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RESEARCH ARTICLE | *Integrative Cardiovascular Physiology and Pathophysiology*

Blood pressure control is not enough to normalize endothelial repair by progenitor cells

Elena M. V. de Cavanagh,[1] Sergio A. González,[2] Felipe Inserra,[3] Pedro Forcada,[4] Carlos Castellaro,[2,5] Jorge Chiabaut-Svane,[2] Sebastián Obregón,[6] María Jesús Casarini,[7] Pablo Kempny,[2] and Carol Kotliar[6]

[1]*Instituto Massone Sociedad Anónima, Buenos Aires, Argentina;* [2]*Cardiometabolic Unit, Cardiology Department, Austral University Hospital, Pilar, Argentina;* [3]*Maimonides University, Buenos Aires, Argentina;* [4]*Cardioarenales, Buenos Aires, Argentina;* [5]*Centro de Educación Médica e Investigaciones Clínicas "Norberto Quirno," Buenos Aires, Argentina;* [6]*Arterial Hypertension Center, Department of Cardiology, Austral University Hospital, Buenos Aires, Argentina; and* [7]*Department of cardiology, Clínica 25 de Mayo, Mar del Plata, Argentina*

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de Cavanagh EM, González SA, Inserra F, Forcada P, Castellaro C, Chiabaut-Svane J, Obregón S, Casarini MJ, Kempny P, Kotliar C. Blood pressure control is not enough to normalize endothelial repair by progenitor cells. *Am J Physiol Heart Circ Physiol* 319: H744–H752, 2020. First published August 14, 2020; doi:10.1152/ajpheart.00333.2020.—Patients presenting with classical cardiovascular risk factors within acceptable or average value ranges often develop cardiovascular disease, suggesting that other risk factors need to be considered. Considering that endothelial progenitor cells (EPCs) contribute to endothelial repair, we investigated whether EPCs might be such a factor. We compared the ability of peripheral blood EPCs to attach to extracellular matrix proteins and to grow and function in culture, between controlled hypertensive patients exhibiting a Framingham score (FS) of $<10\%$ while showing severe vascular impairment (intima-media thickness/diameter, carotid-femoral pulse wave velocity, brachial artery flow-mediated dilation, carotid and femoral atherosclerotic plaque presence; vulnerable group, $N = 30$) and those with an FS of $\geq 10\%$ and scarce vascular changes (protected group, $N = 30$). When compared with vulnerable patients, protected patients had significantly higher early and late-EPC and early and late-tunneling nanotube (TNT) numbers. Significant negative associations were found between vascular damage severity and early EPC, late-EPC, or late-TNT numbers, whereas EPC or TNT numbers and patient characteristics or cardiovascular risk factors were not associated. Except for protected patients, in all controlled hypertensive patients, early and late-EPC and early and late-TNT counts were significantly lower than those in the normotensive subjects studied ($N = 30$). We found that the disparity in vascular status between patients presenting with both an FS of $\geq 10\%$ and scarce vascular changes and those presenting with both an FS of $<10\%$ and severe vascular impairment is related to differences in peripheral blood EPC and TNT numbers. These observations support the role of EPCs as contributors to vascular injury repair and suggest that EPC numbers may be a potential cardiovascular risk factor to be included in the FS calculation.

NEW & NOTEWORTHY As individuals who present with risk factors within acceptable or average value ranges often develop cardiovascular (CV) disease, it has been suggested that other CV risk factors need to be considered in addition to those that are commonly combined in the Framingham score (FS) to estimate the risk of general CV disease. We investigated whether peripheral endothelial progenitor cells (EPCs) and tunneling nanotubes (TNTs) deserve to be considered. Here we report that EPCs and TNTs are significantly lower in controlled hypertensive patients versus normotensive subjects and that the disparity in vascular status between patients presenting with an FS of $\geq 10\%$ with scarce vascular changes and those presenting with an FS of $<10\%$ with severe vascular impairment is related to differences in EPC and TNT numbers. These data point to EPC and TNT numbers as potential CV risk factors to be included in the FS calculation.

endothelium repair; progenitor cells; vascular risk factors

INTRODUCTION

Hypertension is among a group of cardiovascular (CV) risk factors, e.g., diabetes, hypercholesterolemia, old age, physical inactivity, and smoking, that are known to pose mechanical, chemical, and/or immunological challenges to the endothelium, eventually leading to endothelial damage and dysfunction and ultimately to endothelial cell death (12). These endothelial changes initiate a burst of inflammatory responses that underlie the development and perpetuation of atherosclerosis (21), a condition that represents the hallmark of CV disease. Adult vascular endothelial cells are mainly in a quiescent state and display a low turnover; however, these can be activated to give rise to new vessels in response to signals provided by a variety of growth factors and chemokines, as well as by metabolic cues (23, 33). Thus, in the setting of normal vascular functioning, endothelial health is preserved as a result of the proliferation of cells located in the vicinity of injured endothelial cells; however, when endothelial damage exceeds the repair capacity of these perilesional cells, endothelial progenitor cells (EPCs) are needed to facilitate endothelial repair (13). Tissue ischemia and/or endothelial damage promote EPC mobilization from the bone marrow or from an alternative and still undefined location (15), as well as EPC recruitment and incorporation at sites of vascular damage (16). Although several unresolved issues concerning EPCs' source, identification, and repair potential still persist, enthusiasm over exploitation of EPCs to stimulate endothelial repair has not dwindled (32). Two distinct types of EPCs have been identified by in vitro cell culture of the blood mononuclear cell fraction, i.e., early EPCs and late EPCs, the latter also known as outgrowth endothelial cells. Early EPCs, representing alternative activated M2 macrophages, promote vascular repair through the paracrine re-

Correspondence: E. M. V. de Cavanagh (elenacavanagh@yahoo.com.ar).

lease of cytokines, and late EPCs, by differentiating into endothelial cells and incorporating into blood vessels (19, 22). In addition, EPCs can rescue damaged endothelial cells by transferring mitochondria and lysosomes through the recently discovered cell-to-cell communication channels referred to as tunneling nanotubes (TNTs) (30, 31). Studies involving animals (28, 29) and humans (5, 14) support the role of EPCs in the regeneration of the injured vessel wall.

