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The endothelial status reflected by circulating endothelial cells, circulating endothelial progenitor cells and soluble thrombomodulin in patients with mild and resistant hypertension

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ABSTRACT

Background: The aim of the present study was to evaluate endothelial status by measuring the concentration of novel markers of endothelial dysfunction (ED): a number of circulating endothelial cells (CECs), circulating endothelial progenitor cells (CEPCs) and their ratio (CEPCs/CECs) as well as a traditional parameter - soluble thrombomodulin (sTM) in patients with resistant (RH) and mild hypertension (MH).

Materials and methods: Thirty patients with MH and thirty subjects with RH were involved in the study. The control group included thirty-three age and sex-matched normotensive volunteers. We used multicolor flow cytometry for CECs and CEPCs analysis and the commercial human sTM ELISA kit to measure plasma sTM concentration.

Results: An elevated CECs number and a decreased CEPCs/CECs ratio was found in MH as well as in RH patients in comparison with normotensive volunteers. CECs correlated positively with an increased triglycerides in MH patients and an elevated LDL-cholesterol and hsCRP in RH group. Positive correlation between CEPCs and LDL-cholesterol level was observed in both types of hypertension.

Conclusions: The results of the present study suggest that an endothelial alteration accompanies hypertension. The number of CECs reflecting the extent of endothelial damage does not appear to be related to the severity of disease. The drastically decreased ratio between CEPCs and CECs observed in both groups of patients suggests an inadequate process of endothelial regeneration. Among analyzed factors inflammation and lipid abnormalities may have significant contribution in endothelial pathology in hypertension.

1. Introduction

Hypertension is the most common chronic disease in the Western world, with an estimated prevalence in the adult population of > 25% [1] and a major risk factor for cardiovascular and renal diseases, including coronary artery disease, heart failure, stroke, chronic kidney disease (CKD), and death [2–4]. Resistant hypertension (RH) represents extreme phenotype of hypertension, characterized by high uncontrolled

Abbreviations: BMI, body mass index; CECs, circulating endothelial cells; CEPCs, circulating endothelial progenitor cells; CKD, chronic kidney disease; Cr, creatinine; DBP, diastolic blood pressure; ED, endothelial dysfunction; eGFR, estimated glomerular filtration rate; FMD, flow mediated dilation; HDL-C, HDL-cholesterol; HGB, hemoglobin; hsCRP, highly sensitive C-reactive protein; IL-6, interleukine 6; K, potassium; LDL-C, LDL-cholesterol; LYMPH, lymphocytes; MH, mild hypertension; MONO, monocytes; Na, sodium; NEUT, neutrophils; oxLDL, oxidatively modified LDL; PLT, blood platelet; RBC, red blood cells; RH, resistant hypertension; SBP, systolic blood pressure; sTM, soluble thrombomodulin; TAG, triglycerides; TC, total cholesterol; TOD, target organ damage; U, urea; UA, uric acid; vWf, von Willebrand factor; WBC, white blood cells

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blood pressure (BP) despite sustained therapy with at least 3 different classes of antihypertensive medications, including a diuretic agent. RH is associated with earlier changes in organ systems in the body, such as left ventricular hypertrophy (LVH), proteinuria and renal failure, retinopathy and vascular dementia which are grouped under the term target organ damage (TOD) [5–7]. Although a precise cascade of events from the development of hypertension to adverse cardiovascular complications and TOD remains to be elucidated, endothelial damage and/or dysfunction (ED) seems to have a great contribution in their initiation [8–11]. ED defined as an alteration of the endovascular lining of blood vessels and characterized by a pro-thrombotic, pro-inflammatory and pro-constrictive phenotype is a common feature of patients with hypertension including those with RH [12–14]. There is evidence that RH patients have worse ED resulting in an increased arterial stiffness than well-controlled hypertensive patients, which may explain their higher susceptibility to complications, including stroke, acute aortic dissection, myocardial infarction, congestive heart failure and renal failure when compared with other types of hypertensive patients [16]. Since it was demonstrated that ED is implicated in the development of hypertension and that the improvement in its functioning is associated with a better cardiovascular prognosis in hypertensive patients, the endothelium and its parameters were treated not only as markers with prognostic value but also as a new potential therapeutic targets [17,18].

Over the past 25 years many methodological approaches have been developed to measure the (patho-)physiological function of the endothelium in humans [19]. Although the ability of measuring endothelial function has boosted clinical research in this field, its use as a clinical tool in daily practice is not established, nor has any method been recommended in clinical guidelines for planning primary or secondary prevention of vascular disease. Therefore, a marker which could act as a gold standard for clinical assessment of ED is still being investigated. A number of biophysical techniques have been applied to provide robust estimation of ED, however due to the invasive nature, poor reproducibility and problematic standardization their use are limited [20]. An evaluation of various circulating biomarkers of ED has been proposed as an alternative method. Once established, such biomarkers could detect ED in early stages, when the vascular functional tests are still normal [21]. The candidate markers include soluble thrombomodulin (sTM), an endothelial bound protein, released into circulation by proteolytic degradation of endothelial cells after injury by oxidative stress products, such as hydrogen peroxide, or by activated leukocytes [22–24]. However, its concentration similarly to most of others soluble markers is subject to intra-patient variability and also depends on renal function. Recently a lot of attention has been focused on the quantification of circulating endothelial cells (CECs) and circulating endothelial progenitor cells (CEPCs), highly specific and direct cellular markers of endothelial damage/dysfunction associated with an increased cardiovascular risk as well as worsening cardiovascular outcomes [25–27]. CECs represent mature endothelial cells detached from blood vessels due to various types of mechanisms, including apoptosis, mechanical injury, weakening of intracellular connection and endothelial structure injury caused by cytokines/proteases [28,29]. The number of CECs therefore reflects the condition of the endothelium and can be used to assess the extent of blood vessel injury. CEPCs, on the other hand, are immature precursor cells with proliferative potential, mobilize from the bone morrow in the conditions related with an enhanced vascular injury such as trauma, burns, myocardial infarction, stroke or vascular stent implantation [30,32]. The CEPCs number is thought to reflect the body's capacity to repair the endothelium. Moreover the CEPCs/CECs ratio is calculated as an indicator of an imbalance between regenerative and degenerative endothelial processes [33,34].

