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Effects of ACE inhibition on circulating endothelial progenitor cells, vascular damage and oxidative stress in hypertensive patients#

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Conflict of Interest: NONE

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Abstract

Purpose: The pathogenic role of ACE inhibition in hypertensive patients regarding to endothelial progenitor cell (EPC) function is still poorly understood. The aim of the study was to evaluate both EPC number and function and their relationship to carotid intimal medial thickness (IMT) progression.

Methods: We studied 36 newly diagnosed mildly hypertensive patients free of cardiovascular disease and related risk factors without prior or concurrent therapy with ACE inhibitors. Patients were randomized to receive enalapril 20 mg/die (n=18) or zofenopril 30 mg/die (n=18). EPC number and migrating capacity, plasma nitrite/nitrate (NOx) and isoprostane concentrations were evaluated. Carotid intimal media thickness (IMT) was determined by ultrasonography at baseline and after 1 and 5 year follow-up.

Results: EPC number increased during the follow-up with no statistical differences between treatment groups. There was an inverse correlation between circulating EPCs and IMT increase over the time. Plasma nitrite/nitrate decreased during the study without evident differences between the treatment groups. Isoprostanes decreased more markedly in zofenopril treated patients. Multiple linear regression model demonstrated that the carotid IMT was significantly inversely correlated with EPC but not with migratory cells after adjustment for confounders.

Conclusions: The present study demonstrated that EPC levels increased during the follow-up in both groups of newly diagnosed hypertensive patients treated with ACE-inhibitors. These drugs prevented the progression of vascular damage with an inverse correlation between circulating EPC levels and IMT values.

Introduction

In the last decade, there was a growing interest in the regenerative medicine afforded by the findings related to endothelial progenitor cells (EPCs) from bone marrow origin.[1,2] Thus, the pathobiology of EPC is an emerging field in the clinical setting of cardiovascular diseases. It has been hypothesized that EPC depression can be causally linked to the atherogenic process.[3] EPCs constitute a circulating pool of cells able to repair the dysfunctional endothelium whereas a reduced EPC pool may determine the inability to maintain adequate endothelial homeostasis. Moreover, the number and the functional activity of EPCs have been reported to decrease with hypertension, diabetes mellitus and the coexistence of multiple classical cardiovascular risk factors. [4-11]. Levels of EPCs are also inversely correlated to progression of coronary heart disease.[12] There are some preclinical studies suggesting that ACE inhibition may have potential effects on EPC. [13-15] Since ACE inhibitors are widely used in cardiovascular therapy, this promoted further investigation into the effects of such compounds on circulating EPC and their role in vascular healing, repair, and post natal neovascularization. ACE inhibitors may have a pivotal role in the management of atherosclerotic-related diseases independent of their vasodilating and hypotensive effects. Experimental studies show that these drugs can attenuate the development of atherosclerosis in a wide range of species, the most effective being those containing the antioxidant sulfhydryl group.[16-20]

Here, we used a previous clinical trial [21] on ACE inhibition and vascular damage to study EPC number and function and the relation to carotid intimal medial thickness (IMT) progression over the time.

Methods

Participants and measurements

We studied 36 newly diagnosed mildly hypertensive patients; this population comes from a previously published ongoing study [21] involving 48 patients and started in 2001. Exclusion criteria included the presence of other classical risk factors for coronary heart disease (eg, diabetes/impaired glucose tolerance and hyperlipidemia); prior or concurrent therapy with ACE inhibitors, antiplatelet drugs, or anticoagulants; history of ischemic events; or refusal to grant informed consent. Patients were enrolled if they had systolic BP >160 mmHg and/or diastolic BP >95 mmHg. Hyperlipidemia was defined as having total cholesterol >220 mg/dL, or LDL cholesterol >140 mg/dL, or triglyceride >150 mg/dL, and/or taking antihyperlipidemic medications. We used a simple randomization scheme with treatment assignment determined by computerized random number generation in a sequential manner and then provided to the clinical investigators in sealed envelopes. Follow-up measurements were scheduled at 1 and 5 years. The trial protocol followed the principles outlined in the Declaration of Helsinki and approved by the Local Ethical Committee.

