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Circulating Endothelial Progenitor Cells and Endothelial Microparticles in Patients With Arterial Erectile Dysfunction and Metabolic Syndrome

SANDRO LA VIGNERA, ROSITA CONDORELLI, ENZO VICARI, ROSARIO D'AGATA, AND ALDO E. CALOGERO

From the Section of Endocrinology, Andrology, and Internal Medicine, and Master in Andrological, Human Reproduction, and Biotechnology Sciences, Department of Internal Medicine and Systemic Diseases, University of Catania, Catania, Italy.

ABSTRACT: Blood endothelial progenitor cells (EPC) and microparticles (EMP) have been proposed as markers of endothelial dysfunction. The aim of this study was to evaluate both EPCs and EMPs in patients with arterial erectile dysfunction (ED) and metabolic syndrome (MetS). To accomplish this, 100 patients (ages 45–60 years) with ED and MetS (Adult Treatment Panel III [ATP III] 1999 criteria) and 17 healthy men (ages 44–57 years) were selected. EPC ($CD45_{neg}/CD34_{pos}/CD144_{pos}$) and EMP ($CD45_{neg}/CD144_{pos}$/Annexin V_{pos}) blood concentrations were evaluated by flow cytometry, before and after administration of tadalafil (20 mg) on demand for 3 months. Before treatment, EPCs and EMPs were significantly higher in patients compared with healthy men. EPCs increased significantly after tadalafil administration, whereas EMPs did not differ significantly. EPCs correlated positively or negatively with body mass index and with some cavernous artery indices, both before and after tadalafil administration. EMPs showed only positive correlations with body mass index and some cavernous artery indices, both before and after tadalafil administration. Patients with arterial ED and MetS have higher EPCs and EMPs compared with healthy men; hence, these cells may be regarded as markers of cavernous artery dysfunction. Tadalafil administration increased EPCs but not EMPs, suggesting that this compound may play a role in the endothelial repair response.

Key words: Endothelial dysfunction, phosphodiesterase V inhibitors, international index of erectile function.

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Patients with metabolic syndrome (MetS) are more prone to developing cardiovascular diseases (CVD), such as coronary artery disease (CAD), cerebrovascular disease, and peripheral artery disease (PAD; Fadini et al, 2006). All of these conditions share endothelial dysfunction, which has a well-established role in the pathogenesis of both atherosclerosis and plaque instability (Widlansky et al, 2003; Sugiyama et al, 2004). Recently, a large body of literature has shown that arterial erectile dysfunction (ED), a manifestation of PAD, has a similar pathophysiologic mechanism (Matfin et al, 2005). Hence, ED is more common in patients with overt or silent CAD, and it is increasingly being regarded as an early clinical manifestation of a generalized CVD (Feldman et al, 2000; Russel et al, 2004; Baumhakel et al, 2006; Gazzaruso et al, 2008). Therefore, MetS components may also favor the onset of ED (Gündüz et al, 2004; Demir et al, 2006).

Endothelial dysfunction may be evaluated in many ways, and recently this has been done by estimating the number of circulating endothelial precursor cells (EPC) and endothelial microparticles (EMP). EPCs are subpopulations of leukocytes that may differentiate into mature endothelial cells both in vitro and in vivo (Real et al, 2008). The relevant contribution of these cells in the processes of re-endothelialization at sites of endothelial injury and neovascularization has also been confirmed (Asahara et al, 1997; Werner et al, 2003; Kong et al, 2004; Urbich and Dimmeler, 2004). The capability of circulating EPCs to repair the capability of the damaged endothelium suggests that these cells play a key role in maintaining endothelial homeostasis. As a result, the number of EPCs may reflect the "vascular health" of an individual, and it is has been shown to be an independent predictor of CVD (Güven et al, 2006).