The aim of the present study was to evaluate the endothelial status by measuring the concentration of a traditional soluble marker - sTM as well as by determining the number of CECs and CEPCs and their ratio in patients with resistant hypertension (RH) versus patients with mild hypertension (MH) and the normotensive control. The second purpose was to analyze the correlation of markers of endothelial injury with demographical data and various biochemical parameters to recognize the factors potentially contributing to endothelial pathology in hypertension.

2. Material and methods

2.1. Patients

The study was carried out in a group of hypertensive patients (38 men and 22 women), aged 21–73 (mean age 55.57 ± 12.91) who had been admitted to the Department of Hypertension at the University of Medical Sciences in Poznan. The study protocol conforms to the ethical guidelines of the World Medical Association Declaration of Helsinki. The study was approved by the Local Bioethical Committee of Poznan University of Medical Sciences (no. 163/17). All patients qualified for this study underwent a detailed interview and a clinical examination. All study participants fulfilled the study criteria, undersigned written consent forms and completed the study. Based on the interview carried out before study, the patients were divided into two groups: patients with mild hypertension (MH) including 20 men and 10 women (mean age 52.87 ± 13.55) and with resistant hypertension (RH) comprised 18 men and 12 women (mean age 58.27 ± 11.85). Resistant arterial hypertension was recognized when in spite of the use of at least 3 antihypertensive agents (including a diuretic) in maximum doses, it was impossible to achieve the target values of arterial blood pressure (< 140/90 mmHg). In all MH and RH patients abdominal ultrasound examinations, computed tomography of the abdomen and Doppler ultrasound of the renal arteries were performed to exclude secondary causes of arterial hypertension. All participants of the study underwent transthoracic echocardiography. The exclusion criteria were the following: secondary hypertension, white coat hypertension, myocardial infarction and revascularisation within 6 months before the study, stroke and transient ischaemic attack (TIA) within 6 months before the study, congestive heart failure with grade III-IV according to New York Heart Association grading (NYHA), chronic kidney disease (eGFR < 30 mL/min), addiction to alcohol and psychotropic substances, active cancer, or diabetes.

The control group consisted of 33 subjects (25 men and 8 women), aged 27–61 (mean age: 41.87 ± 6.99), all of whom were normotensive blood donors in Regional Blood Center in Poznan.

2.2. Blood pressure measurements

In all patients clinical blood pressure (BP) measurements were performed three times at rest, in a supine position, in standard condition, using validated an upper-arm blood pressure monitor (Omron 705IT).

2.3. Sample collection

Blood samples were drawn at early morning, from the arms of MH and RH patients, in the recumbent position after 10 min of rest. Two vacuum neutral tubes of blood were collected from each patient into ethylenediaminetetraacetic acid (EDTA) anticoagulant. After 30 min, the EDTA tube, which was drawn as first, centrifuged at 3.000 rpm for 15 min and the obtained plasma was stored at temperature of −80 °C until all of assays were performed. The EDTA test tube collected as second, was designed for flow cytometric analysis in order to minimize the number of endothelial cells phenotypically equivalent to CECs exfoliated during venipuncture procedures.

2.4. Laboratory analysis

2.4.1. Biochemical parameters

Hematological parameters such as: white blood cells count (WBC), red blood cells count (RBC), hemoglobin (HGB), blood platelet (PLT), neutrophils (NEUT), lymphocytes (LYMPH) and monocytes (MONO) were determined by Medonic M20 automatic analyzer (Clinical Diagnostic Solution, Inc., USA). Blood biochemical analysis was performed using EasyRA analyzer (Medica, USA) and included the determination of uric acid (UA), total cholesterol (TC), triglycerides (TAG), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), creatinine (Cr), sodium (Na), potassium (K), glucose and urea (U). eGFR was estimated from serum creatinine, sex, age, and race using the CKD-Epi equation. The sTM concentration was measured using enzyme-linked-immunosorbent assay (Gen-Probe Diaclone SAS, France).

2.4.2. Multicolor flow cytometry analysis for CECs and EPCs

2.4.2.1. Monoclonal antibodies used for CEC and CEPC analysis in multicolor flow cytometry. The mouse anti-human monoclonal antibodies were used for analysis: PE/Cy7 anti-human CD34 (BioLegend, London, United Kingdom), FITC anti-human CD146 (BioLegend), apc/Cy7 anti-human CD45 (BioLegend), and PE/Cy5 anti-human CD106 (BioLegend).

2.4.2.2. Multicolor flow cytometry analysis. Multicolor flow cytometry analysis was performed according to the method published by Szpera-Goździewicz et al. [33] with some modifications. 4 mL of blood collected into EDTA tubes were used for flow cytometric analysis. The blood was transferred into polypropylene test tube and mixed. Next, the appropriate concentrations/volumes of conjugated mouse anti-human monoclonal antibodies: CD34, CD146, CD45, and CD133 was/were added. Simultaneously, the test tube containing 100 μL of blood only for the determination of the background of autofluorescence and non-specific antibody response was prepared. Both tubes were incubated for 40 min in the dark at room temperature. Next, 1 mL of buffered lysing solution (FACS lysing solution, Becton Dickinson) diluted 1:10 was added into both test tubes, followed by incubation in the dark at room temperature for 10 min. In the next step, 2 mL of phosphate buffer (pH.7.4) were added into both test tubes, followed by centrifugation 1500 rpm for 4 min at 4 °C, and decanting the supernatant. After the removal of the supernatant from the second wash and centrifugation, cell pellet was resuspended in 500 μL of phosphate buffer (pH.7.4; 0.01 M). The samples thus prepared were stored in the dark at room temperature until analysis, which was carried out within 1 h. The evaluation of nucleated cells was carried out on 6-color FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA). The data were analyzed using BD FACSDiva software. The number of CD45 (−), CD34 (+), CD146 (+), CD133 (−) cells per 1,000,000 analyzed nucleated cells was defined as CECs, whereas CD45(−), CD34 (+), CD146 (+), and CD133 (+) cells as CEPCs (Fig. 1). The calibration of flow cytometer and the control of fluid stability were conducted each time before analysis.

