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SYMPATHETIC PREDOMINANCE IS ASSOCIATED WITH IMPAIRED ENDOTHELIAL PROGENITOR CELLS AND TUNNELING NANOTUBES IN CONTROLLED-HYPERTENSIVE PATIENTS

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Running Head: Endothelial progenitors, autonomic activity and hypertension

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ABSTRACT

Objective. Early-endothelial progenitor cells (early-EPC) and late-EPC are involved in endothelial repair, and can rescue damaged endothelial cells by transferring organelles through tunneling-nanotubes (TNT). In rodents, EPC-mobilization from the bone marrow depends on sympathetic nervous system activity. Indirect evidence suggests a relation between autonomic derangements and human EPC-mobilization. We aimed at testing whether hypertension-related autonomic imbalances are associated with EPC impairment. **Results.** Thirty controlled-essential hypertensive patients [SBP/DBP=130(120-137)/85(61-88) mmHg; 81.8% male], and twenty-healthy normotensives [(114(107-119)/75(64-79) mmHg; 80% male] were studied. Mononuclear cells were cultured on fibronectin- and collagen-coated dishes for early-EPC and late-EPC, respectively. Low-(LF) and high-frequency (HF) components of short-term heart rate variability were analyzed during a 5-min rest, an expiration/inspiration maneuver, and a Stroop color-word-test. Modulations of cardiac sympathetic and parasympathetic activities were evaluated by LF/HF (%) and HF-power (ms^2), respectively. In controlled-hypertensive patients, the numbers of early-EPC, early-EPC that emitted TNT, late-EPC and late-EPC that emitted TNT were 41%, 77%, 50% and 88% lower than in normotensives ($p<0.008$). In controlled-hypertensive patients, late-EPC number was positively associated with cardiac parasympathetic reserve during the expiration/inspiration maneuver (Rho=0.45, p=0.031) and early-EPC with brachial flow-mediated dilation (Rho=0.655; p=0.049); also, late-TNT number was inversely related to cardiac sympathetic response during the stress-test (Rho=-0.426, p=0.045). EPC exposure to epinephrine or norepinephrine showed negative dose-response relationships on cell adhesion to fibronectin and collagen; both catecholamines stimulated early-EPC growth, but epinephrine inhibited late-EPC growth. **Conclusions.** In controlled-hypertensive patients, sympathetic overactivity/parasympathetic underactivity were negatively associated with EPC,

suggesting that reducing sympathetic/increasing parasympathetic activation might favor endothelial repair.

Key Words: endothelial regeneration, autonomic balance**,** parasympathetic

Introduction

Current evidence supports the concept that circulating bone marrow-derived endothelial progenitor cells (EPCs) contribute to the repair and regeneration of the injured endothelium(15). Tissue ischemia and/or endothelial damage promote EPC mobilization from the bone marrow, and EPC recruitment and incorporation at sites of vascular damage (15). Two distinct types of EPC have been identified by *in vitro* cell culture of the blood mononuclear cell fraction, i.e., early-EPC and late-EPC, the latter also known as outgrowth endothelial cells. Early-EPC –representing alternative activated M2 macrophages- promote vascular repair through the paracrine release of cytokines, and late-EPC by differentiating into endothelial cells and incorporating into blood vessels (20, 26). In addition, EPC can rescue damaged endothelial cells by transferring mitochondria and lysosomes through the recently discovered cell-to-cell communication channels referred to as tunneling nanotubes (TNT) (34, 35).
Studies in rodents showed that the sympathetic nervous system plays an essential role in EPC mobilization from the bone marrow (22), and that catecholamines are involved in the modulation of post-ischemic revascularization (31), suggesting that autonomic dysregulation can impair vascular endothelial repair. In humans, the relation between sympathetic activation and EPC egress from the bone marrow was indirectly suggested by reports showing a negative relation between the number of circulating EPC and both psychosocial stressors (13) and depression scores (8), which are known to be associated with autonomic derangements. In this context, it is noteworthy that *in vivo* endothelial repair capacity is reduced in prehypertension and hypertension patients (16), and that sympathetic overactivation has been calculated to be present in approximately 50% of essential hypertension patients (10, 30).

Considering all the above, we postulated that autonomic imbalances characterized by sympathetic overactivation and/or parasympathetic inhibition would be associated to impaired endothelial repair capacity in treated hypertensive patients. To test this

notion, in this study we assessed the relations between the number and function of EPCs and autonomic status in patients under treatment for hypertension that had achieved goal blood pressure, and also investigated *ex vivo* EPC responses to added epinephrine and norepinephrine.

Materials and Methods

Patients

The study adhered to the principles outlined 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 90 individuals that 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. Thirty treated essential hypertensive patients (Hypertensive group) that had achieved goal blood pressure were included. Patients were asked to interrupt their antihypertensive medications for 24 h in advance of the day when they underwent anthropometric, metabolic (fasting glycemia and lipid profile), autonomic and flow-mediated dilation evaluations. Twenty healthy normotensive volunteers (Normotensive group), that were not receiving any medication, were studied to obtain reference values for EPC and TNT. Office blood pressure was measured with a calibrated and validated semiautomatic oscillometric device (Omron HEM-781CPINT, Omron Healthcare, Inc., Lakeside Drive Bannockburn, IL) according to JNC7 (9), with an appropriate cuff size and by averaging the second and third readings after the patients had been seated for 5 min.

Assessment of autonomic function.

In Hypertensive patients, heart rate variability (HRV) was measured in the morning after 40 minutes of supine rest in a quiet room, at 22 °C. Frequency domain analysis of short-term HRV was performed by using an autoregressive model (Kubios software, Finland) in 3 settings: basal (during a 5-min supine rest), during a deep breathing

maneuver (E/I maneuver, with 6 metronomic breaths/minute), and while performing a mental stress test (Stroop color-word test). Beat-to-beat R-R intervals (RRI; i.e. HRV) and systolic blood pressure (SBP) were continuously monitored throughout these maneuvers (1),(29).

Spectral analysis of HRV data yielded the low (0.05-0.15 Hz, LF) and high (>0.15 Hz, HF) frequency components of total power. For the assessment of autonomic balance, the cardiac parasympathetic reserve was evaluated by analyzing HF (ms^2) during both the 5-min rest period and the E/I maneuver, whereas the cardiac sympathetic response was assessed by analyzing the LF/HF ratio (%) during the 5-min rest and the mental stress test.

