Inhibition of Endothelial Progenitor Cells in the first 24 hours of an Acute Ischemic Cerebrovascular Event

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ABSTRACT: **Background:** Endothelial progenitor cells may have a role in ongoing endothelial repair. Impaired mobilization or depletion of these cells may contribute to progression of vascular disease. Our hypothesis was that endothelial progenitor cells would be suppressed in patients with acute cerebrovascular event based on our previous study that found severe endothelial dysfunction in those patients.

Objectives: To study the ability of patients with acute stroke to build colonies of endothelial progenitor cells.

Methods: We studied the number of colony-forming units in endothelial progenitor cells (CFU-EPCs) from the peripheral blood of 22 male patients with a first-time acute stroke (age 58.09 ± 9.8 years) and 13 healthy men (34 ± 6.7 years), 8 female patients with a first-time acute stroke (54.6 ± 10.3 years) and 6 healthy women (38.3 ± 11.6 years). Endothelium-dependent function was assessed by high-resolution ultrasonography of the brachial artery that measured the change in diameter of the artery by flow-mediated diameter percent change (FMD%). All patients had strokes demonstrated by a brain computed tomography (CT) scan done on admission. Peripheral blood was drawn soon after admission and was processed for endothelial progenitor cells in culture.

Results: Thirty patients without known cardiovascular risk factors and who did not take any medications were admitted with a first-time acute stroke. All demonstrated a strong correlation between CFU-EPCs grown in culture and endothelial dysfunction ($r = 0.827$, $P < 0.01$). Endothelial dysfunction with an FMD% of -2.2 ± 9.7% was noted in male patients vs. 17.5 ± 6.8% in healthy males ($P = 0.0001$), and -7.2 ± 10.1% in female patients vs. 25.1 ± 7.1% in healthy females ($P = 0.0001$). CFU-EPCs were 5.5 ± 6.3 in men with stroke vs. 23.75 ± 5.3 in healthy males ($P = 0.0001$), and 7.6 ± 4.9 in women with stroke vs. 22.25 ± 6.7 in healthy females ($P = 0.0004$).

Conclusions: Patients with acute stroke had an impaired ability to grow CFU-EPCs in culture and exhibited endothelial dysfunction. The novelty of this study was the discovery of the phenomenon of depressed numbers of EPCs and the poor ability to grow colonies of EPCs in the first 24 hours of the cerebrovascular event.

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KEY WORDS: stroke, endothelial function, colony-forming units of endothelial progenitor cells (CFU-EPCs), flow-mediated diameter percent change (FMD%)

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We demonstrated previously that patients with an acute cerebrovascular event had severe endothelial dysfunction and platelet activation during the first week of the event [1]. Possible mechanistic pathways that may explain the activation of platelets following an acute ischemic stroke include recent infections, inflammation and activation of a symptomatic atherosclerotic plaque, atherothrombosis after plaque eruption, and ischemic neuronal damage, which induce expression of inflammatory factors. Our previous trial demonstrated an independent association between vascular response and platelet activation in acute stroke [1]. The concept of endothelial dysfunction should be extended beyond blood vessels into the vascular wall and even into the level of endothelial stem cells. A meta-analysis found that coronary and peripheral endothelial dysfunction can predict cardiovascular events similarly [2]. Moreover, the fact that cardiovascular events may occur remotely from the site in which the endothelial dysfunction was detected demonstrated the systemic nature of endothelial dysfunction and its key role in prediction of cerebrovascular and cardiovascular events [2].

Endothelial progenitor cells can be isolated from circulating mononuclear cells in the peripheral blood. Laboratory evidence suggests that these cells express a number of endothelial-specific cell-surface markers and exhibit numerous endothelial properties [3-5].

We hypothesized that endothelial progenitor cells are inhibited in patients who suffered an acute cerebral vascular event. To test this hypothesis, we measured the ability of patients with acute ischemic stroke to build colonies of endothelial progenitor cells in culture in the first 24 hours of admission.

PATIENTS AND METHODS

This prospective study was approved by the ethics committee of Padeh Medical Center. Only patients with a first acute ischemic stroke were recruited to the study. The selected patients did not have renal failure, type 2 or type 1 diabetes mellitus, coronary artery disease, or any known chronic inflammatory or autoimmune disease. They did not have any of the traditional risk factors for cardiovascular disease, including hypertension and hypercholesterolemia. All were conscious and signed a consent form before enrollment to the study. All patients were examined by a vascular neurologist on their admission, and brain computed tomography (CT) was performed in the first 3 hours of admission. All vascular studies and the processing of the blood cells were performed within the first 24 hours of admission.