2.5. Statistical analysis

The statistical analysis were conducted using GraphPad Prism software 6.0 (GraphPad Software, San Diego, CA). The normality of quantitative variables were tested using the Kolmogorov-Smirnow or Shapiro-Wilk test. Any parameter not following the normal distribution was presented as a median and interquartile ranges and analyzed using non-parametric Mann-Whitney test. Categorical data and proportions were compared using Chi-square or Fisher's exact test, as appropriate. Normally distributed, continuous variables were presented as a mean and standard deviation and analyzed using the Student's *t*-test. Multiple group comparisons were performed by one-way analysis of variance or Kruskal-Wallis test, respectively. The Pearson or the Spearman correlation coefficient was used to test the strength of any association between different variables. The optimal diagnostic threshold values of analyzed parameters in differentiating hypertensive patients with endothelial injury were assessed using receiver operator characteristic curve methodology (ROC). In all cases, P value ≤ 0.05 was considered significant.

3. Results

3.1. Clinical characteristic of RH and MH group

No significant difference was found between RH group and MH group in terms of age, gender or BMI (Table 1). The average WBC, RBC, NEUT, LYMPH, MONO and PLT counts were not significantly different between RH and MH group (Table 1). No significant changes in the concentration of glucose, hemoglobin, sodium and potassium in either MH or RH group were observed. (Table 1). Among parameters of lipids metabolism only TC concentration was significantly increased in RH group (Table 1). Moreover, RH group demonstrated a significantly higher concentration of hsCRP compared to MH group (Table 1). In both groups of patients renal functions were assessed by measuring Cr, U, and UA concentration and by calculating an eGFR value. An increased concentration of U and a decreased eGFR value were observed in RH patients in comparison with MH group (Table 1). Moreover, the value of SBP as well as DBP were statistically higher in RH patients.

3.2. The markers of endothelial injury in MH and RH group versus normotensive control and their correlation with demographical data and biochemical parameters

The sTM concentration was significantly higher in RH group as compared to MH [6.47 ± 3.26 vs. 5.06 ± 1.30, $P = 0.038$], however its level did not differ significantly in comparison to normotensive control [6.47 ± 3.26 vs. 5.57 ± 1.49, $P = 0.257$] (Fig. 2). No difference in CECs number between MH and RH group was observed [126 (67–198) vs. 113 (64–233), $P = 0.915$], however when compared to normotensive control the CECs number was significantly increased in both groups of patients [126 (67–198); 113 (64–233) vs. 50 (18–78), $P < 0.0001$] (Fig. 3). Neither between MH and RH group [167 (106–411) vs. 164 (101 − 320), $P = 0.373$] nor versus normotensive control any difference in CEPCs number was found [167 (106–411); 164 (101–320) vs. 153 (102 − 232), $P = 0.417$] (Fig. 4). The CEPCs/CECs ratio was observed to be statistically decreased in both groups of hypertensive patients as compared to normotensive control [1.60 (1.01–2.25); 1.35 (1.09–1.98) vs. 3.24 (2.03–14.11, $P = 0.001$] (Fig. 5).

In MH group sTM correlated negatively with SBP and CECs number correlated positively with TAG (Table 2). A significant positive correlation was also found between CEPCs number and hsCRP and LDL-C and between CEPCs/CECs ratio and glucose concentration (Table 2). In RH group neither clinical parameter correlated with sTM (Table 2). CECs number correlated positively with hsCRP and LDL-C and negatively with HDL-C (Table 2). In RH as in MH a significant positive correlation was observed between CEPCs number and LDL-C (Table 2). CEPCs/CECs ratio correlated significantly with SBP and Na (Table 2). Moreover, in both types of hypertensive patients CECs correlated positively with CEPCs (Figs. 6, 7).

3.3. Sensitivity and specificity of CECs and CEPCs/CECs circulating endothelial cell to detecting hypertensive patients with endothelial damage

ROC analysis revealed that a CECs cut-off value of 60 cells/4 mL of blood sample had an initial sensitivity of 80% and specificity of 62% for differentiating hypertensive patients with endothelial perturbation (Fig. 8). ROC analysis revealed that a CEPCs/CECs cut-off value of 2.72 had an initial sensitivity of 82% and specificity of 67% for distinguish hypertensive patients with endothelial damage (Fig. 8). The area under