In this scenario, accumulating evidence indicates that the traditional concept of atherosclerosis as the result of direct damaging actions by CV risk factors on the vessel wall needs to be extended by incorporating data on the endothelial repair capacity of EPCs and other progenitor cells (3, 21).

Drawing from the results of the Framingham Heart Study, Framingham researchers developed the Framingham risk score (FS) to assess an individual's chance or likelihood of developing CV disease. The FS combines data on CV risk factors to estimate the risk of general CV disease or one of its components, including heart failure, cerebrovascular disease, peripheral artery disease, and coronary artery disease (CAD), over the following 10 years (10). However, as pointed out by D'Agostino et al. (9), individuals who present with risk factors within the acceptable or average value range often develop CV disease, suggesting the existence of other risk factors that need to be considered.

In agreement with the latter observation, after studying an Argentinean population of around 1,500-treated hypertensive patients, we identified two groups that called our attention to *1*) individuals who presented with an FS of $<10\%$ while showing severe vascular impairment [as assessed by evaluating carotid intima-media thickness (IMT) and diameter, the presence of carotid and femoral atherosclerotic plaques, carotid-femoral artery pulse wave velocity (PWV), and brachial artery flow-mediated dilation (FMD)] and *2*) individuals who presented with an FS of $\geq 10\%$ and scarce vascular changes. To explain this puzzling observation, we hypothesized that the disparity in vascular status between these groups was related, at least partly, to their different capacities for endothelial repair. To investigate this concept and considering that to preserve a healthy endothelium, circulating EPCs need to both recognize/attach to an exposed vascular extracellular matrix and proliferate, we compared the ability of EPCs isolated from peripheral blood to attach to extracellular matrix proteins, as well as to grow and function in culture between patients belonging to the aforementioned *groups 1* and *2*.

MATERIALS AND METHODS

Patients. The study was conducted in accordance with the ethical principles contained in the Declaration of Helsinki and was approved by the School of Biomedical Sciences-Austral University Ethics Committee. Written informed consent was obtained from all subjects. A total of 400 individuals who consulted consecutively at the Center of Hypertension of the Austral University Hospital were screened for the following exclusion criteria: previous cardiovascular events, diabetes, smoking history, and cancer. Patients were instructed to abstain from any meal or caffeine for at least 12 h and to interrupt their antihypertensive medications for 24 h in advance of the day when they underwent anthropometric, metabolic (fasting serum glucose level and lipid profile), and noninvasive vascular status evaluations (IMT and diameter, the presence of carotid and femoral atherosclerotic plaques, PWV, and FMD). One hundred twenty-treated essential hypertensive patients who had achieved the goal blood pressure ($<140/90$ mmHg) were included. Thirty healthy normotensive volunteers with three or more measurements of blood pressure below 120/80 mmHg (normotensive group) and not receiving any medication were studied to obtain reference values for EPCs and TNTs.

Office blood pressure was measured with a calibrated and validated semiautomatic oscillometric device (Omron HEM-781CPINT, Omron Healthcare, Inc., Bannockburn, IL) according to JNC7 (7) with an appropriate cuff size and by averaging the second and third readings after the patients had been seated for 5 min.

Study Design. FSs were calculated for the hypertensive patients by using the following risk factors: sex, age, total cholesterol, HDL cholesterol, blood pressure, diabetes status, and smoking behavior. Also, to be able to quantify vascular status in hypertensive subjects and to assign patients to different groups according to their levels of vascular functional impairment or structural damage, we designed a numerical system by combining information on several parameters of vascular disease [for the numerical system used, see *Quantification of vascular status*]. The study design (Fig. 1) shows that on the basis of the FS and QVS values obtained for each patient, the subjects were assigned to the following groups ($N = 30$ in each group): vulnerable, FS of $<10\%$ and QVS = 3, 4, or 5; protected, FS of $\geq 10\%$ and QVS = 0, 1, or 2; control for vulnerable, FS of $<10\%$ and QVS = 0, 1, or 2; and control for protected, FS of $\geq 10\%$ and QVS = 3, 4, or 5.

Vascular status testing. Vascular status was assessed noninvasively by ultrasound imaging (GE Vivid 5) to evaluate carotid IMT, carotid diameter, carotid-femoral artery PWV, brachial artery FMD, and the presence of carotid and femoral atherosclerotic plaques, according to current ultrasound guidelines (17). The studies were conducted in a quiet windowless room with patients lying in a comfortable supine position without any visual or auditory stimulation. The ultrasound images were processed with a Hemodyn 4M device (Dinap SRL, Argentina), provided with an automated vascular edge-detection module suitable for the assessment of carotid IMT and carotid diameter. IMT was determined on the right and left common carotid arteries (posterior wall), and the maximum IMT value observed was used for analysis.

Carotid-femoral PWV was measured in the supine position with a Hemodyn 4M apparatus (Dinap SRL, Argentina), a Complior-based device that measures PWV through a synchronic method, by placing two mechanotransducers at the homolateral carotid and femoral pulses. The distance between these points was measured parallel and lateral to the left body side, expressed in meters (direct distance), multiplied by 0.8, and used for PWV calculations. The result was the average of two to three 10-s speed measurements, with a standard deviation of $<10\%$, and was expressed in meters per second.