Carotid ultrasonography measurements

All carotid ultrasonography measurements were made by 2 investigators (G.B. and A.L.) to eliminate interobserver variability, blinded to treatment randomization, as previously described. [21] Carotid artery IMT was measured by high-resolution B-mode ultrasonography, with a 7.5-MHz high-resolution transducer (Sonos-5500 system; Philips Medical Systems, Andover, MA) as described previously. [22,23] The carotid arteries were carefully examined for wall changes in the longitudinal and transverse views. The common carotid artery, carotid bulb, and internal and external arteries were examined. Presence of carotid atherosclerotic lesions (plaques or shadowing) was determined from scans of all right and left carotid artery segments (common carotid artery, bifurcation, and internal carotid artery). The presence of plaques was defined during ultrasound

reading based on wall thickness and arterial wall roughness, loss of alignment, or protrusion into the lumen. Calcification or mineralization, another indicator of atherosclerosis, was based on acoustic shadowing. Right and left common carotid artery wall areas were calculated as the total artery area minus the lumen area assuming a circular lumen and an outer artery structure that was either circular or elliptical. The IMT of the far wall was defined as the distance from the lumen-intima interface (leading edge of the first echogenic line) to the media-adventitia interface (leading edge of the second echogenic line). We measured the IMT of the common carotid arteries bilaterally at 3 points (at 1, 2, and 3 cm proximal to the carotid bifurcation); the averaged value was taken as the carotid IMT. IMT values for the 3 different projections were averaged to obtain the mean maximum IMT (MM-IMT).

Lesions of apparent plaque appearing either as faint gray echoes (soft plaques) or bright white echoes (calcified plaque) protruding into the lumen, were always excluded from the IMT measurement.[24]

Blood pressure measurements

Blood pressure (BP) was measured at baseline and during the 2 subsequent examinations with a mercury sphygmomanometer in a standardized fashion, after 10 minutes of rest with the subject in the supine position.[25] Systolic and diastolic BPs (mmHg) were defined according to Korotkoff sounds I and V. All BP measurements were done just before carotid ultrasound measurements and were performed after drug administration by a physician who was blinded as to the patients' profiles and treatment assignments.

Evaluation of plasma nitrite/nitrate and isoprostane concentrations

Plasma nitrite/nitrate (NO_x) levels were measured with the classical Griess method, as previously described.[25] Amounts of plasma nitrite were estimated by a standard curve obtained from enzymatic conversion of NaNO₃ to nitrite. Isoprostanes (8-iso-PGF₂α) were measured from

plasma samples by using a commercially available immunoassay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.[25]

Measurement of EPC number (culture assay)

Mononuclear cells were isolated by density gradient centrifugation with Biocoll (Biochrom, Berlin, Germany) from 20 mL peripheral blood, as previously described.[26] After isolation, 4×10^6 mononuclear cells were plated on 24-well culture dishes coated with human fibronectin (Sigma-Aldrich, Munich, Germany) and maintained in endothelial basal medium (Cambrex, Walkerville, MD, USA) supplemented with endothelial growth medium and 20% fetal calf serum. After 4 d in culture, non-adherent cells were removed by thorough washing with phosphate-buffered saline (PBS).

Measurement of functional capacity of EPCs (migrating capacity)

Isolated EPCs were detached using 1 mmol/L ethylenediaminetetraacetic acid in PBS (pH 7.4), harvested by centrifugation, resuspended in 500 μ L endothelial basal medium, counted, and placed in the upper chamber of a modified Boyden chamber (2×10^4 cells; BD Bioscience, Milan, Italy). The chamber was placed in a 24-well culture dish containing endothelial basal medium, 20% fetal calf serum, and human recombinant vascular endothelial growth factor (VEGF) (50 ng/mL; R&D Systems, Minneapolis, MN, USA). After 24 h incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with 4',6'-diamidino-2-phenylindole. Cells migrating into the lower chamber were counted manually in several random microscopic fields. [26] Measurements were blinded to treatment randomization

Statistical analysis

Data are presented as mean \pm SD. ANOVA for repeated measure was used to test the effect of treatment (enalapril vs zofenopril) over the time (Baseline, 1 year and five years) on systolic and diastolic BP, IMT, EPCs, Migratory cells, NOx and PGE_{2alpha}, in the table are reported statistical significance for the treatment effect, time effect and interaction between treatment and time. In order to assess the association between IMT versus EPC and/or Migratory cells controlling for confounder effect of treatment, time and other variables, we used linear regression analysis with robust variance estimation clustering for each patients in order to compensate for the correlation between the repeated measures.[27] A p value < 0.05 was considered as statistically significant. Analysis was performed using STATA 10 software.

