Detrimental Impact of Low Birth Weight on Circulating Number and Functional Capacity of Endothelial Progenitor Cells in Healthy Children: Role of Angiogenic Factors

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Objective To provide a comprehensive assessment of the relationship of birth weight with both endothelial progenitor cell function and angiogenic factors in children.

Study design Anthropometric measures, biochemical profile, endothelial progenitor cell number, endothelial progenitor cell colony-forming units, vascular endothelial growth factor-A, and nitric oxide plasma levels of 58 children aged 7-11 years were determined.

Results A positive correlation was observed between birth weight and circulating endothelial progenitor cell number ($r = 0.461$; $P = .001$), endothelial progenitor cell colony-forming units ($r = 0.512$; $P < .001$), vascular endothelial growth factor-A ($r = 0.407$; $P = .002$), and nitric oxide ($r = 0.547$; $P < .001$) levels, whereas the adjustment for prematurity, family history of cardiovascular disease, and systolic blood pressure levels did not modify these associations.

Conclusion Low birth weight was associated with a decrease in the circulating/functional capacity of endothelial progenitor cells among healthy children, independent of traditional cardiovascular risk factors. This detrimental impact was accompanied by lower circulating levels of angiogenic factors. *(J Pediatr 2019;206:72-7).*

Low birth weight is a risk factor for the development of cardiovascular disease (CVD).[1-4] To date, the underlying mechanisms linking low birth weight with cardiovascular injuries are not fully understood; however, a role for circulating endothelial progenitor cells has been reported.[5-7] A study conducted by Ligi et al found a significant decrease in the functional capacity of endothelial progenitor cells in newborns with low birth weight along with an observed decrease in the angiogenic capacity of endothelial progenitor cells associated with accelerated senescence.[5] These progenitor cells are involved in multiple processes of endothelial physiology, including postnatal neovascularization and re-endothelization.[8] The ability of endothelial progenitor cells to mobilize from the bone marrow into the bloodstream and differentiate into mature endothelial cells changes in response to specific stimuli, including metabolic alterations, hypoxia, and increased shear stress.[8-10] Moreover, this process can be initiated through the upregulation of angiogenic factors, such as nitric oxide (NO) and vascular endothelial growth factor (VEGF).[11-13]

Understanding the physiological aspects involved in the association of low birth weight with an increased risk of developing hypertension and cardiometabolic complications later in life is important, because vascular abnormalities can start early in childhood. Thus, the present study aimed to evaluate the effect of birth weight on circulating levels and functional capacity of endothelial progenitor cells and examine its relationship with NO and VEGF-A concentrations in a group of healthy children aged between 7 and 11 years.

Methods

This study was conducted in a group of children aged 7-11 years identified in the Youth Healthcare Centre located near the Federal University of São Paulo (UNIFESP, São Paulo, SP, Brazil). A total of 74 children (43 boys and 31 girls) were selected. Previous studies have reported that overweight/obesity and hormonal status could modulate endothelial progenitor cell number

APC	Allophycocyanin
BMI	Body mass index
CVD	Cardiovascular disease
FITC	Fluorescein isothiocyanate
MNC	Mononuclear cell
NO	Nitric oxide
PE	Phycoerythrin
SBP	Systolic blood pressure
VEGF	Vascular endothelial growth factor

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and function. We excluded 5 pubescent girls and 11 children (8 boys and 3 girls) who were overweight or obese. Therefore, 58 children (35 boys and 23 girls) remained eligible for this study. These children had no clinical signs or laboratory markers of endocrine, renal, or CVD. Personal and family medical histories were obtained using a questionnaire completed during an interview with the parents. The prenatal data from the mother's recall was confirmed with medical records. The Ethics Committee of the Federal University of São Paulo approved the study protocol (Number: 220.565). All parents and children signed the written informed consent/assent forms.

Anthropometry

Body weight and height were measured in light clothing without shoes using a standard balance beam scale. Body mass index (BMI) was computed using the formula weight (kg)/height (m^2). Waist circumference was measured using inextensible tape and recorded at a level midway between the lower rib margin and the iliac crest at the end of normal expiration. To remove any bias, the same researcher performed all the measurements.