Quantification of vascular status. To quantify vascular damage, after recording the normal or abnormal status of IMT, the presence/absence of atherosclerosis plaques, PWV, and FMD, we designed a numerical system by assigning a value to each outcome and combining the data, as explained next. The normal cutoff values used for each parameter were taken from consensus guidelines (24, 26). Accordingly, the following values were used as abnormality limits: IMT higher than the 90% confidence interval of the 10-yr IMT stratification in a sample of normal subjects from the Atherosclerosis Risk in Communities (ARIC) study, PWV higher than that calculated with the Asmar formula (2), and a poststimulation brachial artery diameter that is $\leq 5\%$ of the basal diameter for FMD; this limit was defined after having conducted a pathological FMD prevalence analysis in our patient population. Abnormal IMT, PWV, and FMD were each assigned a value equal to 1, as opposed to a value of 0 if these were within normal limits. The presence and absence of atherosclerotic plaques were assigned values of 2 and 0, respectively. As a result, the QVS used here ranged from 0 to 5, where 0 represents the absence of vascular damage, 5 corresponds to abnormal findings in all the vascular damage parameters studied, values between 1 and 2 correspond to mild to moderate vascular changes, and values 3, 4, or 5 correspond to fairly severe to severe vascular alterations.

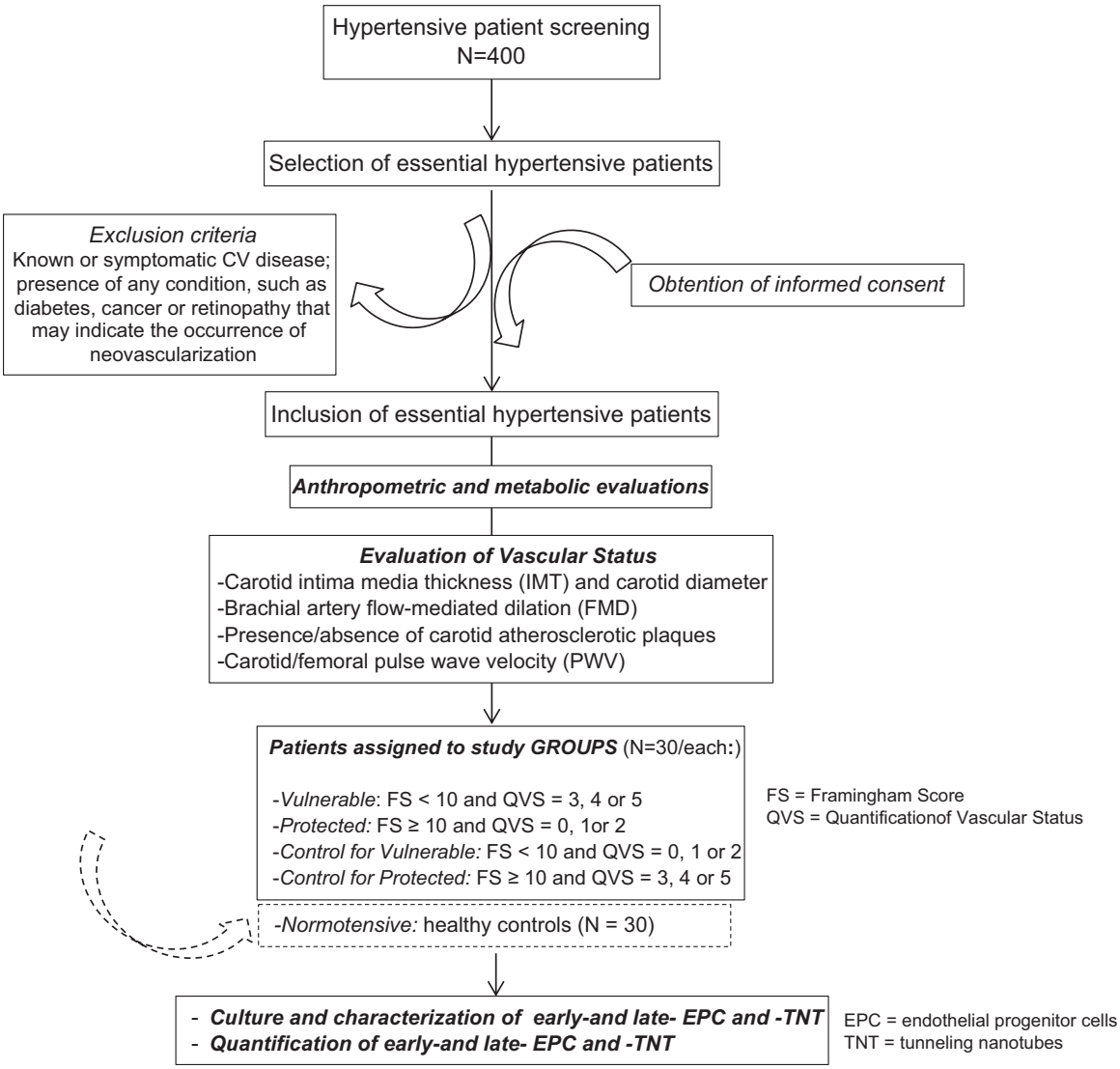

Fig. 1. Study design.

Brachial artery FMD. To evaluate endothelial function status in treated hypertensive patients, brachial FMD was measured in the morning under fasting conditions, with patients lying in the supine position in a quiet room kept at 22°C using a high-resolution device (Esaote Caris 7230, Genova, Italy). The International Brachial Arterial Reactivity Task Force guidelines were followed (6). In brief, brachial artery diameter measurements were obtained with a 10-MHz transducer positioned perpendicular to the vessel in the upper arm by using a stereotactic clamp to ensure that the measurements were made in the same arterial segment and to avoid transducer displacement. Ultrasonic gel was used as the transmitting medium. Brachial artery blood flow velocity was obtained continuously by pulsed Doppler signal, in the arm opposite to that used for blood extraction. After positioning a blood pressure cuff in the upper arm, baseline vessel diameter and blood flow were acquired. The cuff was then inflated to ≥10 mmHg above systolic blood pressure to occlude arterial flow for 3 min. Continuous recordings of the longitudinal image of the artery were obtained starting 30 s before and up to 2 min after cuff deflation. To assess hyperemic flow, the Doppler signal was registered immediately after cuff release for a maximum of 15 s. The information obtained was processed with a Hemodyn 4M instrument (Dinap SRL, Argentina). The flow-mediated dilator response, expressed as a percentage of the baseline brachial artery diameter, was used as an estimation of endothelium-dependent vasodilation. This method is routinely used in our laboratory.