STUDY POPULATION

Thirty patients – 22 men (mean age 58.09 ± 9.8 years old) and 8 women (mean age 54.6 ± 10.3 years old) – without known cardiovascular risk factors were admitted with a first-time acute stroke. None of them received thrombolysis. All had a first mild lacunar stroke and were conscious without any cognitive impairment. The control group comprised 19 healthy volunteers – 13 men (mean age 34.3 ± 6.7 years old) and 6 women (mean age 38.3 ± 11.6 years old). All signed a consent form. None of the patients or the volunteers had any of the traditional cardiovascular risk factors.

VASCULAR STUDIES

- **Flow-mediated diameter percent change (FMD%)**

All measurements of brachial artery diameter and FMD% were performed in the morning, in a quiet and dark room, and at controlled ambient temperatures between 20°C and 26°C. Studies were conducted after an overnight fast of at least 10 hours (water was permitted), with the subject supine, and after 10 minutes of rest. The subject's right arm was comfortably immobilized in the extended position, allowing for ultrasound scanning of the brachial artery 5–10 cm above the antecubital fossa. At each examination, the recording of vessel images was followed by inflation of a cuff to a supra-systolic pressure (40–50 mmHg above systolic pressure) for 5 minutes. Subsequently, the cuff was deflated, and the brachial artery diameter was imaged and recorded for 3 minutes. An FMD% of more than 10% is considered a normal response. Lower than 10% FMD% reflects endothelial dysfunction, which means a high likelihood of developing a cardiovascular event in the future. Subjects with negative FMD% results (constricted artery after stress and not dilated as expected) have the worst prognosis.

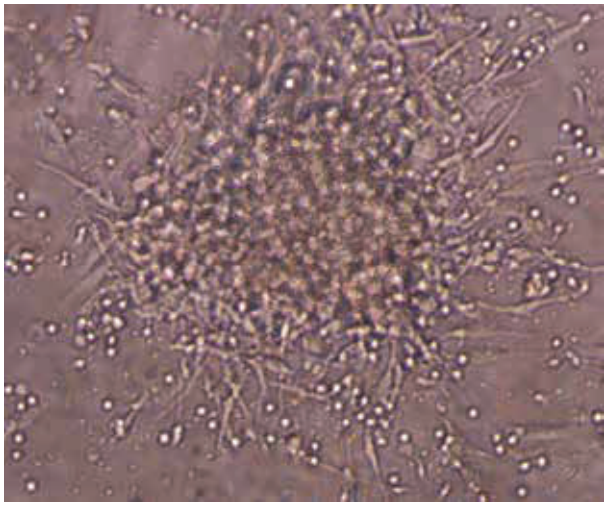
GROWTH OF CFU-EPCS IN CULTURE

The investigator who performed the laboratory experiments was blinded to the patients' clinical data. Venous blood samples were drawn from an antecubital vein into ethylene diamine tetra acetic acid-containing tubes; 40 ml of blood were processed. Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation, washed twice in phosphate-buffered saline with 5% fetal bovine serum, and resuspended in media (EndoCult basal media with supplements, StemCell Technologies, Vancouver, Canada, for EPC colony-forming assay). Cells were plated on human fibronectin-coated plates (BIOCOAT, Becton-Dickinson Labware, Bedford, Mass, USA) at a density of $5x10^6$ cells/well and incubated at 37^0C in humidified 5% CO_2. After 48 hours the non-adherent cells were re-plated onto fibronectin-coated 24-well plates at a density of $1x10^6$ cells well. After 5 days, CFUs (defined as a central core of rounded cells surrounded by elongated, spindle-shaped cells) were counted manually in 8 wells of a 24-well plate. The average number of CFUs per well is represented. A colony of EPCs consisted of multiple thin, flat spindle-like cells emanating from a central cluster of rounded oval cells. A central cluster alone without associated emerging cells was not considered a colony. Colonies were counted manually in eight wells by observers who were unaware of the subjects' clinical profiles, and the average number of all the wells that were counted was the number of colonies for the patient. A colony was defined as multiple thin, flat spindle-like cells emanating from a central cluster of rounded oval cells (the "sunflower" image) [Figure 1].