EPC culture and characterization

A 40-mL sample of venous blood was used for the isolation of EPC 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 amphothericin B (0.25 ug/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) 6 well dishes (BioCoat cellware, Becton Dickinson, Bedford, MA) for early-EPC ($5x10^6$ cells/well) and late-EPC ($2x10^7$ cells/well), respectively. After 48 h, the non-adherent cells were discarded, and the adherent cells were cultured during 9 days or 21 days for early-EPC and late-EPC, respectively (27). 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 anti-human CD14 (Cat. No. C7673, Sigma-Aldrich) and VEGFR-2 (Cat. No. V3003, Sigma-Aldrich) antibodies, followed by FITC-conjugated anti-Mouse IgG (Cat. No. F5687, Sigma-Aldrich). TNT were identified by microscopy and fluorescent phalloidin staining (Alexa Fluor® 594 phalloidin, Cat. No. A12379, Molecular Probes, Life Technologies, Carlbad, CA) on days 9 and 21 for early-EPC and late-EPC, respectively. Digital images and Cell C software (Tampere University of Technology, Finland) were used to count the cells in 6 randomly selected microscopic fields/well, by one operator that was blinded as to the corresponding subject´s group. TNT were quantified manually on digital images by one observer that 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 x 100 / Data Mean]- were 5.0 % and 5.3 %, respectively. The inter-assay CV% were 7.5 % and 7.1 % for EPC and TNT counting, respectively.

Effects of epinephrine and norepinephrine on cultured EPC

Early- and late-EPC were cultured in the presence of epinephrine hydrochloride (Cat. No. E4642, Sigma-Aldrich) or norepinephrine bitartrate (Cat. No. A9512, Sigma-Aldrich) that were dissolved in phosphate buffered saline (PBS) and added to the culture medium at the final epinephrine concentrations reported for human plasma (11) at standing, during exercise and in response to other types of stress (25, 100 and 400 ng/L, respectively; n= 6 independent assays each) and at plasma norepinephrine levels reported at standing and during exercise (100 and 200 ng/L, respectively) (17). Control cells were added with PBS. To test the effects of these catecholamines on EPC adhesion to fibronectin and collagen, epinephrine or norepinephrine were added to the mononuclear cell culture medium at the time of seeding onto the respective culture dishes, and adherent EPC were counted 48 h later; whereas to test their effects on

EPC growth, mononuclear cells were seeded onto fibronectin or collagen in the absence of epinephrine or norepinephrine, and 48 h later -after having discarded non-adherent cells- the catecholamines were added to adherent EPC. The catecholamine supplemented media were replaced every 48 h, and early- and late-EPC were counted on days 9 or 21, respectively, as was described in “EPC culture and characterization”.

Brachial artery flow-dependent endothelium-mediated dilation

To evaluate endothelial function status in treated hypertensive patients, brachial artery flow-dependent endothelium-mediated dilation was measured in the morning under fasting conditions, with patients supine 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 (7). 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 minutes. Continuous recordings of the longitudinal image of the artery were obtained starting 30 s before and up to 2 minutes after cuff deflation. To assess hyperemic flow, the Doppler signal was registered immediately after cuff release for a maximum of 15 seconds. The information obtained was processed with a Hemodyn 4M instrument. 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.

Statistical analysis

Since the data were not normally distributed, differences between the Hypertensive and Normotensive groups in median values of EPC and TNT and of anthropometric variables were tested by the nonparametric Mann–Whitney *U* test.

Correlations between EPC parameters and autonomic evaluation parameters were assessed both by Spearman rank order correlation test (for not normally distributed data) and by simple linear regression after natural logarithmic transformation of the variables. Multivariate linear regression analysis with either EPC or TNT as predicted variables and autonomic evaluation, anthropometric/metabolic parameters and blood pressure as predictor factors was assessed according to the forward-stepwise method. Those parameters appearing clearly uncorrelated to EPC or TNT numbers by the Spearman test and simple linear regression were excluded from the multivariate analysis.

RESULTS

Subjects´ characteristics

The characteristics of the study subjects are shown in Table 1. The Hypertensive and Normotensive groups showed no differences in age and gender distribution, BMI, LDL- and HDL-cholesterol levels, and glycemia. In the Normotensive group systolic and diastolic blood pressures were significantly lower than in the Hypertensive group. Also, in the Hypertensive group plasma triglycerides and total cholesterol were higher than in the Normotensive group, although the differences reached statistical significance only for triglycerides.

Cultured early-EPC, late-EPC, and TNT identification

Early-EPCs and late-EPCs were obtained from human peripheral blood according to established protocols. After *ex-vivo* culture, each type of EPC homogeneously exhibited their distinctive morphology, i.e., early-EPC were spindle-shaped cells and

late-EPC exhibited a cobblestone appearance. After immunophenotyping, early-EPC were identified as CD14+, CD45+, AcLDL+, UEA1+, VEGFR-2+ cells on day 9 of culture, and late-EPC as CD14-, CD45-, AcLDL+, UEA1+, VEGFR-2+ cells (day21) (Figure 1A). Therefore, as expected, early-EPC –but not late-EPC- expressed the haematopoietic markers CD14 and CD45, indicating that late-EPC are committed to the endothelial lineage.

To ascertain that CD14 and CD45 positive cells were not a mixture of live and dead cells, we first stained early-EPC with DiI-acetylated LDL that is taken-up through receptor-mediated endocytosis and functions as a live cell marker. Since we could not co-stain cells with CD45 fluorescent and DiI-acetylated LDL antibodies, because they both emitted light in the red region, we used an indirect approach consisting of two steps: 1) we co-stained cells with DiI-acetylated LDL and a green fluorescence CD14 antibody, and found that all CD14+ cells were live cells, since they were also DiI-acetylated LDL+ cells (Figure 1B), and 2) we co-stained cells with CD45 with CD14 antibodies (red and green fluorescence, respectively), and showed that all CD45+ cells were also CD14+ cells (Figure 1B). Figure 1B also shows that early EPC were positive for both VEGFR-2 and CD45.

Tunneling nanotubes were characterized as thin, straight, actin-rich cytoplasmic projections, with a length equivalent to several cell diameters, and crossing from one cell to another (Figure 2).

Ex vivo cultured early-EPC, late-EPC and TNT are reduced in controlled essential hypertensive patients

Table 2A shows the quantification of EPC and TNT (i.e., tunneling nanotubes projected by EPC). TNT counting refers to the total number of EPC emitting tunneling nanotubes per microscopic field. For early-EPC and late-EPC, TNT started to be evident on days 5 and 10 of culture, respectively. In the Hypertensive group, early-EPC and early-TNT,

late-EPC and late-TNT were significantly less abundant than in the Normotensive group (43 %, 78 %, 51 % and 89 %, respectively).

The percentage of early-EPC and late-EPC that emitted TNT was significantly lower in Hypertensive patients than in Normotensive individuals (Table 2B).

Relation between autonomic status and EPC numbers in controlled essential hypertensive patients

Table 3A shows cardiac parasympathetic reserve and sympathetic response as evaluated by analyzing the HF and LF/HF ratio components of heart rate variability in the Hypertensive group, during a 5-min rest, the E/I maneuver, and the mental stress test.