Blood Pressure Evaluation

Blood pressure was evaluated according to the Fourth National Task Force on High Blood Pressure in Children and Adolescents recommendation.[14] Systolic blood pressure (SBP) and diastolic blood pressure levels were measured at the right arm with the child in supine position after being remained seated for 10 minutes by auscultation with an appropriate pediatric cuff size. Three measurements were made at 2-minute intervals, and the mean value was used in the analysis. To remove any bias, the same researcher performed all the measurements.

Blood Collection and Biochemical Variables

After overnight fasting, the blood venous samples were collected (7:00-8:00 a.m.) by venipuncture of a forearm vein into separate vacutainer tubes containing spray-dried K2EDTA anticoagulant or with gel serum separator. The tubes with K2EDTA were processed within 1 hour after blood collection for isolation of endothelial progenitor cells and assessment of NO and VEGF-A. The serum sample was sent to the Clinical Laboratory of the Kidney and Hypertension Hospital for evaluation of glucose, insulin, triglycerides, total cholesterol, and high-density lipoprotein cholesterol by automated routine methods. Low-density lipoprotein cholesterol was estimated using the Friedewald formula. Insulin resistance was calculated using the homeostatic model assessment (HOMA) method, according to the following formula: [glucose (mg/dL)/18 × insulin (mUI/mL)]/22.5.

Endothelial Progenitor Cells Quantification by Flow Cytometer and In Vitro Functional Capacity Assay

Peripheral blood mononuclear cells (MNCs) were isolated using a centrifuge method through a Ficoll-density gradient (2500 rpm for 25 minutes at 20°C). MNCs were recovered, washed twice with phosphate-buffered saline, and addressed to endothelial progenitor cell quantification by flow cytometer using cell surface antigen. Circulating MNCs with $CD34^+$/$CD133^+$/KDR^+ were considered endothelial progenitor cells. Briefly, 1×10^6 MNCs were stained with allophycocyanin (APC)-conjugated anti-KDR (CD309) (Miltenyi Biotec, Bergisch Gladbach, Germany), phycoerythrin (PE)-conjugated anti-CD133 (Miltenyi Biotec), and fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (Miltenyi Biotec). Fluorescent isotype-matched antibodies IgG1-APC/IgG1-PE/IgG1-FITC were used as controls. After incubation, cells were washed in phosphate-buffered saline and fixed in 1% paraformaldehyde solution. Fixed cells were kept in 4°C, in the dark, for 15-20 hours, and then analyzed in flow cytometer (FACSCANTO, BD Biosciences, San Jose, California) by collecting 10^6 events. Data were analyzed using BD FACSDIVA Software (BD Biosciences). Gates were established on the forward- and side-scatter plot corresponding with select MNCs followed by CD34-FITC, CD133-PE, and KDR-APC gate (**Figure 1**; available at www.jpeds.com). The number of endothelial progenitor cells ($CD34^+$/$CD133^+$/KDR^+) was calculated as the number of cells per milliliter of blood. For endothelial progenitor cell functional capacity assay, the peripheral MNCs were individually addressed to colony-forming unit, 5×10^6 MNCs were cultured (at 37°C with 5% CO_2 and 95% humidity) on 6 wells precoated fibronectin (BioCoat, BD Biosciences) seeded with EndoCult medium (EndoCult, Stem Cell Technologies, Vancouver, Canada). After 48 hours, 1×10^6 nonadherent cells were transferred in triplicate onto 24 wells precoated fibronectin and cultured for 96 hours (at 37°C with5% CO_2, and 95% humidity) in EndoCult medium. Next, the endothelial progenitor cells colony-forming units were counted manually by 2 blinded observers in inverted microscope (Nikon Instruments Inc, Melville, New York).

Determination of NO

NO is extremely unstable. Thus, the nitrite and nitrate in plasma samples were re-converted to NO via reaction with vanadium. Briefly, plasma samples were deproteinized with $ZnSO_4$ and NaOH incubated at room temperature and centrifuged at 12 000 rpm for 5 minutes. NO were determined by chemiluminescence using Model 280 Nitric Oxide Analyzer (Sievers Instruments, Boulder, Colorado), which is a highly sensitive detector for measuring NO based on gas-phase chemiluminescent reaction between NO and ozone. Data collection and analysis were performed using the NO Analysis software (version 3.21; Sievers). Results were calculated under the curve of the photomultiplier tube current for each determination paralleling the amount of NO and expressed in micromoles per liter. This assay was performed in duplicate and, to remove any bias, the researcher devised single-blinded analysis techniques.