STATISTICAL ANALYSIS

Data are expressed as means ± SE. Patients were compared with healthy controls in age, vascular reactivity (FMD%), and in the ability to grow endothelial progenitor stem cells with the use of a two-tailed unpaired Student's *t*-test. The chi-square test was used for comparisons of categorical variables. Univariate correlations were performed with the use

Figure 1. CFU-EPC. A central cluster of rounded cells surrounded by radiating thin, elongated, spindle-like flat cells



of Spearman's correlation coefficient. Results were verified by means of the non-parametric Wilcoxon rank-sum test. To identify predictors of changes in colony counts of endothelial progenitor cells in a multivariate setting, we used multiple linear regression (General Linear Model Procedure, SAS) on specific variables. A similar analysis was conducted with respect to determinants of flow-mediated brachial reactivity.

RESULTS

PATIENTS' CHARACTERISTICS

None of the patients had any of the traditional cardiovascular risk factors, nor did they have coronary artery disease and were admitted with a first-time cerebrovascular ischemic event. None of them had thrombolysis; all were admitted to the hospital conscious and signed a consent form before enrollment to the study. The vascular studies were done and the endothelial progenitor stem cells counted in the first 24 hours of admission.

As shown in Tables 1 and 2, the average age was 58.1 ± 9.8 years for men and 54.6 ± 10.3 for women, without gender difference in age. There was a significant difference in vascular responsiveness between men with stroke (FMD% -2.2 ± 9.7) and healthy men (FMD% 17.5 ± 6.8) ($P = 0.0001$), and a significant inhibition of EPC growth in men with stroke (5.5 ± 6.3 colonies per well) compared with healthy men (23.7 ± 5.3) ($P = 0.0001$) [Table 1]. For women, there was a significant difference in vascular responsiveness between women with stroke (FMD% -7.2 ± 10.1) and healthy women (FMD% 25.1 ± 7.1) ($P = 0.0001$), and the ability to grow colonies of EPCs: women with stroke had an average of 7.6 ± 4.9 colonies per well, compared to 22.2 ± 6.7 in healthy women ($P = 0.0004$) [Table 2].

No gender effect was found in relation to vascular responsiveness and growth of colonies (EPC number and function) [Table 3].

FORMATION OF EPC COLONIES AND ACUTE STROKE

Peripheral blood mononuclear cells formed distinct colonies on fibronectin-coated dishes [Figure 1]. We used a phase-contrast micrograph to detect an EPC colony characterized by a central cluster of rounded cells surrounded by radiating thin, flat cells (×200). It was previously demonstrated that endothelial progenitor cells isolated in this fashion exhibit many endothelial characteristics, including expression of CD31, TIE2, and vascular endothelial growth factor receptor [6].

We next assessed whether the level of circulating EPCs correlated with the presence or absence of acute stroke. The numbers of CFU-EPCs were significantly reduced in subjects with acute stroke (men and women, both $P = 0.0001$) [Tables 1 & 2]. In men with stroke the CFU-EPCs were 5.5 ± 6.3 vs. 23.75 ± 5.3 in healthy volunteers ($P = 0.0001$), and 7.6 ± 4.9 in women with stroke vs. 22.25 ± 6.7 in healthy women ($P = 0.0004$) [Tables 1 & 2]. No gender effect was found among patients with stroke ($P = 0.583$) [Table 3].

Table 1. Endothelial progenitor cells & vascular function in men with stroke

	Age		FMD%		CFU-EPC	
	Controls	Patients	Controls	Patients	Controls	Patients
No.	13	22				
Years	34.3 ± 6.7	58.1 ± 9.8	17.5 ± 6.8	-2.2 ± 9.73	23.7 ± 5.3	5.5 ± 6.3
P value	0.0001		0.0001		0.0001	

Table 2. Endothelial progenitor cells & vascular function in women with stroke

	Age		FMD%		CFU-EPC	
	Controls	Patients	Controls	Patients	Controls	Patients
No.	6	8				
Years	38.3 ± 11.6	54.6 ± 10.3	25.1 ± 7.1	-7.2 ± 10.1	22.2 ± 6.7	7.6 ± 4.9
P value	0.02		0.0001		0.0004	