Considering that the data were not normally distributed, the Spearman correlation coefficient was used to assess the strength of the relation between variables. However, given that the robustness of Spearman correlation is lower than that of Pearson correlation coefficient, we also used the latter method after normalization by natural logarithmic transformation of the data.

In the Hypertensive group, late-EPC number was positively related to the cardiac parasympathetic reserve during the E/I maneuver, both when assessed by statistical non-parametric analysis (Table 3B) and after natural logarithmic transformation and linear regression analysis (Figure 3).

In contrast, in the Hypertensive group late-TNT numbers were negatively associated with cardiac sympathetic response during the mental stress test (Table 3B). During the rest period, no relations were found between either early-EPC, early-TNT, late-EPC or late-TNT and cardiac parasympathetic reserve or sympathetic response.

To establish whether risk factors or hypertensive patients´ characteristics other than cardiac parasympathetic reserve and sympathetic response might have partly explained the reduced late-EPC and late-TNT counts, a multiple linear regression analysis was performed. No relations were found between any of early-EPC, late-EPC,

early-TNT, late-TNT and either BMI, glycemia, TG, Total/HDL/LDL Cholesterol, blood pressure (SBP/DBP) or age. Therefore, for multivariate linear regression analysis with either late-EPC or late-TNT numbers as predicted variables, only autonomic evaluation parameters (HF during the E/I maneuver and LF/HF ratio during the stress test, respectively) could be used as predictor factors.

Effects of epinephrine and norepinephrine on cultured EPC

Since correlation analysis is not sufficient proof of causation, early- and late-EPC were cultured in the presence of epinephrine or norepinephrine to test whether plasma catecholamine levels might contribute to the negative association observed in hypertensive patients between sympathetic overactivity/parasympathetic underactivity and EPC and TNT numbers. Exposure of EPC to epinephrine showed a negative dose-response relationship on cell adhesion to fibronectin; thus, at 25 ng epinephrine/L the number of adherent cells 48 h after seeding was 13 % lower (not significant) than early-EPC treated with PBS, but at 100 and 400 ng epinephrine/L cell adhesion was progressively and significantly reduced by 34 % and 63 %, respectively (Figure 4-A1). A negative dose-response behavior was also observed when the cells were exposed to norepinephrine at 100 ng/L and 400 ng/L, which decreased EPC adhesion to fibronectin by 16 % (not significant) and 50 %, respectively, relative to PBS-treated cells (Figure 4-A1). However, when seeded over collagen, cell incubation with epinephrine at 100 ng/L and 400 ng/L or norepinephrine at 200 ng/L slightly but significantly reduced EPC adhesion (by 3.7 %, 7.7 % and 3.6 %, respectively) compared with cells incubated in the presence of PBS (Figure 4-A2).

In contrast, exposure to epinephrine showed a positive dose-response relationship on early-EPC growth, i.e. early-EPC count on day 9 of culture was 17 % and 39 % higher in cells incubated with epinephrine at 100 ng/L and 400 ng/L, respectively, relative to PBS-treated cells, whereas 25 ng epinephrine/L had no effect. Incubation of early EPC with norepinephrine at 100 ng/L and 200 ng/L stimulated cell growth by 43 % and 41

%, respectively, relative to incubation without norepinephrine (Figure 4-B1). When cultured over collagen, incubation with norepinephrine had no effect on late-EPC growth, and only 400 ng epinephrine/L reduced cell growth by 20 % versus PBS-treated cells (Figure 4-B2).

The amount of cultured early-EPCs is positively correlated with flow-dependent, endothelium-mediated dilation

In controlled hypertensive patients, the median value for brachial artery flow-dependent, endothelium-mediated dilation (FMD) was 4.88 %, with minimum and maximum values of 0 % and 19.3 %, respectively.

Ex vivo cultured early-EPC counts were positively correlated with brachial artery FMD [Spearman rho = 0.655; p = 0.049; and linear regression analysis (Figure 5)].

No relation was found between the arterial blood flow response [median (max-min values) = 139 (68-181) ml/min] to reactive hyperemia and FMD (Spearman rho = 0.036; p= 0.8342), indicating that although increases in blood flow-associated shear stress are known to trigger the vasodilatory response, other factors exerted a predominant influence on FMD modulation.

DISCUSSION

The present work shows that in the treated essential hypertensive patients studied, although the blood pressure achieved was within the range recommended by the JNC7 guidelines, a) the number and function of *ex vivo* cultured early- and late-EPC are reduced when compared with normotensive subjects, b) an autonomic profile characterized by sympathetic overactivity/parasympathetic underactivity is negatively associated with EPC and TNT numbers, and c) epinephrine and norepinephrine negatively affect early- and late-EPC adhesion and to a lesser extent stimulate *ex-vivo* early-EPC growth, whereas epinephrine reduces late-EPC growth, d) a positive correlation exists between cultured early-EPC counts and the extent of brachial flow

mediated dilation, indirectly suggesting that EPC alterations are associated with, and can have detrimental effects on, vascular function.

Given that no relations were found between any of early-EPC, late-EPC, early-TNT, late-TNT and plasma triglycerides, total cholesterol, or systolic/diastolic blood pressures it follows that the lower number and function of *ex vivo* cultured early- and late-EPC observed in hypertensive patients relative to normotensive subjects was not dependent on the differences observed in these particular parameters between both groups.

To the best of our knowledge, this is the first report to show that both cultured late-EPC counts and the number of early- and late-EPC that emit TNT are lower in controlled hypertensive patients relative to normotensive subjects. The relevance of TNT formation by EPC is evidenced by puzzling data showing that the marked improvement of vascular repair by transplantation of EPC is accompanied by scarce EPC engraftment at injury sites (37). This apparent contradiction can be explained, at least partly, by recent evidence showing that *in vitro* and *in vivo* the formation of TNT by EPC is responsible for the rescue of prematurely senescent endothelial cells; this involves TNT-mediated transfer of lysosomes from EPC to endothelial cells, which reconstitutes the lysosomal pool of stressed endothelia and improves their viability and function (34). In this context, a previous report showed that essential hypertensive patients have an increased proportion of senescent *ex-vivo* cultured EPC relative to normotensive individuals, and that the extent of hypertension-related organ damage was positively related to EPC senescence (21).

The number of TNT reported here represents the basal level of TNT expression by cells under normal tissue culture conditions. The lower early- and late-TNT numbers observed in cultured cells from controlled hypertensive patients relative to normotensive subjects cannot be extrapolated the *in vivo* situation; however, considering that TNT contribute to cell survival, the present *ex vivo* finding is consistent with the well-documented reduction of *in vivo* endothelial repair capacity that occurs in

prehypertension and hypertension patients. At present, the signals that guide tunneling nanotube formation are not completely understood. It should be mentioned, that apart from EPC, TNT were described in other cell types. Also, TNT can facilitate not only the transfer of lysosomes but also that of mitochondria, plasma membrane components, vesicles, Ca2+, pathogens and electrical signals. This novel type of intercellular communication participates in such vital processes as tissue regeneration, signal transmission, development, and immunity (2) . According to our present results, it can be suggested that in treated hypertensive patients the alterations in TNT formation may negatively affect EPC function despite having achieved adequate blood pressure values through pharmacological treatment.