Determination of VEGF-A

Plasma levels of VEGF-A were measured using Luminex xMAP technology (HAGP1MAG-12K—MILLIPLEX MAP Human Angiogenesis/Growth Factor Magnetic Bead Panel, EMD Millipore, Burlington, Massachusetts) according to the manufacturer's instructions. This assay was performed in duplicate and, to remove any bias, the researcher devised single-blinded analysis techniques.

Statistical Analyses

Statistical analyses were conducted using SPSS version 21.0 for Windows (IBM Corporation, Armonk, New York). Values were expressed as the mean ± standard error or as percentages. Categorical variables were compared using the χ^2 test. Pearson correlations and partial correlations adjusted for prematurity, family history of CVD, BMI, or SBP levels were calculated to examine the association of birth weight to several continue variables. Statistical tests were 2-tailed, and the significance level was set at $P < .05$.

Results

Among the 58 included children (35 boys and 23 girls), the mean birth weight was 2931 g (range, 2200-3680 g), and the mean length at birth was 46.6 cm (range, 38-52 cm). Therefore, children born with very low birth weight were not included in the present study. The incidence of low birth weight (<2500 g) was 20% (n = 12), and prematurity rate was 5.2% (n = 3). The mean values of blood pressure, BMI, glucose, insulin, and lipid profile were all within the normal range. No significant differences were found in all measured variables between girls and boys. Thus, data were reported in total. General characteristics of the children are listed in the **Table**.

No significant correlations were found between birth weight and BMI ($r = 0.102$; $P = .444$), waist circumference ($r = -0.162$; $P = .234$), triglycerides ($r = -0.008$; $P = .950$), total cholesterol ($r = -0.165$; $P = .216$), low-density lipoprotein cholesterol ($r = -0.229$; $P = .084$), high-density lipoprotein cholesterol ($r = 0.078$; $P = .559$), glucose ($r = 0.077$; $P = .566$), insulin ($r = -0.241$; $P = .068$), or HOMA ($r = -0.239$; $P = .070$). Of importance, a statistically significant inverse correlation was found between birth weight and SBP levels ($r = -0.389$; $P = .003$), but no correlation was detected with diastolic blood pressure ($r = -0.173$; $P = .194$). Although BMI could be a confounding factor in SBP analysis, adjustment for BMI did not modify the significance of the correlation between birth weight and SBP levels in our cohort of children ($r = -0.384$; $P = .003$).

Endothelial progenitor cell number correlated significantly with plasma concentration of NO ($r = 0.547$; $P < .001$), VEGF-A ($r = 0.413$; $P = .004$), and SBP levels ($r = -0.306$; $P = .039$). Similar correlations of endothelial progenitor cell colony-forming units with NO ($r = 0.563$; $P < .001$) and VEGF-A ($r = 0.413$; $P = .009$) levels were detected. There was a significant positive correlation between VEGF-A with NO ($r = 0.312$; $P = .018$). Regarding the correlation between circulating endothelial progenitor cells and endothelial progenitor cell colony-forming units, a moderate positive correlation was detected ($r = 0.478$; $P = .007$).

A moderate positive correlation was detected between birth weight and endothelial progenitor cell number ($r = 0.461$; $P = .001$), endothelial progenitor cell colony-forming units ($r = 0.512$; $P < .001$), and NO ($r = 0.547$; $P < .001$), and VEGF-A ($r = 0.407$; $P = .002$) levels (**Figure 2**), whereas the adjustment for prematurity, family history of CVD, and SBP levels did not modify these associations (endothelial progenitor cell number: $r = 0.400$, $P = .008$; endothelial progenitor cell colony-forming units: $r = 0.488$, $P = .002$; NO level: $r = 0.494$, $P = .001$; VEGF-A level: $r = 0.339$, $P = .012$). These findings suggest that the effects of birth weight on endothelial progenitor cell number/colony-forming units and angiogenic factors are detrimental to children with a lower birth weight. No other significant correlations were observed among either endothelial progenitor cell number or colony-forming capacity and age and anthropometric or biochemical variables (all $P > .05$).