EPC culture and characterization. A 40-mL sample of venous blood was used for the isolation of EPCs by density gradient centrifugation (Histopaque 1077, Cat. No. H8889, Sigma-Aldrich, St. Louis, MO). Samples were processed within 2 h after collection. The mononuclear peripheral blood cells were washed once with phosphate-buffered saline and twice in growth medium (BioCoat Endothelial Cell Culture Enviroment, Cat. No. 355054, Becton Dickinson, Bedford, MA) supplemented with 20% fetal-calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL). The isolated cells were resuspended in growth medium and plated onto human fibronectin-coated (Cat. No. 354559) or rat collagen-coated (Cat. No. 354557) six-well dishes (BioCoat Cellware, Becton Dickinson, Bedford, MA) for early EPCs (5×10^6 cells/well) and late EPCs (2×10^7 cells/well), respectively. After 48 h, the nonadherent cells were discarded, and the adherent cells were cultured for 9 days or 21 days for early EPCs and late EPCs, respectively (11). Growth medium was changed every other day, and digital photographs were obtained. To confirm the endothelial phenotype, at the end of the growth curve, cultured cells were characterized by exposure

to DiI-acetylated low-density lipoproteins (Cat. No. L3484, Invitrogen, Carlsbad, CA), FITC-conjugated lectin from Ulex europaeus (Cat. No. L9006, Sigma-Aldrich, St. Louis, MO), and monoclonal antihuman PE-conjugated CD45 (Cat. No. P7687, Sigma-Aldrich). CD14 and vascular endothelial growth factor receptor-2 (VEGFR-2) were revealed by indirect fluorescence immunohistochemistry by using monoclonal antihuman CD14 (Cat. No. C7673, Sigma-Aldrich) and VEGFR-2 (Cat. No. V3003, Sigma-Aldrich) antibodies, followed by FITC-conjugated antimouse IgG (Cat. No. F5687, Sigma-Aldrich), as previously described (11). TNTs were identified by microscopy and fluorescent phalloidin staining (Alexa Fluor 594 phalloidin, Cat. No. A12379, Molecular Probes, Life Technologies, Carlsbad, CA) on *days 9* and *21* for early EPCs and late EPCs, respectively. The details of the antibody immunohistochemistry procedure are provided as supplemental data in Table S1 (all Supplemental material is available at https://doi.org/10.6084/m9.figshare.12612158.v1). Digital images and Cell C software (Tampere University of Technology, Finland) were used to count the cells in six randomly selected microscopic fields per well by one operator who was blinded as to the corresponding subject's group. TNTs were quantified manually on digital images by one observer who was masked to the subject's group. For EPC and TNT counting, intraobserver variabilities,assessed by calculating the coefficient of variation [CV(%) = mean of SD × 100/data mean], were 5.2 and 5.4%, respectively. The interassay CV% was 6.9 and 7.4% for EPC and TNT counting, respectively.

Early and late-EPC capacity to form tubular structures was evaluated with a tube formation assay kit (Millipore, Cat. No. ECM 625) by following the manufacturer's instructions. Briefly, the extracellular matrix was mixed with diluent buffer, and the solution was kept on ice to prevent solidification. Fifty microliters of this solution was transferred to each well of a precooled 96-well culture plate, followed by incubation at 37 °C for 1 h to solidify the matrix solution. Early or late EPCs were suspended in endothelial cell culture medium (Becton Dickinson) and seeded at a density of 5×10^3 cells/well onto the matrix-coated plate. After overnight incubation at 37°C, tube formation was observed under an inverted microscope at ×200 magnification, and digital photographs were obtained. Tube formation by human umbilical vein endothelial cells was used as a positive control.

Statistical analysis. As the data were not normally distributed (Anderson–Darling normality test), differences between the hypertensive and normotensive groups in median values of EPCs and TNTs and of anthropometric variables were tested by the nonparametric Mann–Whitney *U* test.

Correlations between EPC parameters and anthropometric/metabolic parameters were assessed by both the Spearman rank order correlation test (for not normally distributed data) and simple linear regression after natural logarithmic transformation of the variables. The χ^2 test was used to investigate the presence of significant associations between two categorical variables, i.e., vascular alterations (levels, mild/moderate and fairly severe/severe) and numbers of either early EPCs or late EPCs or early TNTs or late TNTs (levels, lower and higher than half the values observed in normotensive patients).

RESULTS

Subject characteristics. The characteristics of the study subjects are shown in Table 1. Age, sex, and glycemia distributions showed no differences among the study groups.

In the normotensive group, systolic blood pressure, body mass index (BMI), and total cholesterol and triglyceride plasma levels were significantly lower than those in the rest of the groups. HDL cholesterol was significantly higher in the normotensive group versus all the other groups studied. In the normotensive group, diastolic blood pressure was significantly lower than that in the protected and control for protected groups.

Cultured early EPC, late-EPC, and TNT identification. The identification of early EPCs, late EPCs, and TNTs has been previously described (26). Briefly, early EPCs and late EPCs were obtained from human peripheral blood by using established mononuclear cell culture protocols. After immunophenotyping, early EPCs were identified as $CD14^+$, $CD45^+$, $AcLDL^+$, $UEA1^+$, and VEGFR-2^+ cells on *day 9* of culture, and late EPCs were identified as $CD14^-$, $CD45^-$, $AcLDL^+$, $UEA1^+$, and VEGFR-2^+ cells on *day 21* of culture. Therefore, as expected, early EPCs, but not late EPCs, expressed the hematopoietic markers CD14 and CD45, indicating that late EPCs are committed to the endothelial lineage. Early EPCs showed their characteristic spindle shape, and late EPCs displayed a cobblestone appearance.