Here we observed that in treated hypertensive patients the cardiac parasympathetic reserve was positively related to *ex vivo* cultured late-EPC levels, whereas cardiac sympathetic response was negatively associated with TNT numbers in late-EPC cultures. These results suggest that normalization of the sympathetic/parasympathetic balance may increase EPC levels and improve their function, thereby enhancing the capacity for endothelial repair in essential hypertensive patients. The latter concept is supported by both the observed negative dose-response relationship on *ex-vivo* EPC adhesion to fibronectin and collagen, as well as epinephrine´s inhibitory effect on late-EPC growth. Although both epinephrine and norepinephrine stimulated early-EPC growth, the magnitude of the effect was smaller than the detrimental influence on EPC adhesion. The observed stimulation of early-EPC growth by catecholamines is a puzzling finding for which we can only provide a speculative reasoning. Since early-EPC represent alternative activated M2 macrophages, we have drawn a potential interpretation from known macrophage responses to catecholamines. The sympathetic nervous system has a dual role –i.e., either anti-inflammatory or pro-inflammatory- on local inflammatory responses mediated by the actions of norepinephrine and epinephrine on immune cell adrenoreceptors (12). In the case of macrophages, the effects of catecholamines on cell functions seem to be complex and subject to

410 inconsistencies. It has been proposed that the discordance between stimulatory and
411 inhibitory actions of catecholamines is dependent upon the activation state of
412 macrophage populations and the associated changes in the expression levels of
413 different adrenoreceptor types. In this context, to clarify the significance of our
414 unexpected observation that catecholamines stimulated the *in vitro* growth of early-
415 EPC, other studies are needed to assess the expression of adrenoreceptors under the
416 present experimental conditions. Finally, considering that early-EPC are supportive
417 cells that by releasing cytokines can modulate the vasculogenic activity of late EPC, it
418 is feasible that in the setting of autonomic dysregulation the stimulation of early-EPC
419 growth by epinephrine and norepinephrine might represent a counterbalancing
420 mechanism aimed at buffering the negative effects of both catecholamines on early-
421 and late-EPC and minimizing any deleterious consequences on vascular maintenance.
422 In recent years, the sympathetic nervous system was identified as a decisive
423 determinant in progenitor cell mobilization from the bone marrow (22). In relation to
424 this, EPC were shown to express functional β2-adrenergic receptors which upon
425 stimulation induce EPC migration, proliferation and differentiation, resulting in improved
426 neoangiogenesis in experimental hindlimb ischemia (14). Interestingly, in chronic
427 obstructive pulmonary disease patients β2-adrenergic receptor expression in early-
428 EPCs was higher than in healthy controls, and treatment of EPC with a β2-adrenergic
429 receptor antagonist (ICI 118551) increased EPC migration and proliferation when
430 compared to treatment with an agonist, norepinephrine (24). Concerning the link
431 between the parasympathetic nervous system and EPC, approximately a decade ago a
432 previously unknown cholinergic angiogenic pathway mediated by nicotinic acetylcholine
433 receptors (nAChRs) on endothelial cells, was described (19). Later, nAChR activation
434 by both local and systemic administration of nicotine was shown to increase the
435 mobilization and/or recruitment of mouse EPC to the site of angiogenesis in ischemic
436 tissues (18); also, nAChRs were identified on fibronectin-cultured human EPCs, and
437 nicotine -via nAChRs - was found to improve human EPCs functional activity (36). The

438 archetypal endogenous nAChR agonist is acetylcholine (ACh). The broad hydrolyzing
439 activity of acetylcholinesterase and butyrylcholinesterases in neuronal and non-
440 neuronal tissues, including vascular cells (23) reduces ACh actions to local paracrine
441 or autocrine effects. Of note, choline -that is released from acetylcholine by
442 acetylcholinesterase- can activate at least one of the nAChRs subtypes, i.e. the
443 homomeric α7 nAChRs (4) ; therefore, after ACh hydrolysis a chance for ACh signaling
444 still remains. Considering the above evidence, it is feasible that ACh (or other longer-
445 lasting nAChR agonists) released from nerve endings may be involved in vascular
446 growth or maintenance. Circumstantial evidence supports the latter concept; thus, as
447 pointed out by Cooke et al. (6), the reduced foot-healing capacity associated to
448 diabetes is preceded by a severe neuropathy, raising the question of whether in
449 diabetic patients the loss of nerve-produced trophic agents, including ACh, might
450 participate in the deterioration of angiogenesis. Finally, although parasympathetic
451 innervation to the bone marrow had not been previously reported, parasympathetic
452 activity was recently detected in the skeletal bone marrow, where ACh functioned to
453 decrease bone resorption (5) .
454 Our observations that peripheral blood mononuclear cells from controlled hypertensive
455 patients gave rise to a lower number of cultured early-EPC than those from
456 normotensive subjects, and that early-EPC numbers are positively related to flow
457 mediated dilation, are in line with previous findings by others (16).
458 Although the antihypertensive treatments were discontinued for 24 h previous to blood
459 sampling, some of the drugs used by the study population (RAAS blockers,
460 dihyropyridine Ca2+ antagonists) may have influenced autonomic balances. However,
461 it should be considered that the cardiac sympathetic response during the mental stress
462 test was negatively associated with late-TNT numbers, but both kinds of compounds
463 have been described to lower sympathetic activity. Therefore, if an autonomic effect did
464 occur, a confounding influence by RAAS blockers or dihyropyridine Ca2+ antagonists
465 would have weakened the above association, instead of strengthening it. Also,

466 although statins have been shown to increase the level of circulating EPC (32), no
467 differences were observed here between hypertensive patients receiving and not
468 receiving statins.
469 Injured tissues release a variety of mediators that attract EPC to the site and ensure
470 their engraftment (25, 28). In this setting, in coronary artery disease patients,
471 hypertension was identified as a major independent predictor of early-EPC blunted
472 migratory capacity (33). Also, compared with healthy individuals, in prehypertension
473 and hypertension subjects *in vivo* endothelial repair capacity by early-EPC is
474 considerable reduced, and this is related to accelerated early-EPC senescence and
475 impaired endothelial function. Of note, early-EPC telomere length is negatively related
476 to systolic blood pressure (16). In addition, both in the rat and in humans, hypertension
477 is associated with increased numbers of senescent circulating EPC (38). EPC
478 mobilization from the bone marrow is dependent on NO-mediated activation of MMP-9.
479 Recent experimental evidence supports the concept that in hypertension the
480 impairment of EPC mobilization results from insufficient bone marrow NO synthase
481 activity, and the consequent deficiency in NO/MMP-9 signaling (3). Interestingly, in
482 experimental renovascular hypertension improvements of *in vivo* repair capacity, *in*
483 *vitro* EPC proliferation, and tube formation and homing signals (VEGF and homing
484 receptor expression) are present in the early phase of the disease; however, this
485 putative early compensatory vascular response is lost with longer lasting hypertension
486 (39). In the present paper, we report additional factors that can interfere with adequate
487 EPC function in hypertension.
488 Our findings may help to explain why hypertensive patients that have achieved target
489 blood pressure levels through pharmacological treatment still display a high residual
490 cardiovascular risk. Of note, a recent review (30) emphasized the concept that
491 sympathetic nervous system activation is frequently responsible for the origin and
492 maintenance of elevated blood pressure in essential hypertension, and that at present
493 sympathetic antagonists are underused as treatments for hypertensive patients. If