Table 3. Correlation between gender, endothelial progenitor cells & vascular function

	Controls	Patients
Age	0.459	0.420
FMD%	0.05 (better in women)	0.271
CFU-EPC	0.637	0.583

FMD% = flow-mediated diameter percent change, CFU-EPC = colony-forming units of endothelial progenitor cells

VASCULAR REACTIVITY MEASUREMENTS

There was a significant difference in the FMD% of patients with acute stroke and the healthy controls. Men with stroke had worse endothelial function than controls (-2.2 ± 9.7% vs. 17.5 ± 6.8%, $P = 0.0001$) [Table 1], and women with stroke also had endothelial dysfunction (-7.2 ± 10.1% vs. 25.1 ± 7.1%, $P = 0.0001$) [Table 2]. No gender effect was found in FMD% among patients with stroke ($P = 0.271$) [Table 3].

A strong positive correlation ($r = 0.827$, $P < 0.01$) was found of vascular reactivity, endothelial function expressed by FMD%, and the ability to grow in culture colonies of endothelial progenitor cells (CFU-EPCs).

DISCUSSION

The novelty of our study is the documented inhibition of EPCs in the first 24 hours of an acute ischemic stroke. We recently reported that patients with acute ischemic stroke had impaired vascular function [1]; however, in the present study we have shown a significant inhibition of PECs in the first 24 hours of an acute ischemic stroke, with a strong correlation between inhibition of EPCs and vascular responsiveness. This correlation between EPCs and vascular responsiveness has

been demonstrated before in healthy men with traditional cardiovascular risk factors [5] and in patients with coronary artery disease [2,6-8].

Most of the EPCs reside in niches within the bone marrow and are released only 'on demand' when a trigger is activated (like ischemia) or there is an increase in oxidative stress and free radicals or other inflammatory triggers (such as activation of nuclear factor kappa-B). These cells are then recruited and transported to remote areas of stress, where they are most needed for regeneration of damaged blood vessels or for building new blood vessels [9]. It has been shown that in atherosclerotic processes there is a lack of endothelial progenitor cells. An impairment and reduction of EPCs are hallmark features of type 1 and type 2 diabetes. EPC alterations might have a pathogenic role in diabetic complications and are associated with macrovascular and microvascular complications of diabetes, highlighting their roles and functions in the progression of the disease [10-12]. In patients with documented coronary artery disease, those with low EPC counts and impaired endothelial colony-forming activity had a higher incidence of cardiovascular events compared to patients with high EPC counts and favorable colony-forming activity. The pathophysiological basis for this finding may be insufficient endothelial cell repair by EPCs.

We postulate that EPCs influence coronary endothelial function, which itself is relevant for the outcome of patients at cardiovascular risk. To test this hypothesis in humans, endothelial function was invasively assessed in 90 patients with coronary heart disease by quantitative coronary angiography during intracoronary acetylcholine infusion. Flow cytometry of mononuclear cells isolated from peripheral blood was performed to assess CD133(+) or CD34(+)/KDR(+) EPC. EPC function was assessed ex vivo by determination of endothelial CFU. Low EPC number as well as impaired endothelial colony-forming activity correlated with severely impaired coronary endothelial function in univariate analysis. Multivariate analysis revealed that only the number of EPCs predicts severe endothelial dysfunction independent of classical cardiovascular risk factors [13-15].

Impaired ability to grow colonies of stem cells is a long-standing process that leads to cardiovascular disease and acute myocardial infarction [6], which may also be associated with cerebral vascular disease leading to acute ischemic stroke [7,8]. Endothelial damage ultimately represents a balance between the magnitude of injury and the capacity for repair. This impaired ability to grow colonies of EPCs in patients with ischemic stroke could be due to a long-standing process of oxidative stress, lack of nitric oxide activity, and a self-perpetuating process of deterioration of the nitric oxide reservoir [16].

Another explanation could be continuous endothelial damage or dysfunction leading to depletion or exhaustion of a presumed finite supply of EPCs [17]. Interestingly, recent studies in animals have suggested that the exhaustion of stem cells may be an important determinant of a number of age-related degenerative conditions [18,19]. Future studies will be needed to determine whether this postulated risk factor-induced exhaustion of circulating EPCs is a factor in the pathogenesis of cerebrovascular disease.