To test their vasculogenic capacity, early and late EPCs were seeded onto a solid gel matrix designed to allow endothelial

Table 1. *Subject characteristics*

Characteristic	Vulnerable	Protected	Normotensive	Control for Vulnerable	Control for Protected	*P* Value
N	30	30	30	30	30	
Age, yr	48 (23–69)	51 (25–67)	49 (22–66)	52 (23–65)	50 (22–68)	0.865
Men, %	80.6	85.0	84.6	81.8	83.9	
SBP, mmHg	130 (107–139)	135 (97–138)	107 (103–119)*	130.5 (107–135)	136 (114–139)	*See legend
DBP, mmHg	81 (68–89)	86 (64–88)	78 (68–87)†	82.5 (63–89)	87 (64–88)	†See legend
Medication, %			None			
ACEi/ARAII	59	63		60	62	
Ca^{2+} channel blockers	29	34		31	29	
Diuretics	10	12		9.8	13	
Statins	48.9	47.8		48.2	51.4	
Other	22	19		21	18	
BMI, kg/m^2	28.0 (20.7–36.0)	27.5 (22.8–34.2)	22.0 (20.1–25.0)‡	28.0 (19–39)	29.0 (22.8–36)	‡See legend
Total Cholesterol, mg/dL	193 (113–262)	176 (114–298)	160 (143–199)‡	192 (121–249)	189 (111–253)	
HDL Cholesterol, mg/dL	50 (38–93)	48 (30–86)	76 (60–91)‡	51 (36–90)	48 (36–60)	
LDL Cholesterol, mg/dL	110 (55–178)	114.5 (41–211	115 (100–128)	120 (53–168)	115.5 (44–170)	
Triglycerides, mg/dL	114 (53–258)	94 (66–208)	84 (59–149)‡	123 (48–483)	119 (79–172)	
Glycemia, mg/dL	94.5 (77–117)	96 (84–99)	95 (86–99)	94 (81–99)	94 (88–99)	

Values are medians (maximum−minimum values). SBP, systolic blood pressure; DBP, diastolic blood pressure; ACEi, angiotensin-converting enzyme inhibitor; ARAII, angiotensin II type I receptor antagonist; BMI, body mass index. *$P < 0.001$ vs. all other groups; †$P < 0.02$ vs. protected and control for protected; and ‡$P < 0.05$ vs. all other groups (Mann–Whitney with Bonferroni correction).

cells to self-assemble into hollow tubular structures (in vitro angiogenesis kit). As expected, late EPCs, but not early EPCs, displayed the capacity to form interconnecting tube networks (Fig. 2*A*).

Tunneling nanotubes were identified as straight, thin, actin-rich cytoplasmic projections that crossed from one cell to another and measured the equivalent to several cell diameters (Fig. 2*B*). In general, TNTs appeared to be projected by a cell of normal morphology toward a round and refractile cell.

Early EPCs, late EPCs, and TNTs are reduced in controlled essential hypertensive patients. In the vulnerable group, the number of early EPCs was significantly lower than that in the protected, normotensive, and control for vulnerable groups (47, 52, and 21%, respectively; Fig. 3*A*). In the protected group, the number of early EPCs was 51 and 49% higher than that in the control for protected and control for vulnerable groups, respectively (Fig. 3*A*). In the normotensive group, early EPCs were 63 and 65% more abundant than those in the control for vulnerable and control for protected groups, respectively (Fig. 3*A*).

In the vulnerable group, the number of late EPCs was significantly lower than that in the protected and normotensive groups (27 and 28%, respectively; Fig. 3*B*). In the protected group, late-EPC counts were 125 and 60% higher than those in the control for protected and control for vulnerable groups, respectively (Fig. 3*B*). Also, in the normotensive group, late EPCs were 126 and 61% more abundant than those in the control for protected and control for vulnerable groups, respectively. Finally, in the control for vulnerable group, the number of late EPCs was 41% higher than that in the control for protected group (Fig. 3*B*).

TNT quantification refers to the number of early or late EPCs that emitted TNTs per microscopic field. In the vulnerable group, the number of early TNTs was significantly lower than that in the protected and normotensive groups (33 and 72%, respectively; Fig. 4*A*).

In the protected group, the numbers of early TNTs exceeded those observed in the control for protected and control for vulnerable groups by 32 and 92%, respectively. In the normotensive group, early TNT counts surpassed by 178 and 300% of those found in the control for protected and control for vulnerable groups, respectively (Fig. 4*A*).

In the vulnerable group, the number of late TNTs was significantly lower than that in the protected and normotensive groups (77 and 85%, respectively; Fig. 4*B*). In the protected group, late TNT counts exceeded those found in the control for protected and control for vulnerable groups by 333 and 116%, respectively. In normotensive subjects, the number of late-TNTs surpassed by 566 and 233% of those observed in the control for protected and control for vulnerable groups, respectively. The number of late-TNT was 100% higher in the control for vulnerable group relative to the control for protected group (Fig. 4*B*).