494 further studies replicate these findings, the next step would be to test whether by
495 antagonizing the sympathetic nervous system it is possible to increase EPC numbers
496 in association with the improvement of endothelial function.
497
498 **Grants**
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503
504
505 **Conflict of Interest**
506 None declared
507

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FIGURE TITLES

FIGURE 1. Immunohistochemical characterization of cultured human early- and late-EPC

FIGURE 2. Characterization of tunneling nanotubes in cultured human early- and late-EPC

FIGURE 3. Relation between autonomic status and EPC or TNT numbers in treated hypertensive patients after natural log transformation of variables followed by linear regression analysis

FIGURE 4. Effects of epinephrine and norepinephrine on cultured EPC adhesion and growth.

FIGURE 5. Relation between flow-dependent, endothelium-mediated dilation and early-EPC numbers in treated hypertensive patients after natural log transformation of variables followed by linear regression analysis

TABLE TITLES

TABLE 1. Subjects' characteristics

TABLE 2. Quantification of cultured early-EPC, early-TNT, late-EPC and late-TNT isolated from peripheral blood in treated essential hypertensive patients and normotensive subjects

TABLE 3. Cardiac parasympathetic reserve and sympathetic response, and the relation between autonomic status and EPC or TNT numbers in treated essential hypertensive patients

FIGURE LEGENDS

Figure 1. Endothelial progenitor cells were obtained after culturing peripheral blood mononuclear cells on either human fibronectin or rat collagen for early- and late-EPC, respectively. The cells were characterized by immunocytochemistry on days 9 (early-EPC) and 21 (late-EPC) of culture. A. Early-EPC –but not late-EPC- expressed the haematopoietic markers CD14 and CD45, indicating that late-EPC are committed to the endothelial lineage. B. Merged fluorescence images show that early-EPC were positive for both CD14 and CD45 and all CD14+ cells were also positive for DiI-acetylated LDL, indicating that the cells were not a mixture of live and dead cells. Also, early-EPC were positive for both VEGFR-2 and CD45.

Figure 2. Tunneling nanotubes (TNT) were identified as thin, straight, actin-rich (Phalloidin staining) cytoplasmic projections, with a length equivalent to several cell diameters, and crossing from one cell to another. A. TNT in live early- and late-EPC; B. TNT in fixed late-EPC

Figure 3. In controlled-hypertensive patients, the cardiac parasympathetic reserve was evaluated by analyzing the high frequency (HF) component of HRV during the E/I maneuver, and the cardiac sympathetic response was assessed by analyzing the LF/HF ratio during the Stroop color-word test. In this study group, late-EPC number was positively associated with cardiac parasympathetic reserve during the E/I maneuver and inversely related to cardiac sympathetic response during the stress test.

Figure 4. A. To examine the effects of catecholamines on (A1) early-EPC and (A2) late-EPC adhesion to fibronectin or collagen, respectively, epinephrine or norepinephrine were added to the culture medium at the time of seeding onto culture dishes, and adherent EPC were counted 48 h later. Data are presented as median (minimum-maximal values) of 6 independent assays. Epi = epinephrine; Norepi = norepinephrine; * P = 0.0235 vs. PBS; † P = 0.0314 vs. 100 ng epinephrine/L; ‡ P = 0.0399 vs. PBS ; ǂ P = 0.030 vs. 100 ng norepinephrine/L; ⊼ P = 0.0399 vs. PBS **B.** To investigate the effects of catecholamines on (B1) early- and (B2) late-EPC growth, epinephrine or norepinephrine were added to the culture medium 48 h after seeding and cells were counted on days 9 or 21, respectively. * 0.0314 vs. PBS; † P = 0.0399 vs. 100 ng epinephrine/L; ⋔ 0.025 vs. PBS; ⊼ P = 0.0399 vs. PBS**.**

Figure 5. In controlled-hypertensive patients, *ex vivo* cultured early-EPC counts were positively correlated with flow-dependent, endothelium-mediated dilation (FMD).

TABLE LEGENDS

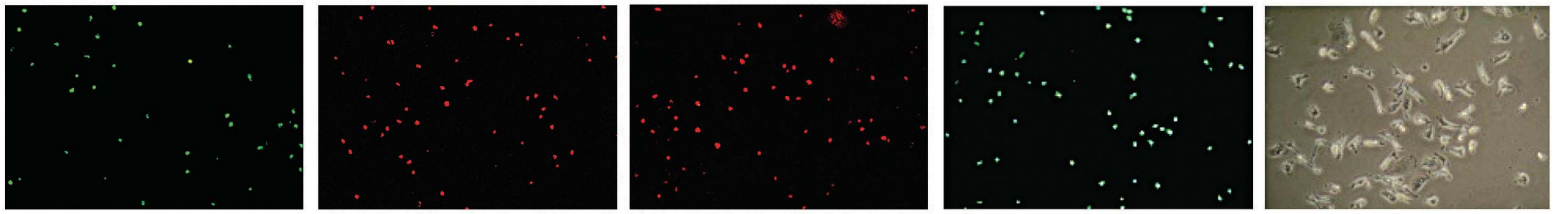
TABLE 1. Values are expressed as median (max - min value).

TABLE 2. A. Shows the numbers of cultured early- (day 9) and late-EPC (day 21) per microscopic field that were isolated from peripheral blood in controlled essential hypertensive patients (Hypertensive) and normotensive individuals (Normotensive) and seeded onto fibronectin (early-EPC) or collagen (late-EPC). **B.** Shows the percentage of cultured early-EPC (day 9) and late-EPC (day 21) that emitted TNT in cells isolated from treated essential hypertensive patients and normotensive subjects**.** Values are expressed as median (max - min value). early-TNT= number of early-EPC that project tunneling nanotubes; late-TNT= number of late-EPC that project tunneling nanotubes. Values are expressed as median (max - min value). early-TNT= number of early-EPC that project tunneling nanotubes; late-TNT= number of late-EPC that project tunneling nanotubes.