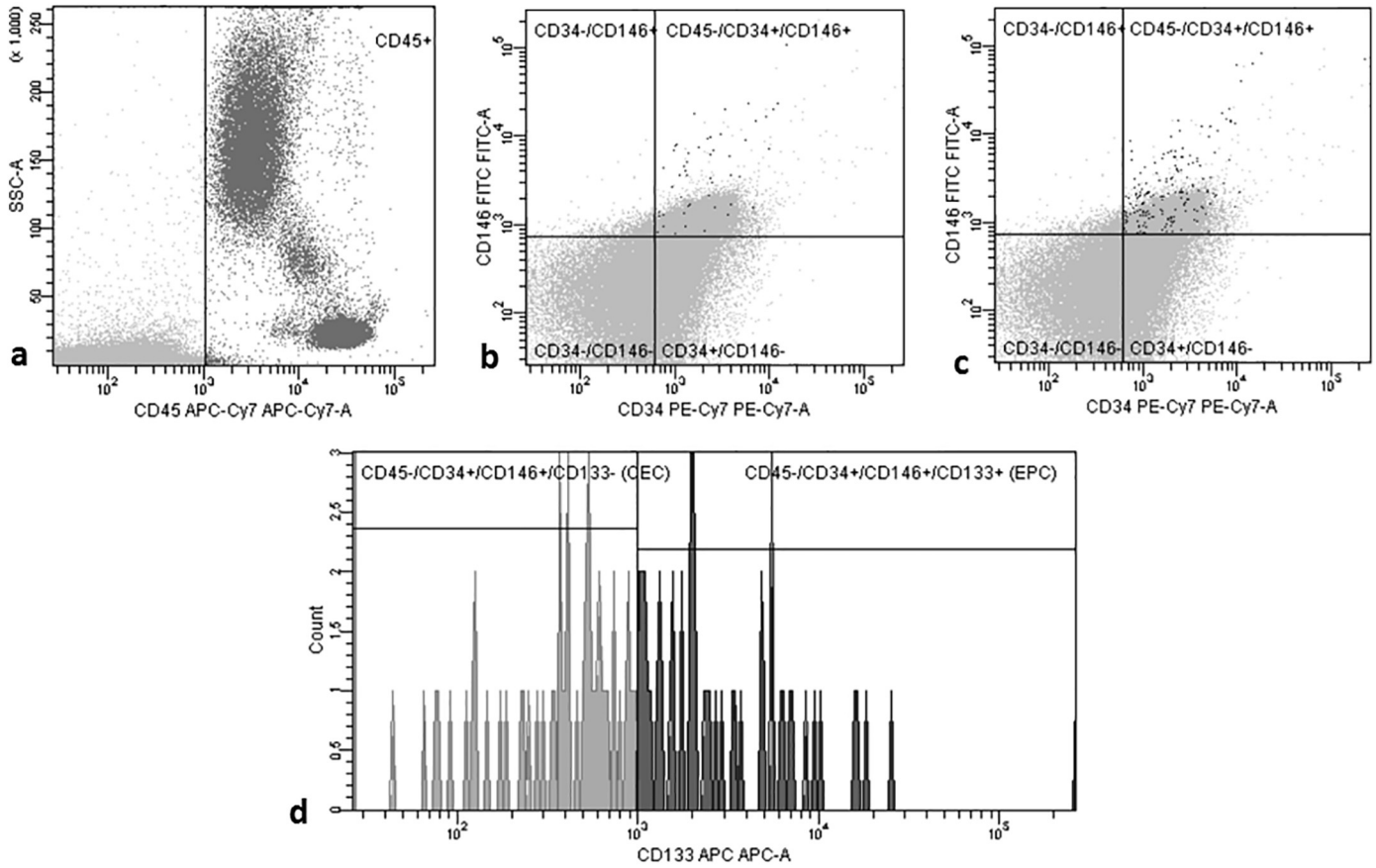

Fig. 1. Detection and characterization of circulating endothelial cells (CECs) and circulating endothelial progenitor cells (CEPCs). Hematopoietic ($CD45^+$) cells were excluded (a). $CD45^-$, $CD34^+$, $CD146^+$, $CD133^+$ cells were considered CEPCs (b). $CD45^-$, $CD34^+$, $CD146^+$, $CD133^-$ cells were considered CECs (c). Representative flow cytometry fluorescence histogram showing population of CECs and CEPCs (d).

the curve (AUC) was 0.82 for CECs count, and 0.76 for CEPCs/CECs ratio.

4. Discussion

Endothelial dysfunction (ED) is considered an early event in the pathophysiology of essenatial hypertension that may contribute to subclinical target organ damage and progression of atherosclerosis [35–37]. Data from the Framingham offspring cohort suggest that the severity of hypertension is positively associated with the degree of endothelial function impairment [38]. Moreover, there is evidence that ED is a marker for future cardiovascular morbid events in initially untreated and uncomplicated subjects with essential hypertension [18]. Whether ED is a cause or an effect of hypertension remains controversial, however, current data support a complex and potentially bidirectional relationship [35]. A major characteristic of ED observed in hypertensive patients is a decreased NO bioavailability which may lead to excess vasomotor tone, vascular inflammation and an increased platelet and monocyte adhesion to vascular wall, the mechanisms contributing to an increased blood pressure [39,40]. Among hypertensive patients, special consideration was given to the high-risk cohort with hypertension that was difficult to treat, including patients with a drug resistance or drug intolerance. Patients with resistant hypertension (RH) display a high prevalence of organ damage (ie. left ventricular hypotrophy, carotid intima-media thickening, carotid plaque, renal failure) [41] which may be a consequence of a worse endothelial status than the well controlled hypertensive patients [16]. Therefore, understanding the link between ED and hypertension seems to be of a great importance. This knowledge may lead to new insights not only into pathology of disease but also drug development, offering patients more effective treatments for the management of arterial hypertension. Moreover, the assessment of endothelial function may be widely used as a screening tool for identifying subjects with a higher cardiovascular risk, decreasing the mortality and morbidity rate among hypertensive patients.

Recently detection of mature endothelial cells in the peripheral blood has emerged as a direct and specific marker for endothelial injury [42,43]. These cells are detached from the vessel walls as an immediate consequence of compromised endothelial integrity and can be detected as CECs in the peripheral blood. Rare in healthy individuals, an increased number of CECs is found in a broad range of diseases and conditions associated with endothelial perturbation or/and damage including various cardiovascular disorders [44–46]. There is evidence that CECs correlate with biophysical and biochemical parameters of endothelial dysfunction, such as flow mediated dilation (FMD), von Willebrand factor (vWf), soluble thrombomodulin and soluble *E*-selectin [47–49]. Dysfunctional or damaged endothelium is replaced by circulating endothelial progenitor cells (CEPCs), mobilized from the bone morrow. The mobilized CEPCs migrate to sites of injured endothelium and differentiate into mature endothelial cells in situ. The imbalance between regenerative and degenerative endothelial processes is reflected by CEPCs/CECs ratio [33,34]. Therefore, in our study, for the first time, we evaluated the endothelial status expressed as the number of CECs, CEPCs and their ratio in two different clinical types of hypertension, including RH which represents the severe type of this disorder, predisposing to target organ damage and serious cardiovascular events, and MH which is associated with low blood pressure elevation and fewer complications.

Our study demonstrates an increased number of CECs in peripheral blood of patients with mild as well as resistant hypertension. However,

Table 1
Clinical characteristic of MH and RH group.