The severity of vascular damage is negatively associated to EPC and TNT numbers. In agreement with our working hypothesis that the disparity in vascular status between the

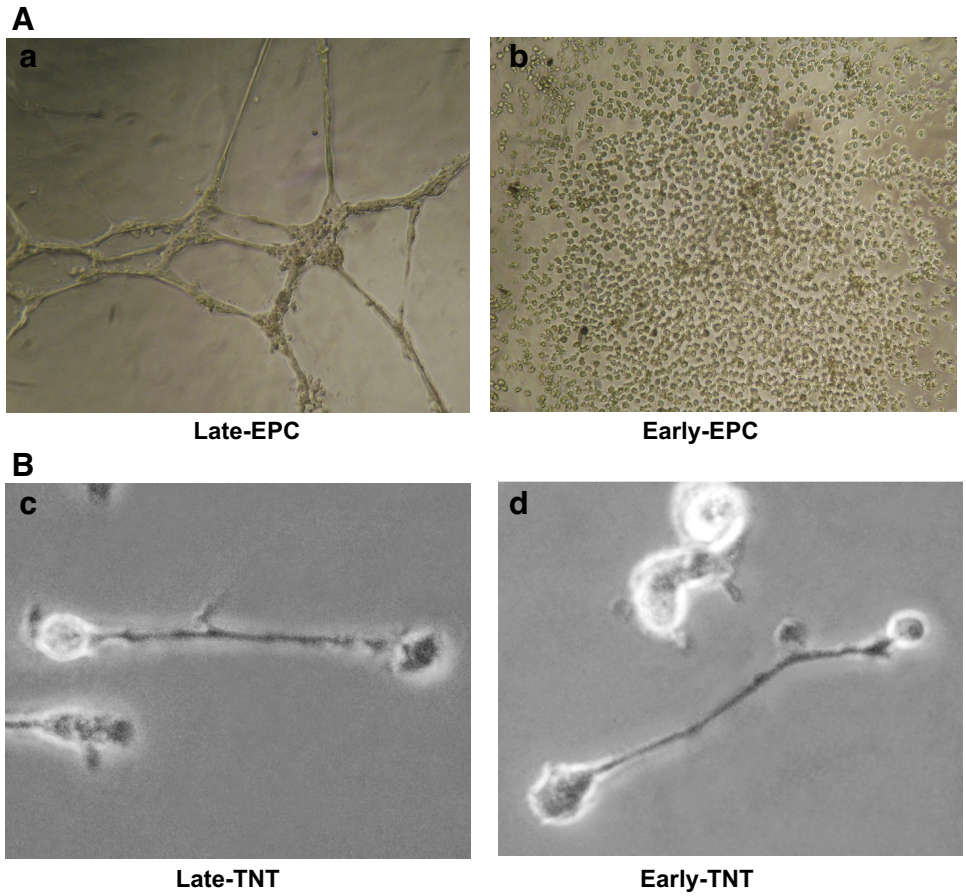

Fig. 2. *A*: early endothelial progenitor cells (EPCs) and late EPCs were seeded onto a solid gel matrix designed to allow endothelial cells to self-assemble into hollow tubular structures (in vitro angiogenesis kit). As expected, late EPCs but not early EPCs displayed the capacity to form interconnecting tube networks (×200). *B*: tunneling nanotubes (TNTs) were identified as thin, straight, actin-rich (phalloidin staining) cytoplasmic projections emitted by both late EPCs (referred to as late TNTs) and early EPCs (referred to as early TNTs), with a length equivalent to several cell diameters, and crossing from one cell to another. Optical microscopy, ×400; zoom, ×3.

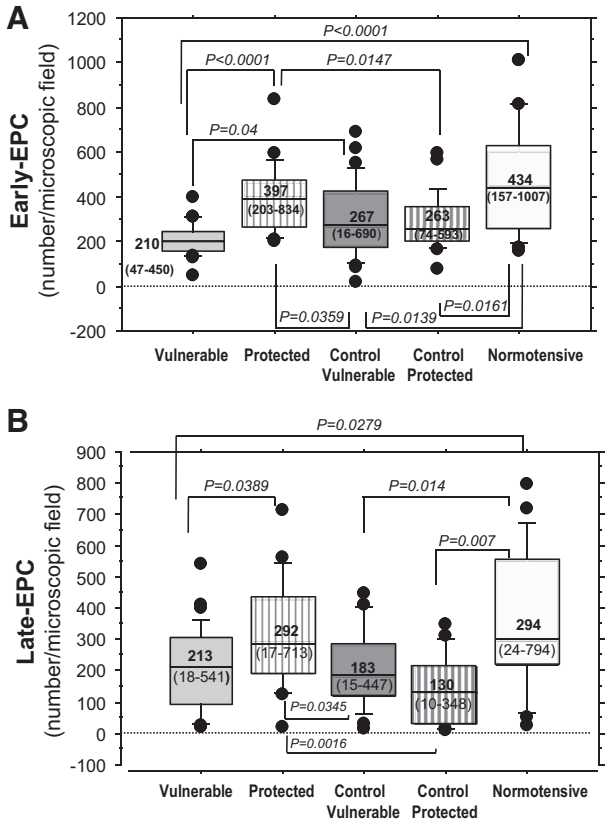

Fig. 3. Early endothelial progenitor cells (EPCs) and late EPCs were counted on culture *days 9* and *21*, respectively. Values are expressed as median (minimum to maximum value range). *A*: early EPCs, $N = 30$ for each group; *B*: late EPCs. Differences between groups were established by using Mann–Whitney statistics with the Bonferroni correction for multiple comparisons. $N = 30$ for each group.

protected and vulnerable groups was related, at least partly, to their different capacities for endothelial repair mediated by EPCs, the χ^2 test revealed significant negative associations between the categorical variables vascular change severity and the numbers of either early EPCs ($P = 0.0003$) or late EPCs ($P = 0.0016$) or late TNTs ($P = 0.0023$) but not early TNTs ($P = 0.0688$). The 2×2 contingency table used included two levels for each variable: on one side were mild/moderate (protected) and fairly severe/severe (vulnerable) vascular alterations and on the other side were lower and higher numbers of EPCs or TNTs relative to half the values observed in normotensive patients. To construct the contingency table, it was necessary to define a cutoff value for the low and high numbers of EPCs and TNTs; for this purpose, we considered it as a reasonable choice to use half the values observed in the normotensive group studied here.

The numbers of EPCs and TNTs do not correlate with patient characteristics or cardiovascular risk factors. Table 2 shows that in the total population of individuals studied ($N = 120$), the numbers of early EPCs, late EPCs, early TNTs, and late TNTs are not related to age, BMI, systolic/diastolic blood pressures, heart rate, glycemia, plasma triglycerides, and total/HDL/LDL cholesterol levels. Consequently, it was unfeasible to perform a multivariate regression analysis.