Table. General characteristics of the study population

Birth weight (g)	2.931 ± 54.77
Birth length (cm)	46.6 ± 0.44
Prematurity (%)	
Yes	94.8
No	5.2
Age (y)	8.6 ± 0.19
Sex (%)	
Female	39.7
Male	60.3
Anthropometry	
BMI (kg/m^2)	15.9 ± 0.17
Waist circumference (cm)	58.2 ± 0.62
Biochemical variables	
TC (mg/dL)	149.6 ± 2.91
Triglycerides (mg/dL)	74.9 ± 1.94
HDLc (mg/dL)	47.6 ± 1.38
LDLc (mg/dL)	89.8 ± 2.83
Glucose (mg/dL)	88.3 ± 1.34
Insulin (μUI/mL)	8.8 ± 0.98
HOMA index	1.9 ± 0.14
Cardiovascular variables	
SBP (mm Hg)	101 ± 1.73
DBP (mm Hg)	62 ± 0.57
CD4$^+$/CD133$^+$/KDR$^+$ (cells/mL)	608.7 ± 37.34
Endothelial progenitor cell colony-forming units (10^6 cells)	16.6 ± 0.95
NO (μmol/L)	38.6 ± 1.38
VEGF-A (ng/mL)	27.2 ± 1.63

DBP, diastolic blood pressure; *HDLc*, high-density lipoprotein cholesterol; *HOMA*, homeostatic model assessment; *LDLc*, low-density lipoprotein cholesterol; *TC*, total cholesterol.
Data expressed as mean ± SE.

Discussion

Our findings indicate a detrimental impact of low birth weight on mobilization and the functional capacity of endothelial progenitor cells as indicated by a decrease in circulating endothelial progenitor cell numbers and endothelial progenitor cell colony-forming units. Our results also highlight a negative consequence of low birth weight on the angiogenic factors.

Cardiovascular complications associated with low birth weight are related, at least in part, to vascular damage.[3,4,15] After endothelial dysfunction and arterial stiffness, capillary rarefaction is the most commonly described vascular diseases in both children and adults with history of lower birth weight.[3,4,15-19] Circulating endothelial progenitor cells are known to play a key role in the maintenance of vascular integrity, angiogenesis, and counteraction to endothelial damage.[20] Moreover, circulating endothelial progenitor cell number is considered a biomarker of endothelial function and a prognostic indicator of cardiovascular morbidity and mortality.[20,21]