The first EPC phenotype was defined by the presence of the following antigens in blood cells: CD34, CD133, and VEGFR2 (or KDR; Asahara et al, 1997). Many articles have reported statistically significant correlations between the number of cells in this EPC subset in

Correspondence to: Dr Sandro La Vignera, Section of Endocrinology, Andrology, and Internal Medicine, Department of Internal Medicine and Systemic Diseases, Catania University, Policlinico "G. Rodolico", S Sofia 78th St, Bldg 4, Rm 2C82, 95123 Catania, Italy (e-mail: sandrolavignera@email.it).

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the blood with the presence of adverse cardiovascular outcomes. However, few studies have attempted to formally compare the functional properties of this EPC subset in hematopoietic and endothelial clonogenic assays to gain more insight into the biology of the phenotype used, namely, to assess the identity of their clonal progeny that participate in vessel repair (Case et al, 2007; Hirschi et al, 2008). No clear evidence has been reported about the existence of specific markers that allow researchers to prospectively isolate EPCs with a phenotype superior to the one thus far described (Hirschi et al, 2008).

Recently, some studies reported that purified blood $CD34_{pos}/CD133_{pos}/KDR_{pos}$ blood EPCs are highly enriched in hematopoietic progenitor cell activity and express the universal hematopoietic cell–surface antigen CD45. Moreover, these cells are unable to give rise to endothelial colony-forming cells in vitro. Indeed, CD45 is not expressed in endothelial cells, even at the mRNA level. Endothelial colony-forming cells were enriched by 368-fold only when $CD45_{neg}/CD34_{pos}$ cells were plated and not when $CD45_{pos}/CD34_{pos}$ cells were used (Case et al, 2007; Timmermans et al, 2007). Therefore, the latter phenotype is a hematopoietic progenitor cell–enriched cell subpopulation, and thus EPCs are restricted to the phenotype $CD45_{neg}/CD34_{pos}$ (Case et al, 2007). A closer look at the biology of these EPCs indicates a progressive loss of CD133 and CD34 antigens and the expression of CD31, VE-cadherin (CD144), and Vwf, following their mobilization into the general circulation (Papayannopoulou, 2004; Schatteman et al, 2007). In fact, CD34 is an adhesion molecule, expressed in hematopoietic stem cells, vascular progenitors, and certain microvascular endothelia (Fina et al, 1990). CD144 is a single-chain, transmembrane protein whose amino acid sequence indicates its homology with cadherins. It is specifically expressed in endothelial cells, where it organizes the adherent junctions, which control permeability and migration and are partially responsible for inhibiting growth by contact (Ryu et al, 2009). The concomitant presence of CD34 and CD144 antigens and the loss of the panleukocyte CD45 antigen ensure that these cells are true stem/progenitor cells of an endothelial nature (Güven et al, 2006; Case et al, 2007; Schatteman et al, 2007; Timmermans et al, 2007). Furthermore, CD144 or VEGFR-2 mRNAs have not been detected in $CD45_{pos}/CD34_{pos}$, but only in all sorted bone marrow $CD34_{pos}/CD45_{neg}$ cells (Timmermans et al, 2007). Thus, the $CD45_{neg}/CD34_{pos}/CD144_{pos}$ phenotype defines a more endothelial committed cell (Case et al, 2007). These EPCs have been shown to be able to form discrete colonies of endothelial cells in culture (Güven et al, 2006). On this account, we chose to evaluate this EPC phenotype.

In addition to EPCs, microparticles of endothelial origin (EMPs) may be found in the general circulation. EMPs are membrane fragments that increase in several pathologic conditions, including atherosclerosis, sepsis, diabetes mellitus, and during the process of apoptosis (Shet, 2008). The main characteristics of these elements are the small dimension (<1.5 μm) and the externalization of phosphatidylserine revealed by annexin V binding (Morel et al, 2008). EMPs are regarded as markers of cardiovascular dysfunction with a prothrombotic role associated with risk factors, such as those that characterize the MetS (Combes et al, 1999; Freyssinet et al, 1999; Diamant et al, 2002; Freyssinet, 2003; Boulanger et al, 2006). The percentage of EMPs is higher in patients with ED and inversely correlated with the International Index of Erectile Dysfunction (IIEF-5) score (Esposito et al, 2007). The presence of diabetes mellitus seems to be associated with a greater elevation of EMPs (Esposito et al, 2008). Many studies have evaluated the correlation between EPCs or EMPs with the severity of CVD. Only a few have assessed both biomarkers simultaneously and correlated them with the arterial condition.