TABLE 3. In hypertensive patients (N=30), autonomic status was assessed by frequency domain analysis of short-term heart rate variability (HRV). **A.** Low-(LF) and high-frequency (HF) components of short-term heart rate variability (HRV) were analyzed during a 5-min rest, an expiration/inspiration maneuver, and a Stroop color-word-test. Values are expressed as median (max - min value). **B.** In hypertensive patients, late-EPC number was positively related to the cardiac parasympathetic reserve during the E/I maneuver, and late-TNT numbers were negatively associated with sympathetic response during the mental stress test. *Increase in HF component of heart rate variability during the E/I maneuver, †Low/high frequency ratio (LF/HF) during the mental stress test.

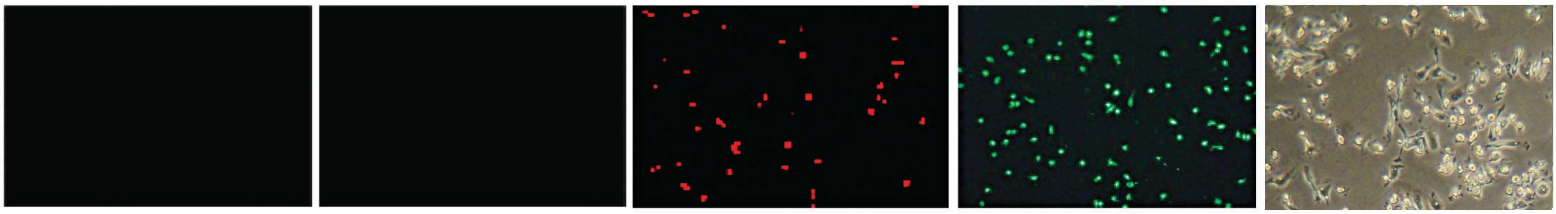
A

Early-EPC (Immunofluorescence, day 9)



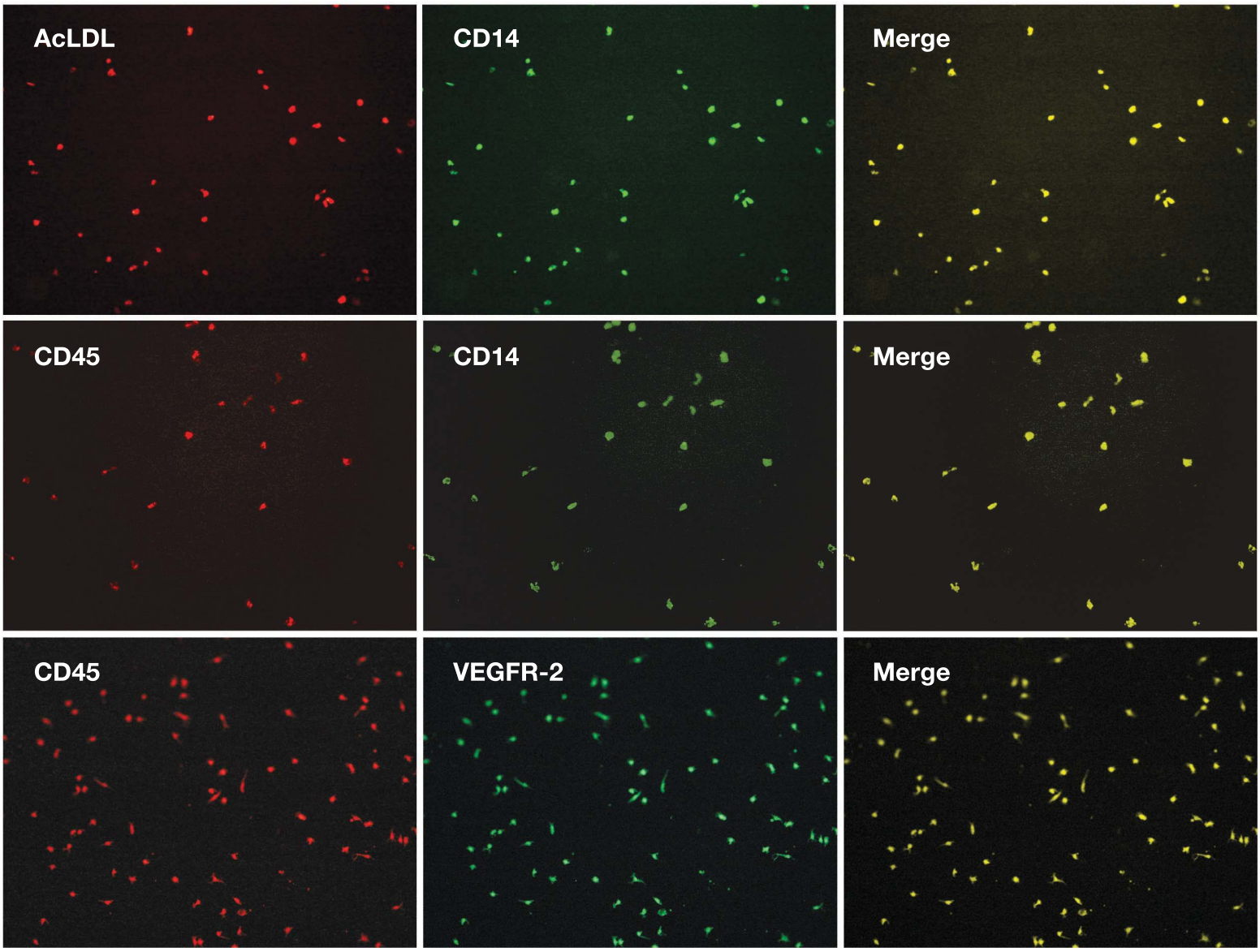
CD14+ | *CD45+* | *Acetylated-LDL+* | *VEGFR+* | *Optical Microscope 400x*

Late-EPC (Immunofluorescence, day 21)