Results

The mean age of the patients was 41.6 ± 2.6 (range 36-46). No significant difference in age, gender, family history of an ischemic event (CHD, peripheral arterial disease, or stroke), history of smoking, sedentary lifestyle, or medication used (calcium antagonist; 2 in enalapril and 2 in zofenopril group) were present at baseline between the 2 groups [21]. Moreover, no differences were present for others risk factors such as total cholesterol, triglycerides, high-density lipoprotein cholesterol, or LDL-cholesterol. [21] No patients had diabetes or were in treatment with statin or aspirin. The compliance to pharmacological treatment was good without withdrawn during the follow-up.

EPCs increased during the follow-up with no statistical differences between treatment groups. **(Table 1 and Figure 1)**. Migrating capacity of EPCs did not change during the study and no differences were evident between the treatment groups. **(Table 1 and Figure 1)**. Plasma nitrite/nitrate (NO_x) decreased significantly during the study while no differences were evident between the treatment groups. **(Table 1 and Figure 1)**. Isoprostanes (8-iso-PGF₂α) decreased significantly during the study more markedly in zofenopril treated patients. **(Table 1 and Figure 1)**. Intimal media thickness increased during the follow-up and this effect was more evident in the group of patients treated with enalapril even if this difference did not reach statistical significance when compared to zofenopril treated group. A statistically significant interaction between treatments and time suggesting a time related effect of pharmacological treatment in reducing IMT progression. **(Table 1 and Figure 1)**. As expected, a significant reduction of systolic and diastolic blood pressure during the study was obtained with no statistical differences between treatment groups also considering the effect of interaction between treatment and time. **(Table 1 and Figure 1)**.

In order to assess the correlation between IMT and EPCs and migratory cells, adjusted for all other covariates, we built a multiple linear regression model with IMT as independent variable and EPC and migratory cells as effect modifiers and all the remaining variables listed in the table as

confounder. The multiple linear regression model showed that the IMT was significantly inversely correlated with EPC but not with migratory cells after adjustment for confounders. (**Table 2 and Figure 2**)

Discussion

The major findings of our study are: 1) an EPC increase during the follow-up in both groups of patients treated with enalapril and zofenopril, 2) an inverse correlation between circulating EPCs and IMT increase over the time, 3) a plasma nitrite/nitrate decrease without evident differences between the treatment groups and 4) a isoprostanes decrease more evidently in zofenopril treated patients.

Migrating capacity of EPCs did not change during the study and no differences were evident between the treatment groups. Multivariate analysis demonstrated that vascular damage was significantly inversely correlated with EPCs levels but not with migrating capacity after adjustment for confounders.

A previous cross-sectional study, demonstrated an association of EPC migration, age and mean arterial blood pressure on carotid intimal thickening, they also found an inverse correlation with EPC number ($r = -0.349$) but with no statistical significance ($p=0.063$) demonstrating an age related decrease in EPC function and a possible effect on vascular remodelling.[28]

Another study [29] demonstrated that a significantly higher carotid mean maximum intima-media thickness, prevalence of carotid plaque and a lower number of circulating CD34⁺/KDR⁺ EPCs was found in stroke patients, compared with controls. In stroke patients, circulating CD34⁺/KDR⁺ EPCs count correlated negatively with carotid IMT, and was an independent risk factor for increased carotid IMT > 1 mm and the presence of carotid plaque.

Another very recent study [30] established that telmisartan shows a more potent anti-inflammatory effects than the ACE inhibitor ramipril and the two drugs did not show a differential effects on EPCs.

Since the expression of ACE may regulate primitive hemangioblasts derived from human stem cells, [14] the renin-angiotensin-aldosterone axis can be therefore exploited to direct the EPC fate during vascular regeneration. This issue may constitute an important clinical implication of ACE therapy on vascular damage occurring in hypertensive patients.