On this basis, the present study was undertaken to evaluate the number of both EPCs and EMPs in patients with MetS and ED of arterial origin. This clinical model seemed to us suitable to gain more insight into the balance between bone marrow endothelial repair mechanism and endothelial dysfunction in the manifestation of the clinical symptoms. To accomplish this, 100 patients with arterial dysfunction–based ED and MetS, established by the Adult Treatment Panel (ATP) III criteria (National Cholesterol Education Program ATP III, 2002), were selected, and the number of circulating EPCs ($CD45_{neg}/CD34_{pos}/CD144_{pos}$) and EMPs ($CD45_{neg}/CD144_{pos}$/Annexin V_{pos}) was determined. Their blood number was reevaluated after treatment with the phosphodiesterase 5 inhibitor (PDE5i) tadalafil, a known stimulator of EPC release (Foresta et al, 2006). A group of men without ED and MetS were enrolled as controls.

Materials and Methods

Patient Selection

One hundred men with arterial ED and MetS were enrolled (age, 54.3 ± 2.5 years; range, 45–60 years). The diagnosis of arterial ED was made when all of the following criteria were fulfilled: 1) IIEF-5 score <21 (Rosen et al, 1999); 2) cavernosal artery peak systolic velocity (PSV) <30 cm/s 10 and 20 minutes after the intracavernosal injection of alprostadil (20 μg) by echo color Doppler (Benson et al, 1993); and 3) acceleration time >110 ms (Speel et al, 2003). The diagnosis of MetS was

made according to the ATP III criteria (National Cholesterol Education Program ATP III, 2002). Patients were excluded if they: 1) had severe hypogonadism (serum total testosterone levels <8 nmol/L (<231 ng/dL; Wang et al, 2009); 2) smoked cigarettes, or 3) had veno-occlusive dysfunction (end diastolic velocity >5 cm/s). They underwent blood withdrawal (10 mL) for EPC and EMP measurements by flow cytometry. They were prescribed tadalafil (Cialis, Lilly ICOS, Florence, Italy) 20 mg on demand for 3 months (60 mg per week, a total of 720 mg for 3 months; taken in the morning between 8:00 and 10:00 AM), and afterwards each patient underwent a second IIEF-5 questionnaire administration, dynamic penile echo color Doppler, and blood withdrawal for routine analysis and EPC and EMP determination. Patients who took less than 700 mg in 3 months and/or reported not having observed the scheme of assumption were excluded.

A group (n = 30) of age-matched (52.7 ± 1.1 years; range, 45–60 years) men without ED and MetS were selected as controls to define the concentrations of EPCs and EMPs in healthy men.

The protocol was approved by the internal institutional review board, and an informed written consent was obtained from each patient.

Assay of EPCs and EMPs

EPC and EMP evaluation was performed in blood following incubation in erythrocyte lysing solution (Versalyse, Instrumentation Laboratory, Milan, Italy) for 1 minute, within 1 to 2 hours after venipuncture. The suspension was then washed twice with phosphate buffer solution and centrifuged, and the pellet was rapidly incubated in phosphate buffer solution containing the appropriate monoclonal antibodies at room temperature for 20 minutes.

EPC Detection

Phycoerythrin covalently bound to Texas red (ECD)–conjugated anti-human CD45 (Instrumentation Laboratory, fluorescein isothiocyanate–conjugated anti-human CD34 (Instrumentation Laboratory), and r-phycoerythrin–conjugated anti-human CD144 (Instrumentation Laboratory) were used for EPC flow cytometry detection.