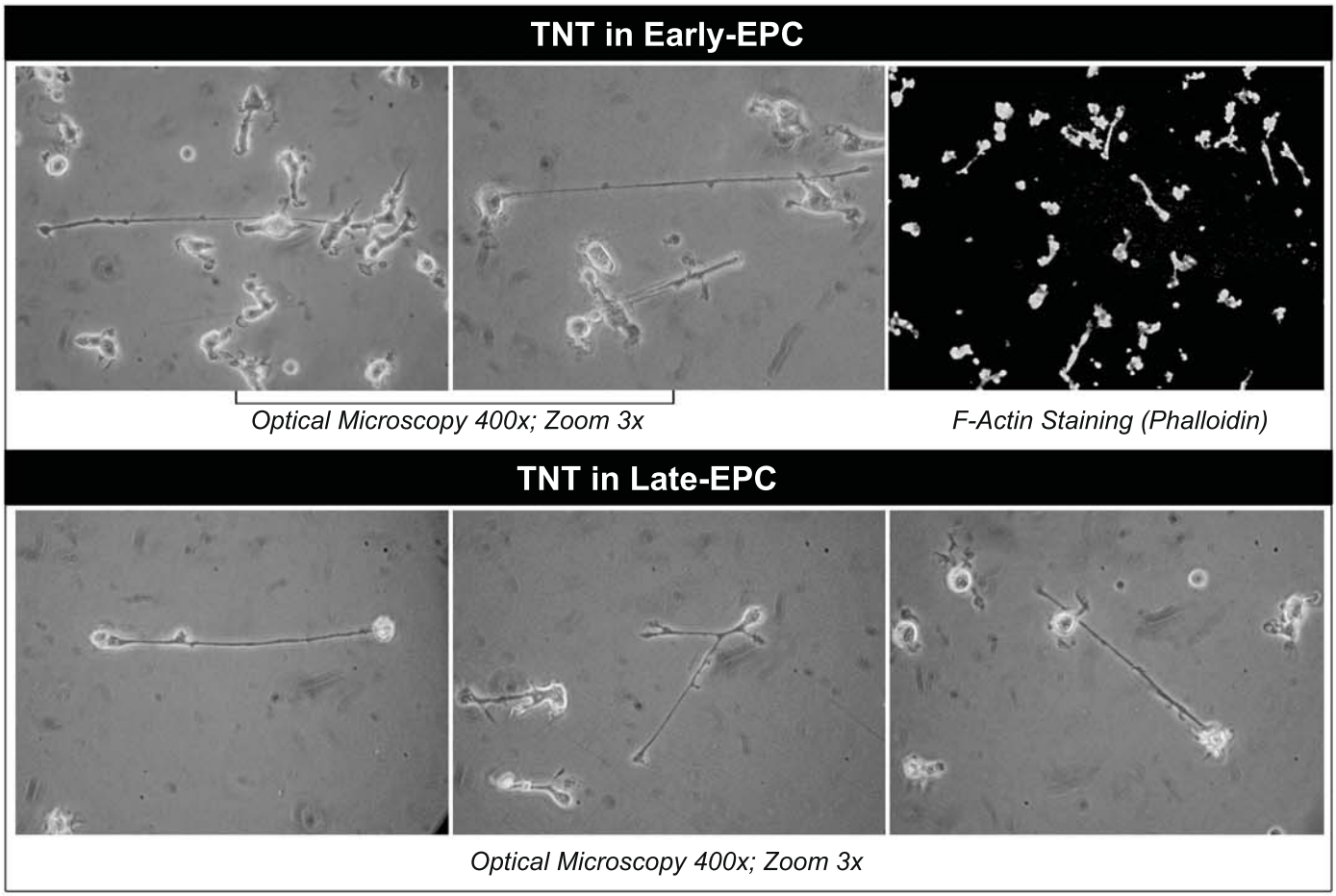


CD14 - | *CD45 -* | *Acetylated LDL+* | *VEGFR+* | *Optical Microscope 400x*

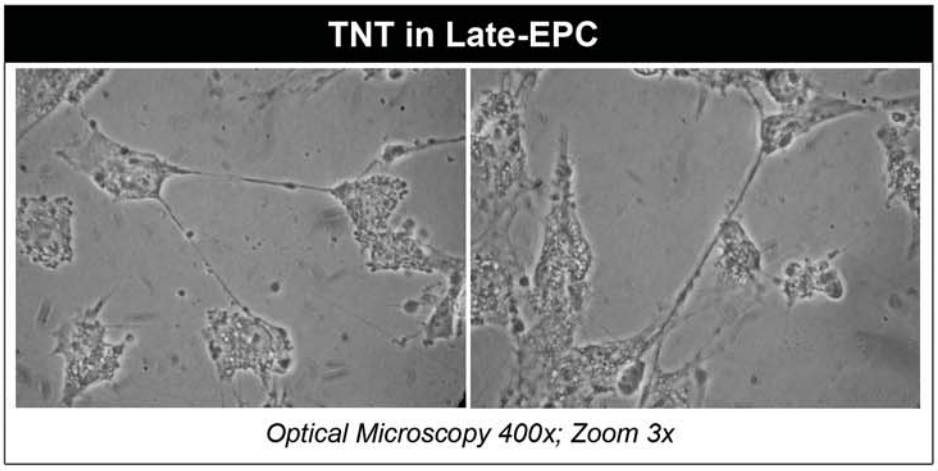
B

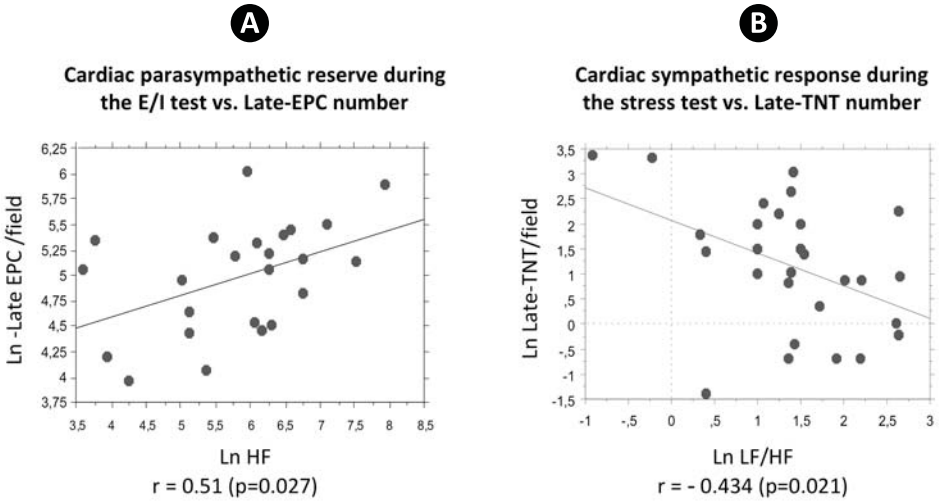


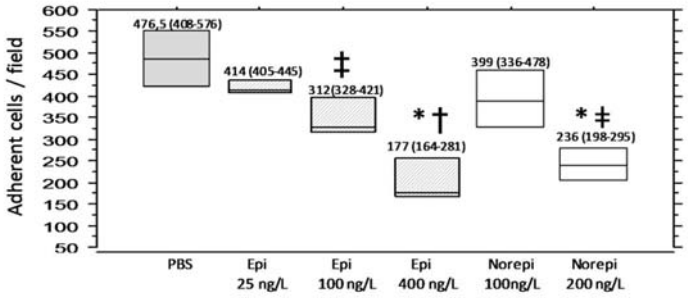
A


TNT in Early-EPC
Optical Microscopy 400x; Zoom 3x
F-Actin Staining (Phalloidin)
TNT in Late-EPC
Optical Microscopy 400x; Zoom 3x