Parameter	MH (n = 30)	RH (n = 30)	p value
Age (years)	53 ± 14	58 ± 12	NS[b]
Gender F/M (n)	11/20	12/18	NS[c]
BMI (kg/m^2)	28 ± 5	30 ± 5	NS[b]
SBP (mmHg)	144 ± 16	173 ± 22	< 0.001[b]
DBP (mmHg)	84 ± 11	95 ± 15	0.002[b]
WBC (10^9/L)	7.04 (5.68–8.88)	6.95 (5.69–8.60)	NS[a]
NEUT (10^9/L)	4.27 (3.29–5.71)	4.42 (3.55–5.85)	NS[a]
LYMPH (10^9/L)	1.64 (1.39–2.37)	1.82 (1.37–2.15)	NS[a]
MONO (10^9/L)	0.46 (0.34–0.53)	0.45(0.28–0.67)	NS[a]
PLT (10^9/L)	223 ± 56	221 ± 51	NS[b]
RBC (10^{12}/L)	4.80 (4.44–5.13)	4.60 (4.34–5.07)	NS[a]
HGB (mmol/L)	9.00 ± 0.87	8.84 ± 0.86	NS[b]
Glucose (mmol/L)	5.60 (5.05–6.41)	5.58 (5.20–6.37)	NS[a]
Na (mmol/L)	141.3 ± 1.99	141.2 ± 3.13	NS[b]
K (mmol/L)	4.30 (3.90–4.60)	4.20 (3.97–4.60)	NS[a]
TC (mmol/L)	4.04 (2.10–4.86)	5.14 (4.04–5.89)	0.009[a]
TAG (mmol/L)	1.19 (0.95–1.55)	1.11 (0.81–1.67)	NS[a]
HDL-C (mmol/L)	1.62 ± 0.38	1.67 ± 0.59	NS[b]
LDL-C (mmol/L)	2.71 ± 1.47	2.78 ± 1.06	NS[b]
hsCRP (mg/L)	1.60 (0.90–3.55)	4.00 (1.55–8.25)	0.046[a]
Cr (μmol/L)	84.2 (65.6–91.6)	83.4 (70.6–112.1)	NS[a]
U (μmol/L)	5.06 (4.15–6.13)	5.66 (5.23–7.27)	0.002[a]
UA (μmol/L)	347 ± 113	345 ± 71	NS[b]
eGFR(mL/min/1.73 m)	86 (76–90)	78 (62–86)	0.030[a]

BMI- body mass index, SBP- systolic blood pressure, DBP- diastolic blood pressure, WBC– white blood cells, NEUT- neutrophils, MONO- monocytes, PLT- blood platelets, RBC– red blood cells, HGB-hemoglobin, TC-total cholesterol, TAG- triglycerides, HDL-C- HDL cholesterol, LDL-C- LDL cholesterol, hsCRP- highly sensitive C-reactive protein, Cr- cretinine, U- urea, UA- uric acid, eGFR- estimated glomerular filtration rate.

[a] Results shown as median and interquartile range, comparison done by using Mann-Whitney test.

[b] Results shown as mean ± standard deviation, comparison done by using unpaired t-test.

[c] Categorical data, Fischer's exact test was used for comparison. NS-not statistically significant.

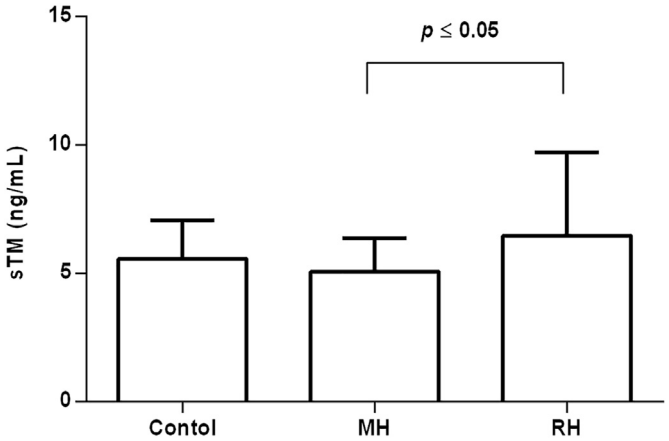

Fig. 2. Concentration of sTM in MH and RH group. Results shown as mean ± standard deviation. Data were analyzed using ordinary one-way ANOVA test followed by the Turkey's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

the gradation in their numbers according to the disease severity was not observed. Patients with RH revealed CECs number comparable with those detected in patients with MH. This fact suggests that not necessarily high blood pressure, resistant to treatment but even a low elevation of this parameter may cause severe endothelial damage. Lyamina et al. taking into consideration young patients with latent and manifest arterial hypertension, obtained results similar to ours, comparable degrees of endothelial damage reflected by an increased CECs

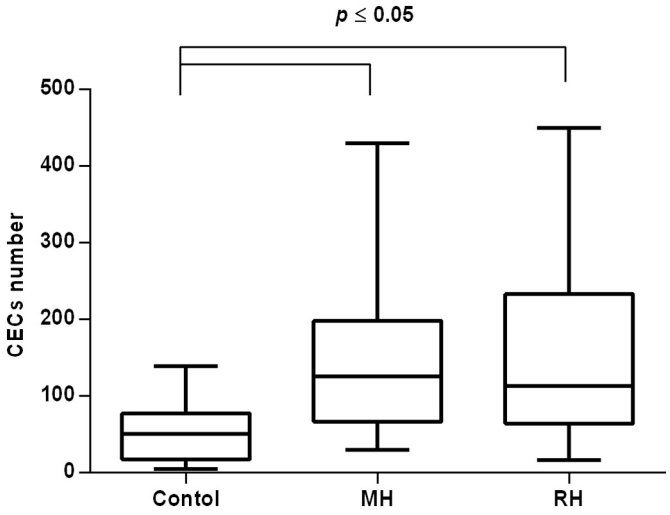

Fig. 3. CECs number in MH and RH group. Box and whisker plots show median (central line), upper and lower quartiles (box) and range excluding outliers (whiskers). Data were analyzed using Kruskal-Wallis test followed by the Dunn's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