Each sample was analyzed by flow cytometry (EPICS XL; Coulter Electronics Instrumentation Laboratory, Milan, Italy) using the following gating strategy (Figure 1):

- histogram 1 reports the forward vs side scatter dot plot: 3 different cell populations were identified: gate F, lymphocytes; gate I, monocytes; and gate J, polymorphonuclear cells;
- histogram 2 reports $CD45_{pos}$ (gate R1) and $CD45_{neg}$ (gate R2) cells; and
- histograms 3 (control) and 4 (patient with ED and MetS) report the $CD45_{neg}$ cells with the dual expression of CD34 and CD144, which were defined EPCs.

EPCs were reported as a percentage of total events.

EMP Detection

ECD anti-human CD45 (Instrumentation Laboratory), r-phycoerythrin–conjugated anti-human CD144 (Instrumentation Laboratory), and fluorescein isothiocyanate–conjugated annexin V (Instrumentation Laboratory) were used for EMP flow detection by flow cytometry. Particles with <1.5 mm size were gated on forward vs side scatter histograms. To exclude microparticles originating from leukocytes, we considered only events within the $CD45_{neg}$ gate. $CD144_{pos}$ events expressing phosphatidylserine in the outer membrane leaflet following annexin V staining were defined EMPs. They were reported as a percentage of total events.

Appropriate isotype controls were used for each staining procedure as negative controls to set the appropriate regions. Flow cytometric analysis was conducted for 600 seconds or 100 000 events, whichever occurred first. The same operator, blinded with respect to the sample origin (control or patient), performed all of the tests throughout the study.

Statistical Analysis

Results are shown as median and percentiles. Statistical analysis was conducted by 1-way analysis of variance (ANOVA) followed by Duncan's multiple range test following logarithm transformation of EPC and EMP data. Correlation analysis was carried out by Pearson's test. Statistical analysis was conducted using SPSS 10.0 for Windows (SPSS Inc, Chicago, Illinois). A P value lower than .05 was accepted as statistically significant.

Results

The 100 ED patients with MetS enrolled in this study had an average IIEF-5 score of 13.7 ± 0.7 (range, 6–19). The frequency of MetS signs is reported in Table 1. A total of 10 patients (33%) had 3 signs of MetS, 15 (50%) had 4 signs, and 5 (17%) had all 5 signs.

At baseline, patients had a significantly higher number of EPCs compared with healthy controls ($P <$ .05, ANOVA followed by Duncan's test; Figure 2). EPCs correlated positively with body mass index, PSV, acceleration time, and cavernous artery intima-media thickness (IMT) (Table 2). After tadalafil administration, EPCs increased significantly compared with baseline ($P <$ .05, 1-way ANOVA followed by Duncan's test; Figure 2). Correlation analysis showed that EPCs correlated positively with body mass index, end-diastolic velocity, acceleration time, and IMT, and negatively with IIEF-5 score, PSV, and resistance (Table 2).

At baseline, patients with ED and MetS had a significantly higher ($P <$ .05, ANOVA followed by Duncan's test) EMP concentration compared to controls (Figure 3). EMPs correlated positively with BMI, PSV, acceleration time and IMT (Table 2). After tadalafil administration, circulating EMPs were slightly higher, but the difference did not reach the statistical

