were higher in the presence of a Coronary Artery Disease story. In a similar study conducted by Guven et al, in 48 patients. there was no relationship between serum creatinine level and EPC, although EPC levels were associated with Coronary Artery Disease [20]. The different results in the literature and the contradictory results of the same study using the same technique show that the methods used in the evaluation of circulating endothelial cells and the properties of the cells have a significant effect on the results. The reason why the difference between healthy controls and CKD patients was not detected in terms of circulating endothelial cells may be a quantitative assessment of circulating endothelial cells only using flow cytometric analysis. It was also the reason why there was no correlation between uremia levels Kt/V and circulating endothelial cells in patient groups. When additional methods. such as cell culture for evaluating migration. proliferation and survey and flow-mediated dilation for evaluating endothelial function are used, the possible relationship between uremia and circulating endothelial cells and endothelial can be determined. which is shown in many studies.

Both activated and resting CEC levels were found to be higher in the third group than in the other patient groups and healthy controls. but this difference was not statistically significant ($p = 0.108$; $p = 0.328$, respectively). If we remember that Group-3 was with baseline Hb level <10 gr/dl and therefore EPO was started in this group; it may be possible to associate high levels of CEC in Group-3 with anemia. In the correlation analysis of Group-3, there was a negative correlation between baseline hemoglobin and baseline activated CEC ($r = -0.601$, $p = 0.018$). Accordingly. it can be said that as Hb decreases. the level of CEC increases. This result is compatible with studies in which circulating endothelial cells were found to be higher in patients with sickle cell anemia and thalassemia than controls [21- 23]. The decrease in resting (1.36-0.84-0.88, respectively) and activated CEC levels (0.27-0.05-0.00 respectively) at baseline, 2nd and 8th weeks is consistent with the increase Hb levels in Group-3 with new-onset EPO treatment at the 2nd and 8th week according to baseline Hb (median values 10.5-10 and 6-10.6, respectively). It strengthens the idea that high CEC levels may be related to anemia. However, in our study, the increase in Hb at 8 weeks was found to be significant at $p = 0.049$. When there is a more significant difference between the Hb values in the group with new-onset EPO treatment, the relationship between CEC- Hb and EPO is thought to be more evident.

Since it is known that the EPC response to EPO is dose-dependent. the differences in doses of EPO used may also contribute to conflicting results of many studies investigating the relationship between EPO and EPC [24]. Our study was an observational study and the patients' natural treatment processes did not interfere and the doses of EPO were not homogeneous. The reason why the difference is not detected between EPO and EPC may be due to the different doses of EPO that was used. It is thought that different results may be encountered when used over therapeutic doses.

When the patient groups in our study were evaluated, there was a significant negative correlation between resting/activated CECs and albumin ($r = -0.270$, $p = 0.046$ and $r = -0.312$, $p = 0.02$, respectively). In the study of Sridevi et al conducted in 2011, a negative correlation between albumin and CEC has been found in mice, consistent with our study [25]. Malnutrition and inflammation are severe in HD patients. and are important determinants of morbidity and mortality [26]. If we remember that albumin is a negative inflammatory marker and an important indicator of nutritional status, to say that low albumin levels associated with the nutritional status of CKD patients may lead to an increase in CEC levels and may be a correct interpretation in terms of a cause-effect relationship.

Furthermore, when the data of the group with new-onset EPO treatment were examined, a significant negative correlation was found between resting/activated CECs and albumin baseline values ($p = 0.027$ and $p = 0.012$, respectively). However, this relationship which was observed in baseline values just before the start of EPO therapy was not detected at the 2nd and 8th weeks of EPO use. When we look at the baseline, 2nd week and 8th-week albumin values in the group with new-onset EPO treatment. we can see that there is no difference between albumin levels. When we looked at the baseline, 2nd week and 8th-week CEC values of this group, it was not statistically significant. but we see that there is a decrease in CEC levels as the week progress. The reason why the relationship between low albumin and high CEC in baseline values is not seen in the following weeks. and a decrease in CEC levels despite there is no change in albumin level may be related to the use of EPO which was at 2nd and 8th weeks. but is not initial.

In our study, there was no difference in CRP levels in terms of patient groups. and there was no correlation between CRP levels and rest / activated CECs & rest /activated EPCs. In agreement with our study, no correlation between CRP and EPC in patients and healthy controls have reported by Groot et al in their study [18]. There was no relationship between CRP and white blood cell count and EPC in the study conducted by Guven et al [20]. Similarly, Schlieper et al. have found no relationship between CRP and EPC in the study conducted with 65 HD patients [27].

There are some limitations to our study. There were age differences between the groups and the endothelial function was not confirmed with functional methods such as flow-mediated dilation.

Conclusions. In conclusion, we found no relations between the therapeutic dose of EPO treatment in HD patients and CEC and EPC levels used as indicators of endothelial dysfunction. The relationship between EPO and endothelial dysfunction can be assessed clearly in long-term studies involving more patients with evaluating EPO's proliferation and mobilization properties as well as EPO correlations with CEC and EPC numbers.

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Author contributions.

T. Bulduk: literature search, blood sampling, collecting, analyzing and interpreting the data, writing the manuscript;

A. U. Yalcin: concept, supervision, management of the research, critical review and data analysis;

O. M. Akay: consultation, literature searching;

S. G. Ozkurt: article redaction, critical review, preparation for submission;

H. U. Teke: consultation and article redaction;

G. Sahin: consultation;

G. Temiz: consultation;

G. Demirel: analyzing the flow cytometry.

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