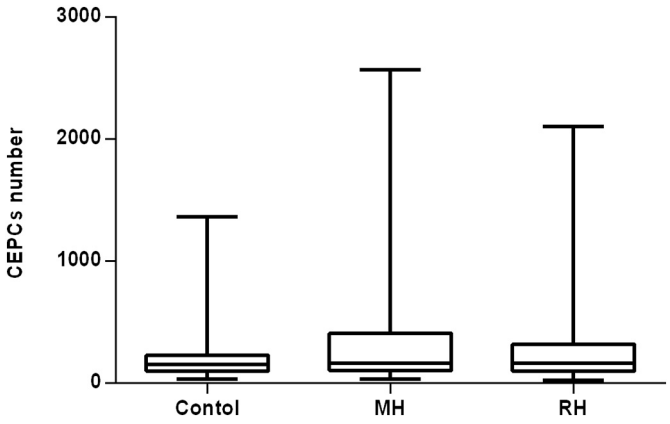

Fig. 4. CEPCs number in MH and RH group. Box and whisker plots show median (central line), upper and lower quartiles (box) and range excluding outliers (whiskers). Data were analyzed using Kruskal-Wallis test followed by the Dunn's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

number in both groups of hypertensive patients [50]. Our results, confirmed by findings of other authors, provide valuable information, namely they demonstrate that ED is an early step in hypertension development as well as indicate that CECs may be an important marker in its early detection. It should be underlined that our study is one of a few evaluating the CECs count among hypertensive patients versus healthy control. Koc et al. noted a significantly higher CECs number among patients with hypertension and chronic kidney disease, when compared with matched, healthy control subjects, thereby confirming our observations [51]. Opposite results were obtained by Nadar et al. who reported no difference in CECs counts between a cohort of 30 hypertensive patients compared with 30 healthy controls [52]. However, the authors recruited a much-lower-risk hypertension population and excluded patients with previous stroke, CAD, or peripheral vascular disease.

In the present study a tendency of CEPCs number to rise in both types of hypertension was observed, however, no statistical significance was reached. Our results are in accordance with the observation of Marketou et al., who did not find any significant difference in the total circulating number of CEPCs between hypertensive patients and healthy control. However, in their study the number of CEPCs

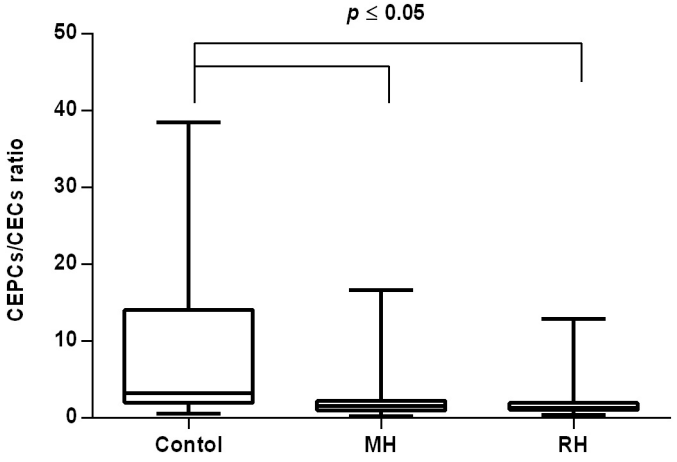

Fig. 5. CEPCs/CECs ratio in MH and RH group. Box and whisker plots show median (central line), upper and lower quartiles (box) and range excluding outliers (whiskers). Data were analyzed using Kruskal-Wallis test followed by the Dunn's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

correlated strongly with arterial stiffness, suggesting that these cells might be a good indicator of arterial remodeling [53]. Delva et al. evaluated not only the number of CEPCs but also their function by testing proliferation and colony-forming ability of CEPCs isolated from peripheral blood of patients with essential hypertension. Neither number of CEPCs nor their function were different from that in normotensive control [54]. Interestingly, in our study, we observed that CEPCs number correlated positively with CECs count in MH as well in RH. This correlation confirms that CEPCs are mobilized in the circulation in response to vascular damage, in order to preserve endothelial integrity. However, this mechanism seems to be insufficient due to the fact that the total circulating number of CEPCs in both groups of hypertensive patients is comparable with those observed in healthy donors in whom normal endothelial homeostasis is rather maintained. Due to the enhanced endothelial injury observed in hypertensive patients and reflected by an increased number of CECs, the number of CEPCs should rise proportionally. Such response did not occur in hypertensive patients, therefore the ratio between CEPCs and CECs drastically decreases, indicating an inadequate process of endothelial regeneration. This decline did not proceed with severity of the disease which means that a deficient regenerative response to endothelial injury occurs in the same extent. We also examined the diagnostic value of CECs and CEPCs/CECs ratio in discriminating hypertensive patients with ED. In our study, the number of CECs higher than 60 cells/4 mL and value of CEPCs/CECs ratio lower than 2.72 were moderately strong predictive factors of endothelial abnormalities among hypertensive group. However, since the sample size in the present study was relatively small, larger clinical studies are necessary for verifying the precision of these initial estimates. Moreover, a comparison between predictive values of our parameters and some reference indicators of ED should be conducted in the future.

Apart from the evaluation of CECs and CEPCs as novel markers of endothelial status, we also assessed soluble thrombomodulin (sTM) as a traditional parameter of ED in order to compare their informative values. The concentration of sTM was elevated in patients with RH compared to MH, however in comparison with normotensive control it reached a similar value. Our results confirm previous studies, showing the lack of a significant difference in sTM level between hypertensive patients and normal subjects [55–57]. The explanation of the lack of spectacular rise in sTM concentration in the condition certainly associated with endothelial injury has been given by authors of other studies, where its level was assessed in patients with pulmonary hypertension [58,59]. They suggest that sTM level appears to be initially

Table 2
The relationship among endothelial dysfunction parameters and clinical variables in MH and RH group.