Usually, endothelial progenitor cells are increased in the first few days following an acute vascular event. We have shown in the past that this phenomenon starts only after a few days (4–5 days), and it seems that the cells are in "shock" in the first 24–48 hours; they then recover and start to increase, reaching a peak at day 7 post-acute vascular event. More than that, we have shown that in patients with endothelial dysfunction and anemia due to chronic inflammation, EPCs did not increase as was expected compared with patients who did not experience long-standing inflammation. In fact, the EPCs were depressed in the first 24 hours, and then partially "recovered," but much less so than the EPCs of patients without the long-standing inflammation [20].

AGE-RELATED EFFECTS ON EPCs

One of the major limitations in our study was the significant difference in age between patients and controls: 58.1 ± 9.8 years vs. 34.3 ± 6.7 in men ($P = 0.0001$), and 54.6 ± 10.3 vs. 38.3 ± 11.6 in women ($P = 0.02$).

A study addressing this issue in patients undergoing coronary artery bypass surgery found that preoperative values of EPCs declined with increasing age. This age-associated decrease could not be explained by differences in atherosclerotic risk factors. Bypass surgery induced a rapid mobilization in EPCs with a peak 6 hours after surgery. Persistently lower levels of EPCs throughout the observation period were observed in patients older than 69 years, which could not be explained by differences in the operative procedure or inflammatory activation [21]. However, according to our own experience that it is not necessarily so: older subjects who maintain an active healthy lifestyle have normal EPC counts (around 50 per well), sometimes far better than in younger subjects who do not adhere to a healthy lifestyle. A recent paper described 421 nonagenarians (306 women and 115 men, mean age 93.1 ± 3.2 years). Those who followed a Mediterranean diet and were in the fourth quartile of the Mediterranean diet score showed significantly higher EPCs than subjects grouped into the other three quartiles. After adjustment for confounders, elderly subjects who were in the highest quartile of adherence to the Mediterranean Diet Score had EPC levels significantly higher than those with lower adherence to the diet. Furthermore, by analyzing different food categories, it was found that daily consumption of olive oil and a higher consumption of fruit and vegetables showed higher CPCs $CD34^+$ and EPCs $CD34^+/KDR^+$ than in subjects without daily or lower consumption of fruits and vegetables [22].

The age difference in our study is a major limitation, but we should remember that it is not just the age difference that is important in relation to EPCs but also the lifestyle and diet of the subjects.

EFFECTS OF MEDICATIONS ON EPCs

Our patients and controls did not take any medications before admission with an acute cerebrovascular event; however, medications may have an effect on EPCs. It has been shown that a single dose of infliximab (anti-tumor necrosis factor-alpha) improved the number and functional properties of EPCs [23].

- HMG-CoA reductase inhibitors (statins) have a favorable effect on EPCs; they induce EPC proliferation and function even after 3 weeks of treatment at different doses. EPC proliferation and differentiation were induced by statins through the Akt pathway. This mechanism may induce eNOS activation and VEGF-induced endothelial cell migration.
- Insulin growth factor 1 (IGF1) has a favorable effect on EPC number and function, a phenomenon that decreases with age. These decreased effects (with aging) could be reversible with growth hormone treatment. It has been shown that IGF1 stimulates the differentiation, migration, and vascular network formation of EPCs in elderly subjects through activation of IGF1 receptor. IGF1 increases eNOS expression, activating PI3 kinase/Akt. Growth hormone induces augmentation of IGF-1, nitric oxide (NO) bioavailability, and EPC number.
- Estrogen has been shown to induce accelerated re-endothelialization of injured arterial segments within 7 days with a significant reduction in carotid medial thickness 14–21 days after injury. This remarkable effect was observed following another observation: 3 days after the carotid artery injury a significant increase of circulating EPCs was documented in animals that received estrogen compared to others that did not receive estrogen. This mechanism, estrogen-mediated re-endothelialization with increased numbers of EPCs, was found to be nitric oxide-dependent because estradiol did not accelerate re-endothelialization or augment EPC mobilization after injury in mice deficient in the enzyme nitric oxide synthase (eNOS-/-). Estrogen induces mobilization of circulating EPCs from the bone marrow, and these cells help to build and restore injured endothelium, supporting re-endothelialization after arterial injury. This is dependent on eNOS expression and, in the absence of NO, estrogen does not affect EPCs. Estrogen induces proliferation and migration, and inhibits apoptosis of EPCs [24].
- Mineralocorticoid receptor (MR) antagonists therapy leads to an increase in early circulating EPC numbers, without significant effect on cell function. These findings may account in part for the beneficial effects of MR antagonist therapy on heart failure pathophysiology and outcomes [25].