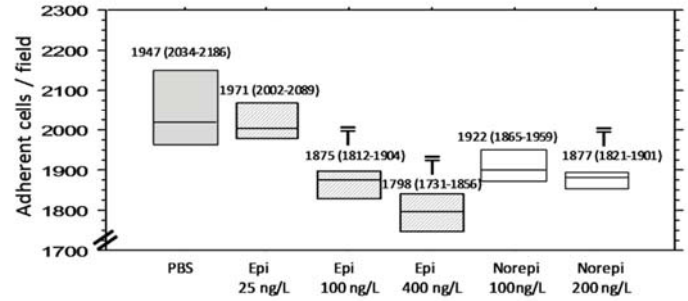
B


TNT in Late-EPC
Optical Microscopy 400x; Zoom 3x

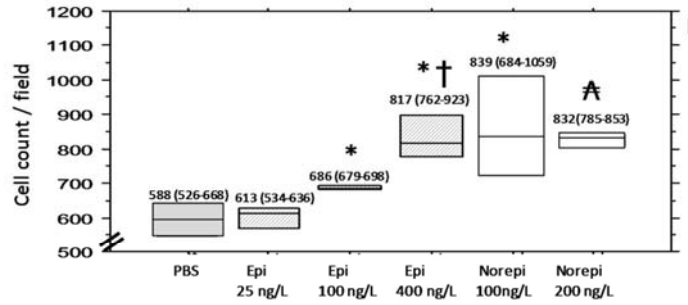

A
Cardiac parasympathetic reserve during the E/I test vs. Late-EPC number
Ln -Late EPC /field
Ln HF
r = 0.51 (p=0.027)
B
Cardiac sympathetic response during the stress test vs. Late-TNT number
Ln Late-TNT/field
Ln LF/HF
r = - 0.434 (p=0.021)



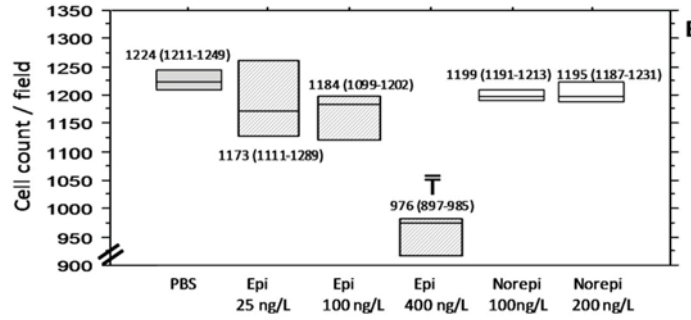
A1. Early-EPC adhesion to fibronectin



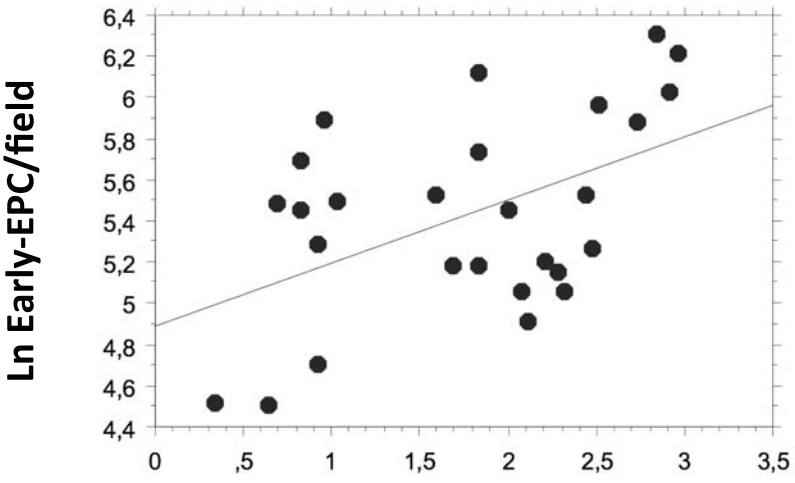
A2. Late-EPC adhesion to collagen



B1. Early-EPC proliferation on fibronectin



B2. Late-EPC proliferation on collagen


Ln Early-EPC/field
6,4
6,2
6
5,8
5,6
5,4
5,2
5
4,8
4,6
4,4
0
,5
1
1,5
2
2,5
3
3,5
Ln FMD

r = 0.679 (p=0.031)

TABLE 1. Subjects' characteristics

Characteristic	Hypertensive (N=30)	Normotensive (N=20)	P value
Age, y	49 (24-69)	50 (27-66)	0.780
Males, %	81.8	80.0	-------
SBP, mm Hg	130 (120-137)	114 (107-119)	0.008
DBP, mmHg	85 (61-88)	75 (64-79)	0.039
Medications, %		None	-------
ACEI/ARAII	63.5		
Ca2+ Antagonist	33		
Diuretics	13		
Statins	17		
Other	18		
BMI, kg/m^2	28.0 (19.1-39.0)	25.0 (18.1-27.8)	0.1189
Total cholesterol, mmol/L	4.96 (3.93-6.44)	3.99 (3.41-5.17)	0.0518
HDL cholesterol, mmol/L	1.34 (1.06-2.33)	1.86 (1.19-2.64)	0.1564
LDL cholesterol, mmol/L	3.06(1.81-4.19)	2.60 (2.12-3.15)	0.2120
TG, mmol/L	1.05 (0.54-2.10)	0.85 (0.74-1.24)	0.0272
Glycemia, mmol/L	5.05 (4.50-6.22)	4.91 (4.78-5.22)	0.4715

Values are expressed as median (max - min value).

TABLE 2.

A.

Type of EPC or TNT (No./microscopic field)	**Hypertensive** (N=30)	**Normotensive** (N=20)	**P Value**
early-EPC	223.5 (87.8 - 551)	392 (248 - 681)	0.0015
early-TNT	13.2 (0.83 - 54.5)	59.7 (24.8 - 99.8)	0.0002
late-EPC	157.6 (52.4 - 409)	324 (215 - 634)	0.005
late-TNT	2.60 (0 - 29.3)	24.2 (19.2 - 44.6)	0.008

B.

	Hypertensive (N =30)	**Normotensive** (N=20)	**P Value**
(early-TNT/early-EPC) x 100, %	5.30 (0 – 17.6)	12.3 (5.89-28.2)	0.012
(late-TNT/late-EPC) x 100, %	2.30 (0 – 13.1)	7.51 (3.0 – 15.6)	0.017

TABLE 3.

A.

Settings at the time of measurement	Parameter	
	HF, ms^2	LF/HF, %
During a 5-min rest	177 (22 - 934)	2.15 (0.40 - 10.5)
During an E/I maneuver	431 (37 – 2791)	10.3 (1.30 – 50)
During a mental stress test	120 (6 – 570)	4.10 (0.40 – 14.1)

B.

Parameters related	**Correlation** (Spearman rho)	**P Value**
Late-EPC number and cardiac parasympathetic reserve*	0.450	0.031
Late-TNT and cardiac sympathetic response†	-0.426	0.045