DISCUSSION

Here we studied two groups of treated hypertensive patients that showed a discrepancy between the calculated Framingham score (FS) and the results of vascular status testing. Vascular testing included the assessments of carotid intima-media thickness (IMT) and carotid diameter, presence/absence of carotid atherosclerotic plaques, brachial artery flow-mediated dilation (FMD), and carotid/femoral pulse wave velocity (PWV). Thus, the vulnerable group exhibited an FS of $<10\%$, which suggests a low risk of developing vascular changes; however, this was accompanied with fairly severe to severe vascular alterations. In contrast, the protected group evidenced an FS of $\geq 10\%$, a value range that suggests a high risk of developing blood vessel deterioration, but these individuals displayed mild to moderate vascular changes; this implies that these patients were protected from the deleterious effects of cardiovascular risk factors.

In this work, the number of TNTs represents the number of EPCs that emitted TNTs per microscopic field. As TNTs have the ability to rescue damaged cells by transferring organelles, ions, or electric currents, those cell cultures where the number of TNTs exceeded the numbers observed in other cultures can be interpreted as possessing superior repair capacity.

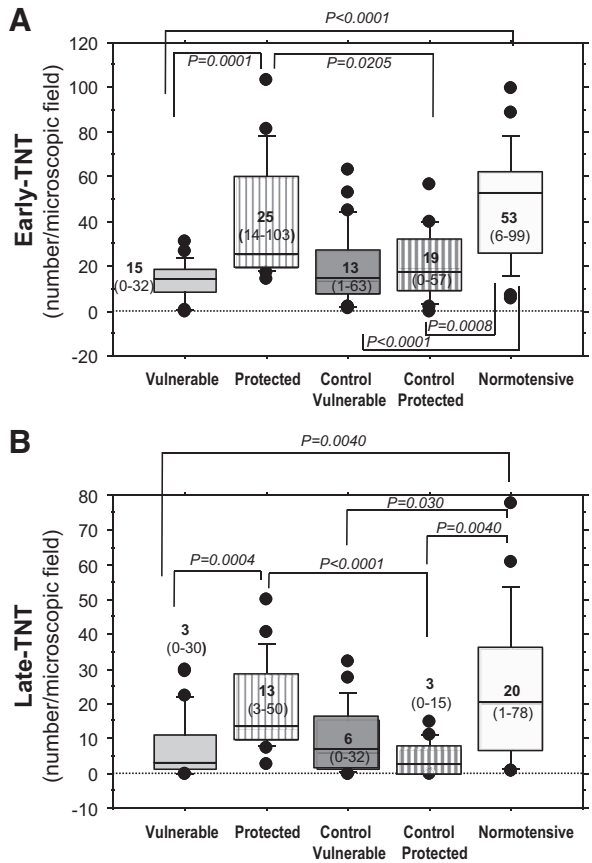

Fig. 4. Tunneling nanotube (TNT) quantification refers to the number of early or late endothelial progenitor cells (EPCs) that emitted TNTs per microscopic field. Early and late TNTs were counted on culture *days 9* and *21*, respectively. Values are expressed as median (minimum to maximum value range). *A*: early TNTs, $N = 30$ for each group; *B*: late TNTs. Differences between groups were established by using Mann–Whitney statistics with the Bonferroni correction for multiple comparisons. $N = 30$ for each group.

Table 2. *Correlations between endothelial progenitor cell parameters and anthropometric/metabolic parameters in the total population of individuals studied (N = 120)*

Correlated Parameter	Rho	*P* Value
Early EPCs		
Age	0.100	0.2777
SBP	−0.086	0.3464
DBP	−0.007	0.9401
Heart rate	0.111	0.2243
Glycemia	0.156	0.0903
Triglycerides	0.031	0.7362
Total cholesterol	−0.015	0.8758
HDL cholesterol	0.049	0.5970
LDL cholesterol	0.016	0.8625
BMI	0.117	0.1985
Late EPCs		
Age	−0.033	0.7223
SBP	−0.146	0.1178
DBP	0.059	0.5312
Heart rate	0.156	0.0969
Glycemia	0.042	0.6559
Triglycerides	0.020	0.4007
Total cholesterol	0.045	0.6375
HDL cholesterol	−0.004	0.9967
LDL cholesterol	0.030	0.7592
BMI	−0.035	0.7100
Early TNTs		
Age	0.114	0.2108
SBP	−0.150	0.1014
DBP	−0.075	0.4137
Heart rate	0.078	0.3933
Glycemia	0.199	0.0310
Triglycerides	0.030	0.7464
Total cholesterol	−0.022	0.8109
HDL cholesterol	0.075	0.4256
LDL cholesterol	0.007	0.9442
BMI	−0.029	0.7547
Late TNTs		
Age	0.037	0.6934
SBP	−0.183	0.0520
DBP	0.008	0.09285
Heart rate	0.162	0.2777
Glycemia	0.051	0.5856
Triglycerides	0.117	0.2234
Total cholesterol	0.008	0.2243
HDL cholesterol	0.101	0.2970
LDL cholesterol	0.127	0.1969
BMI	−0.118	0.2089

Correlations between EPC parameters and anthropometric/metabolic parameters were assessed by both the Spearman rank order correlation test (for not normally distributed data) and simple linear regression after natural logarithmic transformation of the variables. EPC, endothelial progenitor cells; TNT, tunneling nanotubes; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index.

In line with our working hypothesis, which proposed that the disparity in vascular status between the protected and vulnerable groups was related, at least partly, to their different capacities for endothelial repair mediated by EPCs, we found that in the protected group, the numbers of early and late EPCs and early and late TNTs were significantly higher than those in the vulnerable group. In addition, significant negative associations were found between the severity of vascular damage and early EPC or late-EPC or late-TNT numbers. These observations are in agreement with the role of EPCs as contributors to vascular injury repair and indicate that currently unidentified factor(s) exist that are capable of counteracting the blood vessel-damaging effects of the cardiovascular factors included in the calculation of the FS.