ACE inhibitors may have a pivotal role in the management of atherosclerotic-related diseases independent of their vasodilating and hypotensive effects. Experimental studies showed that these drugs can attenuate the development of atherosclerosis in a wide range of species, the most effective being those containing the antioxidant sulfhydryl group. [16-20] Different ACE inhibitors have quite different chemical functional groups, and these structural variations may account for different in vivo and in vitro effects. The ACE inhibitor captopril has a sulfhydryl group to coordinate the zinc ion of the active site, enalaprilat has a carboxylate group, and zofenopril has 2 sulfhydryl groups. [31-32] Sulfhydryl ACE inhibition stimulates the NO activity and decreases oxidative stress in human endothelial cells [33] and in patients with essential hypertension.[21] Zofenopril increases NO production in endothelium, decreases atherosclerotic development, and reduces reactive oxygen species, [34-37] as well as susceptibility to oxidation of plasma low-density lipoproteins (LDL) and formation of oxidation-specific epitopes in the arterial wall and atherogenesis in apolipoprotein E knockout mice. [35] The meaning of NOx levels would be qualitatively rather than quantitatively. However, repeated assays in same subjects have high sensitivity. In the present study, as expected, the untreated state of hypertension was associated to increased levels of NOx, after both treatments, NOx level decreased indicating that a normalization of blood pressure was associated to restoring effects of the NO pathway.

Circulating EPCs could play an important role in accelerating endothelialization at areas of vascular damage, and EPC function would be a viable strategy for assessing reparative ability. Consistently, some studies established that EPCs are affected both in number and function by several cardiovascular risk factors as well as various cardiovascular disorders, such as hypertension, hypercholesterolemia, and coronary heart disease [38].

Our study had some limitations. First, we used plasma storage collected from another clinical trial. [21] Another study limitation would be the limited power of the study due to a small group of patients.

In conclusion, our study demonstrated that EPC levels increased during the treatment in both groups of patients treated with ACE-inhibitors indicating that these drugs prevent vascular damage in newly hypertensive treated patients not only reducing blood pressure but also acting on circulating EPCs.

Author Contributions Dr. Cacciatore, Dr. Vitale and Prof. Napoli played a major role in the writing of the manuscript. Dr. Cacciatore and Dr. Vitale were also responsible for statistical analysis. Dr. Liguori, and Dr. Bruzzese had the overall responsibility for all in-hospital activities, in particular coordination of the clinical team, permissions to access medical records, obtaining informed consent, and clinical protocols. Dr. de Nigris, Dr. Fiorito, Dr. Infante, and Dr. Minucci provided execution and interpretation of clinical laboratory measurements. Prof. Napoli and Prof. Ignarro were involved in the first serie of studies addressing the vascular effects of ACE inhibitors thus providing the rationale and aim of the present study. However, a strong support on the detailed design of the study and its follow-up was done by Prof. Donatelli, Dr. Liguori and Dr. Cacciatore. None of the Authors have conflict of interest in the connection of this study.