MH

Variables	sTM	CECs	CEPCs	CEPCs/CECs
BMI (kg/m$^{2)}$	−0.058	−0.284	0.234	0.313
SBP (mmHg)	−0.424*	0.024	−0.032	0.033
DBP (mmHg)	−0.185	0.099	0.056	−0.044
WBC (10^9/L)	−0.179	0.158	0.134	0.006
NEUT (10^9/L)	−0.240	0.177	0.052	−0.045
LYMPH (10^9/L)	−0.079	0.057	0.221	0.111
MONO (10^9/L)	0.204	−0.083	−0.064	−0.023
PLT (10^9/L)	−0.207	0.272	0.087	−0.067
RBC (10^{12}/L)	0.156	−0.131	−0.255	−0.226
HGB (mmol/L)	−0.098	0.038	−0.122	−0.291
Glucose (mmol/L)	0.144	−0.288	0.041	0.601*
Na (mmol/L)	0.213	−0.288	−0.048	0.310
K (mmol/L)	0.097	0.012	0.149	0.184
TC (mmol/L)	−0.245	0.187	0.054	−0.143
TAG (mmol/L)	−0.097	0.348*	−0.021	−0.397
HDL-C (mmol/L)	−0.017	−0.235	−0.182	0.019
LDL-C (mmol/L)	0.032	0.026	0.376*	−0.111
hsCRP (mg/L)	−0.044	−0.095	0.533**	0.086

RH

Variables	sTM	CECs	CEPCs	CEPCs/CECs
BMI (kg/m$^{2)}$	−0.176	0.159	−0.097	−0.318
SBP (mmHg)	0.142	−0.195	0.180	0.432*
DBP (mmHg)	−0.331	−0.093	−0.112	0.090
WBC (10^9/L)	−0.279	0.218	0.128	−0.074
NEUT (10^9/L)	−0.244	0.247	0.199	−0.023
LYMPH (10^9/L)	−0.298	0.012	−0.167	−0.021
MONO (10^9/L)	−0.210	0.180	0.138	−0.069
PLT (10^9/L)	−0.309	0.288	0.184	−0.097
RBC (10^{12}/L)	−0.235	−0.020	−0.053	−0.154
HGB (mmol/L)	−0.295	−0.184	−0.207	−0.142
Glucose (mmol/L)	0.038	−0.251	−0.335	−0.164
Na (mmol/L)	0.081	−0.101	0.126	0.388
K (mmol/L)	−0.038	0.065	0.028	−0.027
TC (mmol/L)	0.078	0.179	0.225	−0.137
TAG (mmol/L)	0.274	0.018	−0.062	−0.095
HDL-C (mmol/L)	0.041	−0.358*	−0.068	0.083
LDL-C (mmol/L)	−0.045	0.348*	0.395*	−0.010
hsCRP (mg/L)	−0.236	0.459*	0.152	−0.062

Results shown as Spearrman's rank correlation coefficient, *$p \leq 0.05$, **$p \leq 0.01$.

BMI- body mass index, SBP- systolic blood pressure, DBP- diastolic blood pressure, WBC– white blood cells, NEUT- neutrophiles, MONO- monocytes, PLT- blood platelets, RBC– red blood cells, HGB-hemoglobin, TC-total cholesterol, TAG- triglycerols, HDL-C- HDL cholesterol, LDL-C- LDL cholesterol, hsCRP- highly sensitive C-reactive protein.

increased with acute vascular injury, perhaps trough cleavage from cell surface, and then decreased with the subsequent down-regulation of production as the process becomes chronic. Dohi et al. demonstrated an increased sTM level in hypertensive patients with atherosclerotic changes [60] which may suggest that its concentration better reflects the process of atherosclerosis development and progression rather than endothelial damage caused by hypertension per se.

We did not found any correlation between sTM and novel markers of endothelial damage. This fact did not exclude any of them as a reliable indicator of endothelial status but rather suggests that their concentration in circulation are influenced by different factors and mechanisms. The tendency of sTM to fall seems to be associated with an increased BP, especially in patients with MH. This observation is in accordance with a previously presented explanation given by Sakamaki et al. stating that chronically elevated BP probably leads to a decrease in TM synthesis altering anticoagulant and fibrinolytic function of endothelium which may predispose hypertensive patients to intravascular

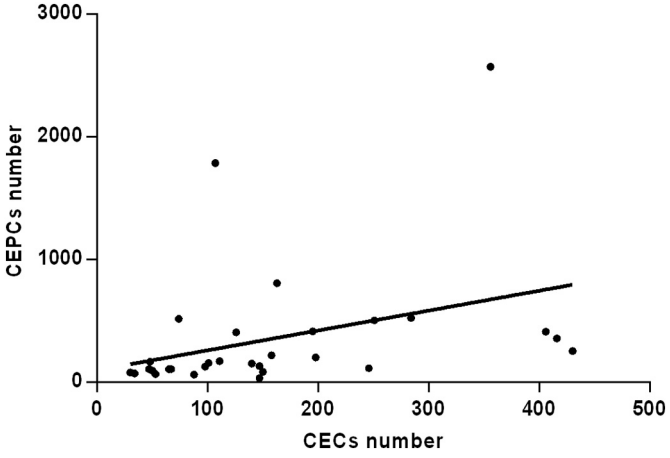

Fig. 6. Correlation between CECs and CEPCs number in MH group. (Spearman correlation coefficient $r = 0.599$, $P < 0.001$).

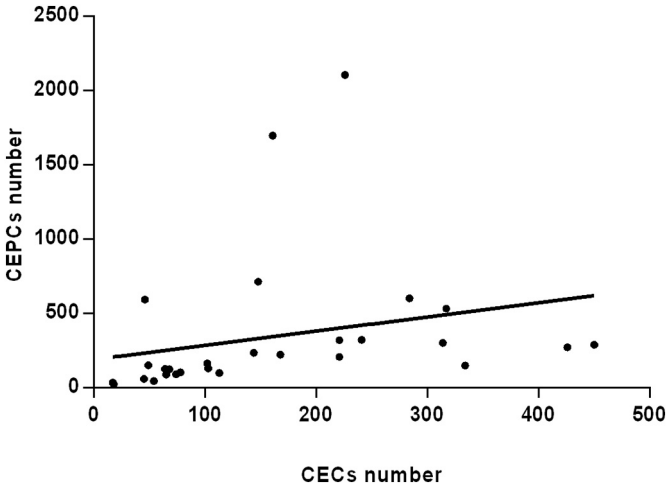

Fig. 7. Correlation between CECs and CEPCs number in RH group. (Spearman correlation coefficient $r = 0.671$, $P < 0.001$).