CULTURE ASSAY VS. FLOW CYTOMETRY MEASUREMENTS OF EPCs

In our study we used the culture assay to grow colonies of EPCs and not the flow cytometry assay (FACS). EPCs can be measured by FACS or by counting EPC colonies. Both methods were equal in estimating the number of EPCs in the peripheral blood. Culture growth of colonies had an advantage because it reflects not only the number of EPCs but also their ability to create colonies; and the morphology of the colony reflects its viability and ability to form "tube-formation" and blood vessels eventually. A healthy subject (human) can grow (on average) 50 colonies per well. In this study we chose to work with the culture growth assay since we wanted to study not only the number of EPCs but also their function and viability.

STUDY LIMITATIONS

The small size of the study did not permit us to determine whether low levels of endothelial progenitor cells can accurately predict future cerebral vascular events. Another major limitation is the significant age difference between patients and controls that may affect the results of our study.

CONCLUSIONS

Patients with first-time acute stroke without known cardiovascular risk factors had an impaired ability to grow CFU-EPCs in culture in the first 24 hours of the acute ischemic event. Inability to grow colonies of endothelial progenitor cells may be the mechanism leading to impaired angiogenesis in patients with cerebrovascular disease and may partly explain the mechanism of endothelial dysfunction observed in these patients. Improving EPC number and function in patients with acute ischemic stroke may provide new therapeutic options in the future.

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Capsule

Licensing interleukin-9 production

Although the biological roles of T helper 1 (TH1), TH2, and TH17 cells are reasonably well established, the functions of interleukin-9 (IL-9)-secreting TH9 cells remain elusive. Several studies have documented the presence of TH9 cells in both humans and mice. **Micossé** and colleagues studied human TH cells ex vivo. They proposed that TH9 cells are a subpopulation of TH2 cells that transiently up-regulate IL-9 and reported that the transcription factor peroxisome proliferator-activated receptor γ (PPARγ) is a key regulator of IL-9 production. These results call for a closer examination of the ontogeny of IL-9-producing TH cells using cytokine reporter mouse strains.

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Eitan Israeli

Capsule

Patients with systemic lupus erythematosus show an increased arterial stiffness that is predicted by IgM anti–β2-glycoprotein I and small dense high-density lipoprotein particles

Parra et al. investigated the metabolic and immunologic factors associated with the presence of central arterial stiffness as measured by the augmentation index (AIx). They conducted a cross-sectional study of 69 female patients with systemic lupus erythematosus (SLE) compared with a control group of 34 healthy women. The anthropometrical variables, the vascular studies, and the analytic data were obtained the same day. The AIx was assessed by peripheral arterial tonometry. The analysis of lipoprotein populations was performed using nuclear magnetic resonance (NMR) spectroscopy. Arterial stiffness was increased in patients with SLE compared with control subjects (mean ± SD 20.30 ± 21.54% vs. 10.84 ± 11.51%, $P = 0.0021$). Values for the AIx were correlated with the Framingham risk score ($r = 0.481$, $P < 0.001$), carotid intima-media thickness ($r = 0.503$, $P < 0.001$), systolic blood pressure ($r = 0.270$, $P < 0.001$), and age ($r = 0.365$, $P < 0.001$). Patients receiving anti-malarial drugs had a lower AIx (mean ± SD 11.74 ± 11.28% vs. 24.97 ± 20.63%, $P = 0.024$). The AIx was correlated with the atherogenic lipoproteins analyzed by NMR. The immunologic variables associated with the AIx were C4 ($r = 0.259$, $P = 0.046$) and IgM anti-β2-glycoprotein I (IgM anti-β2GPI) ($r = 0.284$, $P = 0.284$). In the multivariate analysis, age (β = 0.347, 95% confidence interval [95% CI] 0.020–0.669, $P = 0.035$), IgM β2GPI (β = 0.321, 95%CI 0.024–0.618, $P = 0.035$) and small dense high-density lipoprotein (HDL) particles (β = 1.288, 95%CI 0.246–2.329, $P = 0.017$) predicted the AIx.

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