It is interesting to note that, with the exception of the protected group, in all the controlled hypertensive patient groups (vulnerable, control for vulnerable, and control for protected), early and late-EPC and early and late-TNT counts were significantly lower than those in the normotensive group. This finding is compatible with the concept that EPCs and TNTs are associated with an endothelium repairing function.

Also, as expected, in the protected group, EPC and TNT counts were significantly higher than those in the control for protected group (those patients with an FS of $\geq$10 and showing fairly severe to severe vascular changes). Nonetheless, according to our working hypothesis, it was expected that the control for vulnerable group, i.e., those hypertensive patients exhibiting an FS of $<$10% and mild to moderate vascular changes, would exhibit higher ECP/TNT counts relative to the vulnerable group. In the control for vulnerable group, this turned out to be the case only for early EPCs, as these cells surpassed those observed in the vulnerable group by 27%; however, the numbers of late EPCs, early TNTs, and late TNTs showed no differences between those two patient groups. In addition, as reported in more detail later, in the control for vulnerable group, but not in the other groups studied, early TNT numbers were significantly higher in statin-treated versus nontreated patients. Therefore, it is feasible that although all medications were interrupted for 24 h in advance of the day when blood samples were obtained for the isolation of EPCs/TNTs, the aforementioned unexpected finding may be related to the heterogeneous antihypertensive agents and statins that the patients were receiving, particularly considering that a 24-h interruption of the patient's antihypertensive medications may not be long enough to "wash out" acute effects of the drugs. Thus, several studies have shown that angiotensin II-receptor blockers improve EPC counts and function in hypertensive rats and in coronary artery disease (CAD) patients and when added to cultured EPCs; the same effect was observed for angiotensin II-converting enzyme inhibitors both in hypertensive and CAD patients and for calcium channel blockers in hypertensive individuals [reviewed in Lee and Poh (20)].

Contrasting data have been published in relation to statin effects on EPCs. Some studies reported that statin therapy is associated with higher numbers of circulating EPCs, whereas others found lower counts of circulating EPCs in patients under prolonged statin treatment [reviewed in Sandhu et al. (25)]. Here, we analyzed separately each of the study groups and found no differences in the numbers of early and late EPCs or late TNTs when comparing between those patients receiving and not receiving statin therapy. However, in the control for vulnerable group, but not in the other groups studied, early TNT numbers were 116% higher (P = 0.0240, Mann–Whitney) in statin-treated versus nontreated patients (Table S2).

In the population of essential hypertensive patients studied, neither age nor any of the cardiovascular risk factors evaluated (SBP/DBP, heart rate, glycemia, BMI, and plasma triglycerides and cholesterol) had an impact on the numbers of early and late EPCs or early and late TNTs. Again, this observation may have resulted from the diversity of antihypertensive compounds that the patients were receiving. Concerning the distribution of sexes within the study groups, it is interesting to note that sample subjects were predominantly men, which is not surpris-

ing considering that the age medians in the four groups studied varied from 48 to 52 yr, the age median for menopause is in the range of 50 to 52 yr for white women in industrialized countries (18), atherosclerosis in women mainly evolves following menopause, and the incidence of CV disease is higher in men than in age-matched women, although these sex-related differences decline following menopause because of the gradual loss of vascular protection provided by sex steroids (27).

EPCs have been extensively studied and at present are being tested as cell-based therapies for revascularization approaches (4, 8). In addition, a recent study showed that platelets obtained from healthy hamsters improved the function of late EPCs obtained from animals with experimental atherosclerosis (1).

It can be concluded that although this is an observational study, which as such does not allow to draw any cause-effect conclusions, the present results showing that those hypertensive patients presenting with higher EPC/TNT numbers are protected from vascular deterioration strongly encourage further research aimed at establishing low EPC/TNT count cutoff values so as to intervene before vascular alterations are evident, optimizing cultured EPC function before autologous cellular transplantation in regenerative medicine and identifying nonpharmacological or pharmacological treatments, including antihypertensive agents or other compounds that influence lipid and carbohydrate metabolic disarrangements and could improve EPC/TNT numbers and/or functions.

In previous work (11), we found that in controlled hypertensive patients, sympathetic overactivity and parasympathetic underactivity were negatively associated with EPCs, suggesting that reducing sympathetic activation and increasing parasympathetic activation might favor endothelial repair. This finding may have helped to explain why hypertensive patients who have achieved target blood pressure levels through pharmacological treatment still display a high residual cardiovascular risk. The present study advances knowledge in the field by confirming that controlling hypertension is not enough to attain EPC numbers/functions associated to normotensive levels and that in controlled hypertensive patients, vascular status is dependent, at least partly, on peripheral blood EPC and TNT numbers. As a whole, these observations underscore the need for increasing research efforts in this topic, especially considering that CV disease is the leading global cause of death.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

E.A.M.d.C., S.A.G., F.I., P.J.F., C.E.C., J.A.C.-S., S.O., and C.K. conceived and designed research; E.A.M.d.C., S.A.G., M.J.C., and P.K. performed experiments; E.A.M.d.C. and S.A.G. analyzed data; E.A.M.d.C., S.A.G., F.I., P.J.F., C.E.C., J.A.C.-S., S.O., and C.K. interpreted results of experiments; E.A.M.d.C. prepared figures; E.A.M.d.C. drafted manuscript; E.A.M.d.C., F.I., P.J.F., C.E.C., and M.J.C. edited and revised manuscript; E.A.M.d.C., S.A.G., F.I., P.J.F., C.E.C., J.A.C.-S., S.O., M.J.C., P.K., and C.K. approved final version of manuscript.

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