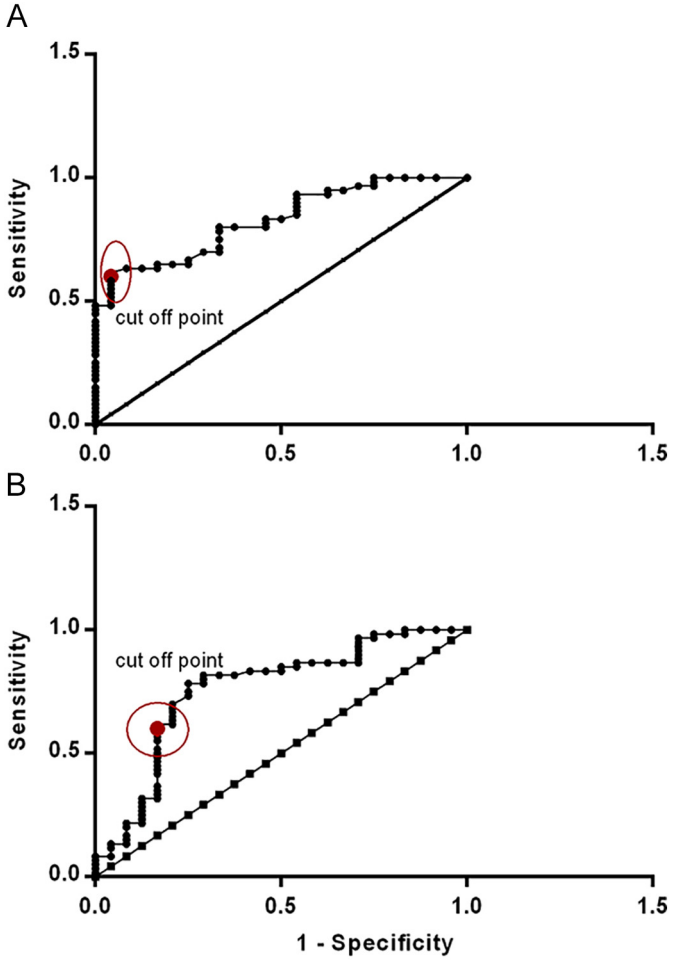

Fig. 8. A. Receiver characteristic (ROC) curve of CECs for distinguish hypertensive patients with endothelial abnormalities. CECs cut-off value of 60 cells/4 mL showed a sensitivity of 80% and specificity of 62%. Area under the curve was 0.82.
B. Receiver characteristic (ROC) curve of CEPCs/CECs ratio for distinguish hypertensive patients with endothelial abnormalities. CEPCs/CECs ratio cut-off value of 2.72 showed a sensitivity of 87% and specificity of 67%. Area under the curve was 0.76.

thrombosis [59]. The number of CECs is found to be effected by lipid abnormalities. CECs correlated positively with an increased TC in MH patients and an elevated LDL-C in RH group. Moreover, in patients with RH an increased CECs number was also associated with a decreased HDL-C. This finding suggests that the alternation in lipid metabolism may play a significant role in endothelial damage in hypertension. It is not surprising, because many clinical investigations indicate that atherogenic lipids, particularly LDL, sensitive to oxidation, as well as TAG cause gross disruptions of the normal function of the endothelium [61–64]. These disruptions include the loss of endothelium-dependent relaxation, stimulation of the expression of cell adhesion molecules, loss of anticoagulant surface, enhanced endothelial cells apoptosis [62]. Atherogenic lipids, by inducing endothelial injury probably activate various mechanisms of its regeneration, protecting from further vascular damage. One of them may be an increased mobilization of CEPCs, which was confirmed in the present study by a positive correlation found between their count and LDL-C level in both types of hypertension. Watt et al., who observed the same association between oxLDL and CEPCs in patients with coronary heart disease postulated conclusions similar to ours: a rise in CEPCs number as a host-repair response, induced by the damaging effects of increased circulating oxLDL on the vasculature [65]. Moreover, a positive association observed between CECs and hsCRP in RH patients suggests, that not only lipid abnormalities but also systemic inflammation may be an important factor responsible for endothelial dysfunction in this type of disorder. It is well known that CRP via activation of expression of various cytokines and immunoglobins such as: IL-6, intracellular adhesion molecule 1, tumor necrosis factor α, mediates enhanced leukocyte adhesion to endothelium resulting in its damage [66,67]. Recently Devaraj et al. reported that CRP may directly alter endothelial integrity by promoting CECs release from aortic endothelium in vitro and in vivo [68]. This effect appears to be mediated by Fc γ receptors, CD32 and CD64, and is due to NO deficiency induced by CRP. The close association between CECs and hsCRP found, for the first time in RH, suggests that analogous mechanism may take place in the pathology of this disorder.

We are aware of the limitations of this study. First, it can be agreed that the potential of endothelial cells to reflect ED could be additionally proven by finding association between CECs and/or CEPCs and some biophysical methods, such as flow-mediated vasodilatation (FMD) which, for now remains the most reliable noninvasive technique to measure endothelial function. However, such relationship was found previously by other authors and therefore it has not been established as the main aim of the present work [69–71]. Secondly, it should be noted that various methods of isolation and quantification of subpopulation of endothelial cells are described in the literature and as yet there is no standardized approach. This fact makes it difficult to properly interpret and analyze the obtained results. In spite of limitations, the present

work calls attention to the important aspect of CECs and CEPCs as a markers of ED, which may be useful in the exploration of the vascular effects of hypertension. Future studies aimed at distinguishing cells origin, identifying the mechanism by which they are generated, and associating their presence or absence with clinical endpoints may help to further define the vascular pathophysiology of patients with hypertension.

5. Conclusions

In conclusion, our results suggest that an endothelial alteration accompanies hypertension. Moreover, our data support the concept that measurement the number of CECs along with CEPCs may be a useful biomarkers for evaluating endothelial dysfunction in hypertensive patients. These interesting yet preliminary results, should they be verified by larger studies, not only provide important pathophysiologic clues pertaining to endothelial injury in hypertension, but also justify the conduct of prospective studies to ascertain the clinical utility of this novel diagnostic methodology.

Declaration of interest

None.

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