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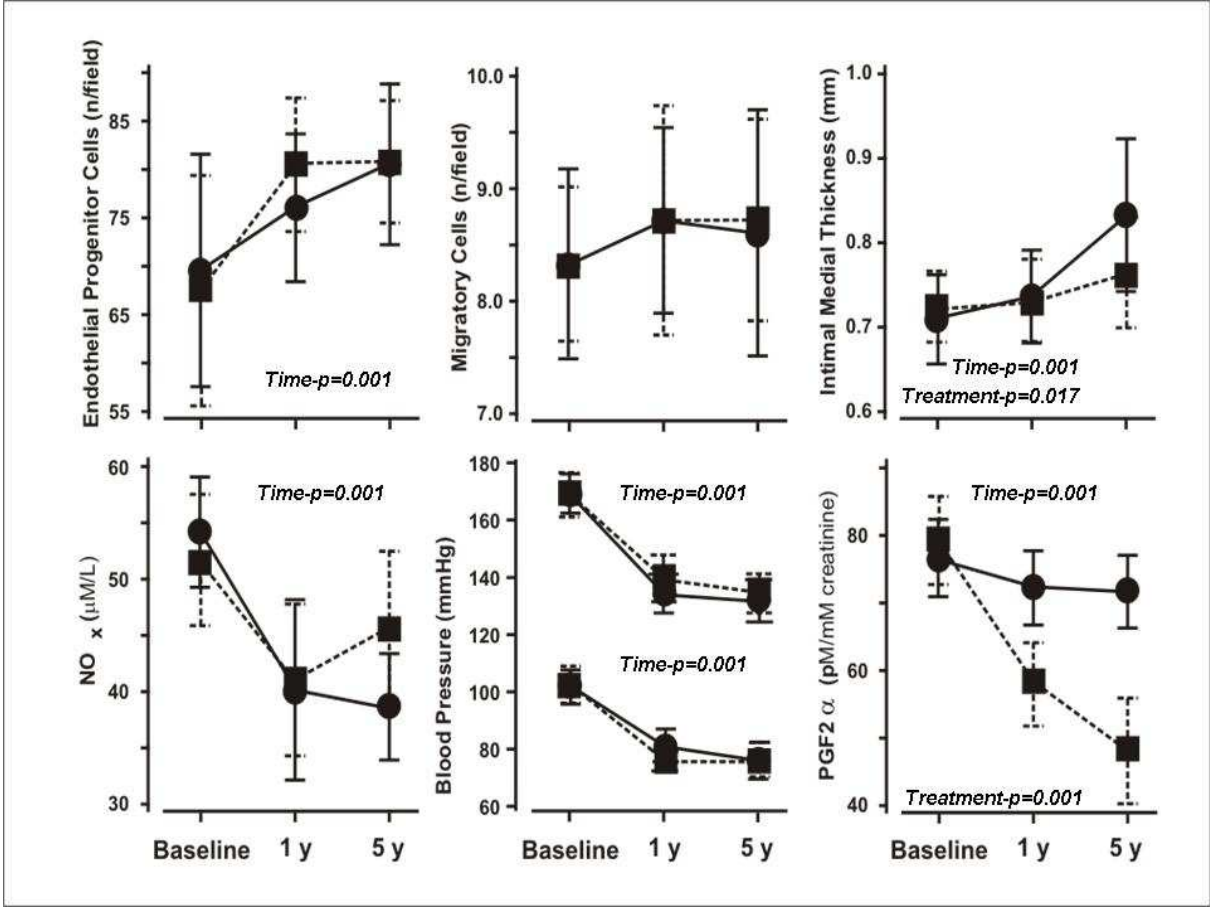
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LEGEND TO FIGURES

Figure 1. Modifications during the study of parameters in enalapril-treated patients (*circle & continuous line*) and zofenopril-treated patients (*square & dashed line*). Data presented are mean \pm SD. Enalapril-treated patients (N=18) and Zofenopril-treated patients (N=18)

Figure 2: Multivariate regression analysis evaluating the independent effect of EPC number on carotid IMT progression.

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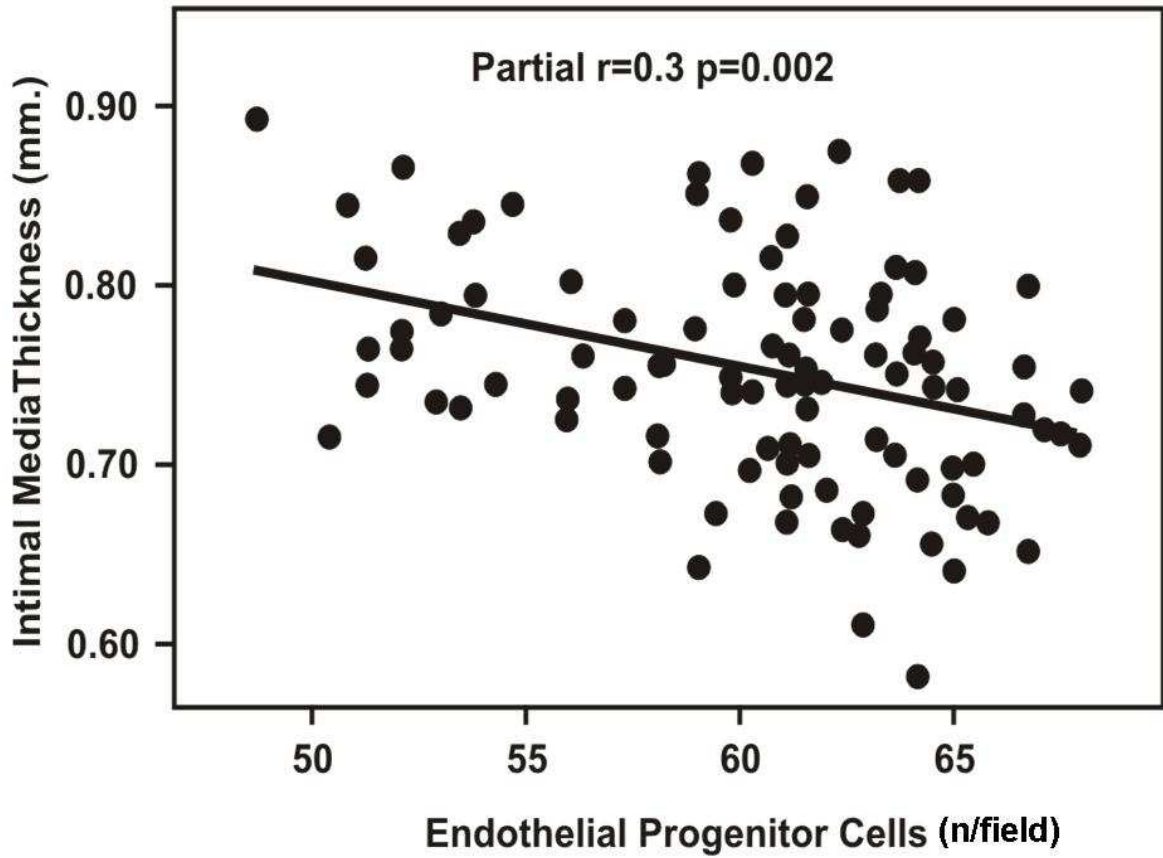