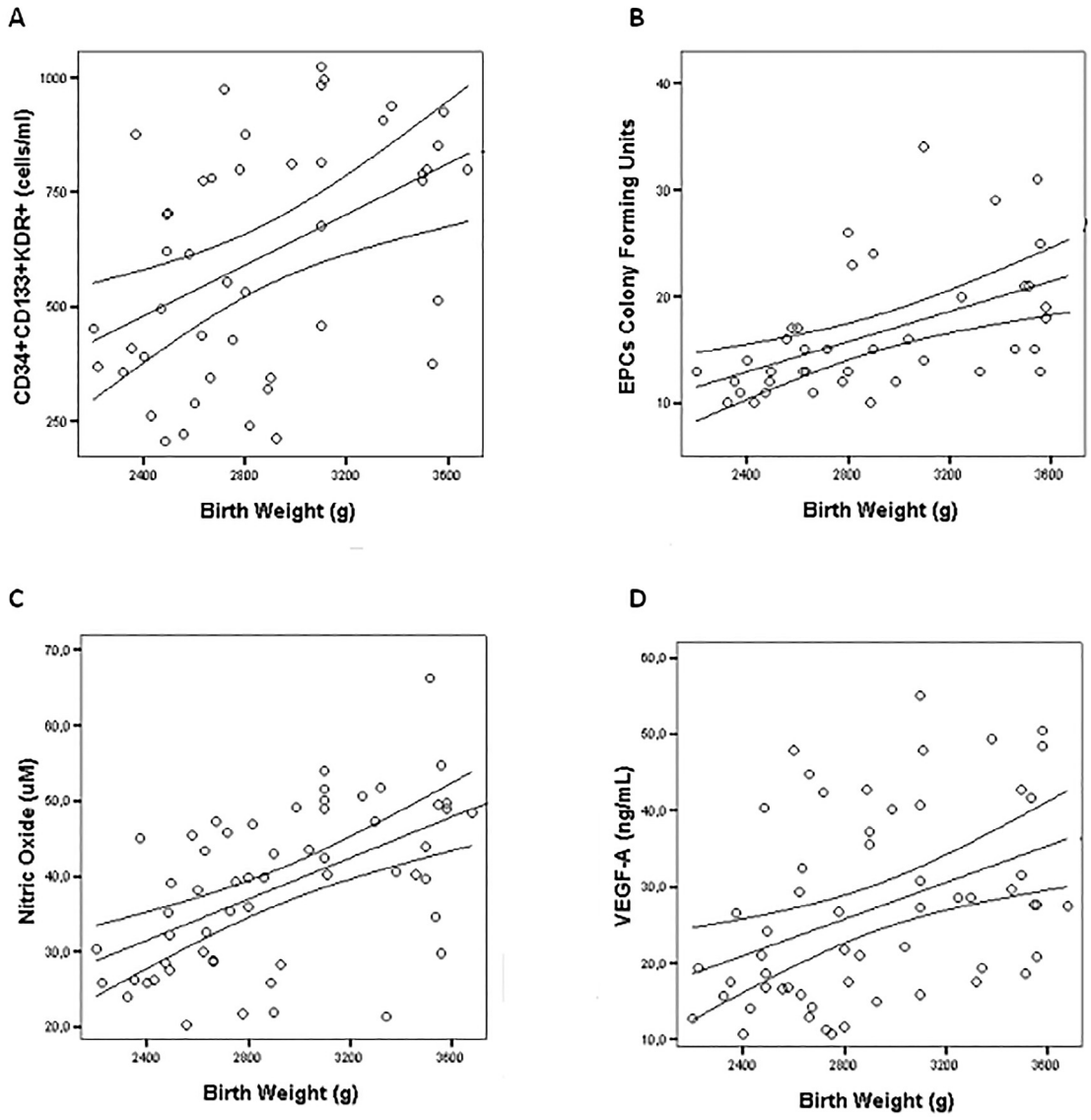

Figure 2. Scatter plots showing the correlation between birth weight with **A,** circulating endothelial progenitor cells defined by the coexpression of CD34, CD133, and KDR; **B,** endothelial progenitor cell colony-forming units; **C,** NO and **D,** VEGF-A levels. The lines represent the weighted regression with its 95% CI. SSC-A, side scatter plot.

Previous studies evaluating the influence of birth weight on endothelial progenitor cells demonstrated an impairment of endothelial progenitor cell functional capacity.[5-7] In a sample of preterm and term neonates, Ligi et al found that those with low birth weight had decreased endothelial progenitor cell colony-forming units when compared with those born with normal weight.[5] Authors found a decrease in the angiogenic properties of the endothelial progenitor cells from the umbilical cord of preterm neonates with low birth weight, a state characterized by decreased capacity to form tubes, to migrate, and to proliferate.[5] This adverse condition can be also related to accelerated endothelial progenitor cell senescence. Indeed, this hypothesis is consistent with that observed by Vassallo et al.[22] These authors found lower expression of Sirtuin 1 in endothelial progenitor cell cultures isolated from preterm neonates.[22] This Sirtuin 1 disability can accelerate endothelial progenitor cell senescence, because this key molecule is a chromatin-silencing factor with nicotinamide adenine dinucleotide-dependent deacetylase activity.[22] In the present study, both circulating endothelial progenitor cell number and endothelial progenitor cell colony-forming units were positively associated with birth weight, which was maintained after adjustment for prematurity, family history of CVD, and SBP. These data suggest a negative influence of low birth weight on circulating and functional capacity of the endothelial progenitor cells and may represent a predictive biomarker of adverse cardiovascular events in children with lower birth weight.

The underlying mechanism linking endothelial progenitor cells and birth weight remains unclear, but is likely mediated, at least in part, by an imbalance between antiangiogenic and proangiogenic factors. Ligi et al reported upregulated expression of genes with antiangiogenic properties in the endothelial progenitor cell colony-forming units isolated from the umbilical cord blood of neonates with low birth weight.[5] Moreover, the investigators demonstrated that serum samples isolated from the umbilical vein of neonates with low birth weight were able to impair proliferation and migration capacities of the endothelial progenitor cell colony-forming units from neonates with normal birth weight.[6] Interestingly, we observed that circulating levels of both VEGF-A and NO correlated positively with birth weight, endothelial progenitor cell number, and endothelial progenitor cell colony-forming units, thereby highlighting the key role of proangiogenic factors in modulating birth weight-induced changes in endothelial progenitor cells. No clear evidence of how low birth weight can lead to impairment of the angiogenic factors during childhood was detected. One possible mechanism can be the epigenetic modification in specific pathways involved in vascular growth and development. An inadequate intrauterine environment is known to influence the late development of CVD, and this susceptibility is strongly related to a modification in the epigenetic inheritance. This hypothesis is supported by recent findings showing that circulating endothelial progenitor cells isolated from the umbilical cord blood of preterm neonates with low birth weight presented hypermethylation in 4 CpG islands located in the promoter region of the AMOT gene, which is involved in the angiogenesis process.[23] Although this finding may advance the understanding of endothelial progenitor cell dysfunction associated with low birth weight, the complex relationships among birth weight, epigenetic modifications, and vascular dysfunction still require further investigation.