Table 1: Repeated Measure of Analysis of Variance in Enalapril (18-patients) and Zofenopril (18 patients) groups during the study.

Variable	Baseline	1 year	5 year	Treatment	Time	Interaction Treatment*time
SBP – Enalapril	169.1±7.0	134.3±6.7	131.8±7.4	P=0.092	P=0.001	P=0.245
SBP – Zofenopril	168.6±7.9	139.6±8.3	134.5±6.8			
DBP- Enalapril	101.9±5.8	81.3±5.6	75.9±6.6	P=0.199	P=0.001	P=0.050
DBP – Zofenopril	102.6±8.3	76.0±3.5	76.2±6.1			
IMT – Enalapril	0.71±0.05	0.74±0.05	0.83±0.09	P=0.074	P=0.001	P=0.017
IMT – Zofenopril	0.72±0.04	0.73±0.05	0.76±0.07			
EPC- Enalapril	69.6±12.0	76.1±7.6	80.6±8.4	P=0.573	P=0.001	P=0.341
EPC- Zofenopril	67.5±11.9	80.7±7.0	80.9±7.3			
Migr – Enalapril	8.3±0.9	8.7±0.8	8.6±1.1	P=0.819	P=0.169	P=0.959
Migr- Zofenopril	8.3±0.7	8.7±1.0	8.7±0.9			
NO- Enalapril	54.1±4.9	40.1±8.0	38.6±4.7	P=0.122	P=0.001	P=0.009
NO – Zofenopril	51.6±5.8	41.1±6.8	45.6±6.9			
8-iso-PGF2α Enalapril	76.4±5.6	72.4±5.4	71.6±5.4	P=0.001	P=0.001	P=0.001
8-iso-PGF2α Zofenopril	79.1±6.6	57.8±6.1	48.1±7.8			

Legend: SBP = Systolic Blood Pressure; DBP=Diastolic Blood Pressure; IMT= Intimal Media Thickness; EPC=Endothelial Progenitor Cells; Migr = Migratory Cells; NO = Plasma nitrite/nitrate; 8-iso-PGF2α = Isoprostane.

Table 2: Multivariate linear regression analysis with robust variance estimation clustering for each patient in order to compensate for the correlation between repeated measures and IMT as dependent variable.

Variable	B coefficient	Robust SE	P
EPC #	- 0.0018	0.0005	<i>0.001</i>
Treatment #	0.0198	0.0152	0.203
Time #	0.0861	0.0181	<i>0.001</i>
Treatment * Time #	- 0.0699	0.02	<i>0.001</i>
EPC Migration	0.0054	0.0065	0.41
NOx	- 0.0005	0.0012	0.678
8-iso-PGF2α	- 0.0024	0.0011	<i>0.04</i>
Systolic BP	0.0013	0.0008	0.1
Diastolic BP	0.00005	0.0008	0.95
Age	0.0013	0.0018	0.46
Gender	0.0019	0.012	0.87

Variable retained after backward hierarchical model.