The modest effect noted in our study of the birth weight on the plasma levels of VEGF-A, NO, and circulating number/functionality of endothelial progenitor cells may partially explain prior reports that demonstrated an association between low birth weight with capillary rarefaction, endothelium dysfunction, and arterial stiffness.[3,4,15-19] It is, therefore, tempting to speculate that individuals with low birth weight have a dysfunction or reduced number of endothelial progenitor cells owing to the decrease of important endothelial progenitor cell-mobilizing agents (eg, VEGF-A and NO). This hypothetical clinical scenario could lead to impairment of endothelial integrity/repair and decreased angiogenesis. These processes probably coexist and promote the long-term development of the vascular abnormalities related to low birth weight.

It is important to interpret these data within the context of our experimental design and the limitations of this study. First, this study evaluated a small sample of children. Despite this, our study was able to reach statistical significance between correlated measures. Second, our findings need to be reproduced in other populations, and a longitudinal follow-up is necessary. Third, we cannot rule out the role of epigenetic patterns in our results. Additionally, residual confounding factors for known determinants of the endothelial progenitor cell activation and/or mobilization may still exist, particularly with respect to factors involved in early alteration of the signaling pathways regulating insulin and matrix metalloproteinases.

In conclusion, our study revealed a positive correlation of birth weight with circulating endothelial progenitor cell number, endothelial progenitor cell colony-forming units, and NO and VEGF-A levels in healthy children, reinforcing the importance of birth weight on vascular outcomes during childhood. Further studies are necessary, because many questions still need to be answered regarding molecular regulatory pathways involved in the correlation of birth weight, endothelial progenitor cells, and angiogenic factors. ■

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Data Statement

Data sharing statement available at www.jpeds.com.

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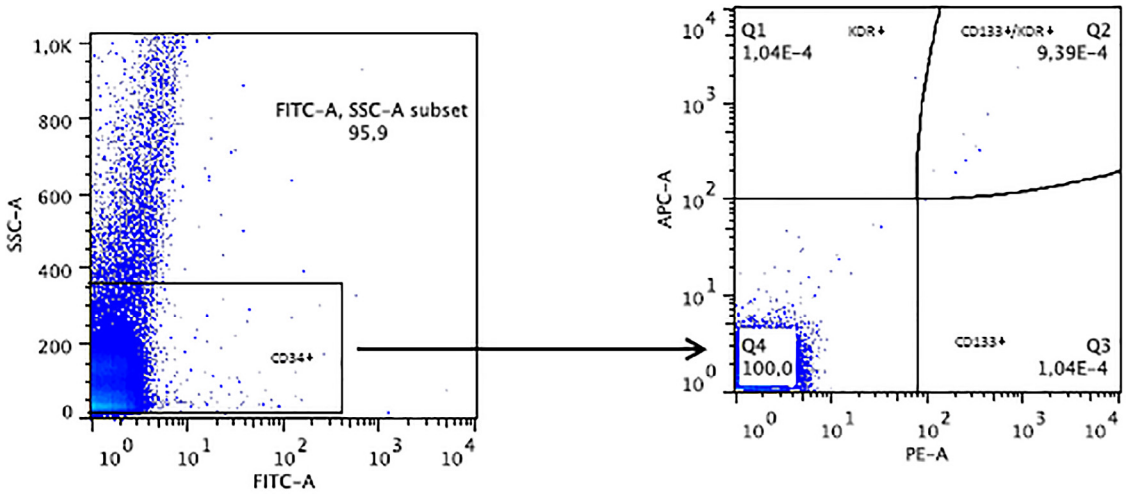

Figure 1. Flow cytometry gating strategy of CD34/CD133/KDR staining. Cells were considered as endothelial progenitor cells when they were triple positive for CD34/CD133/KDR.