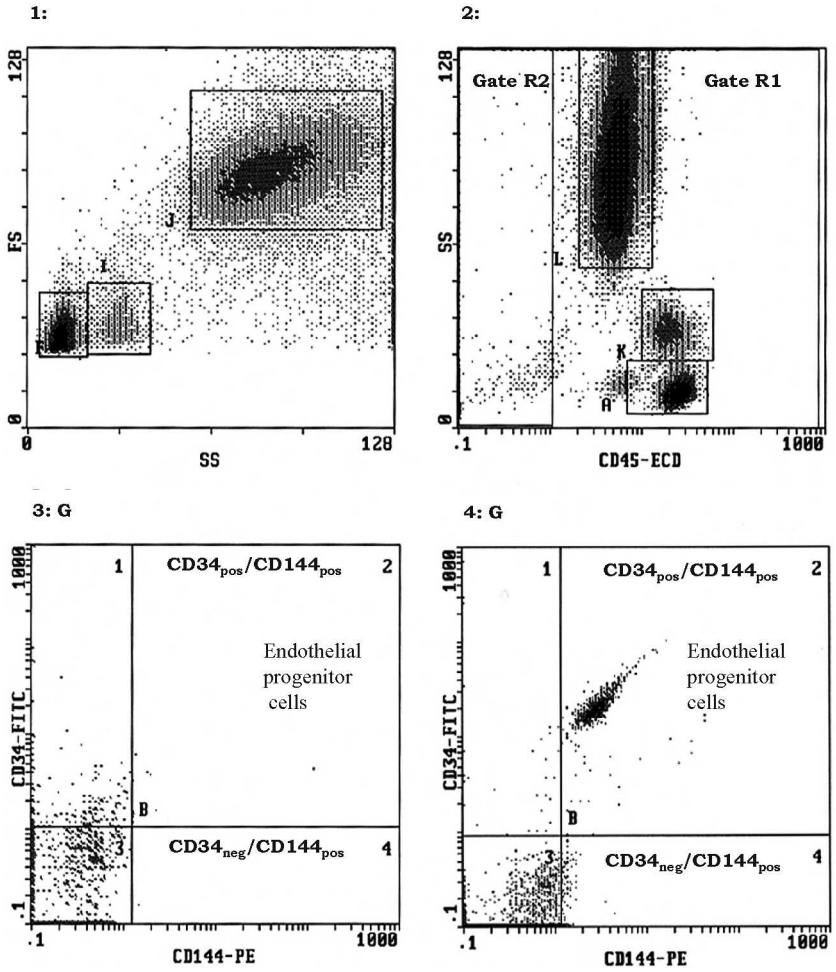

Figure 1. Representative flow cytometric scattergrams showing the gating strategy used in this study. Histogram 1 reports the forward vs side scatter dot plot: three different cell populations can be identified (gate F, lymphocytes; gate I, monocytes; gate J, polymorphonuclear cells). Histogram 2 reports the $CD45_{pos}$ (R1) and $CD45_{neg}$ (R2) cells. Histograms 3 and 4 show cells expressing both CD34 and CD144 antigens. A denser cluster of endothelial progenitor cells ($CD45_{neg}/CD34_{pos}/CD144_{pos}$) can be observed in a patient with erectile dysfunction and metabolic syndrome (histogram 4) compared with a healthy control man (histogram 3). The dots represent the flow cytometric events.

significance (Figure 3). EMPs correlated positively with BMI, acceleration time and IMT (Table 2).

A direct significant correlation between EPCs and EMPs was found at baseline ($r = 0.46$; $P < .05$), and after tadalafil administration ($r = 0.39$; $P < .05$).

Table 1. *Number and percentage of patients with arterial erectile dysfunction and signs of metabolic syndrome*

	No. of Patients (%)
Waist circumference >102 cm	100 (100)
Cholesterol HDL <40 mg/dL	73 (73)
Fasting glucose levels ≥110 mg/dL	68 (68)
Triglycerides ≥150 mg/dL	67 (67)
Hypertension ≥130/85 mm Hg	63 (63)

Abbreviation: HDL, high-density lipoprotein.

Discussion

This study evaluated the number of circulating EPCs and EMPs in patients with arterial dysfunction–based ED and MetS because few data are available in these patients, whereas studies have been conducted in ED patients with diabetes mellitus (Baumhakel et al, 2006) or obesity (Esposito et al, 2009). In addition, these studies have assessed only one of these markers of endothelial dysfunction, whereas enough evidence has now accumulated to suggest that the simultaneous measurement of both EPCs and EMPs would be more useful to evaluate their role as biomarkers of CVD and atherosclerotic complication and progression. In addition, the measurement of EPCs and EMPs in response to PDE5i administration has been poorly investigated.

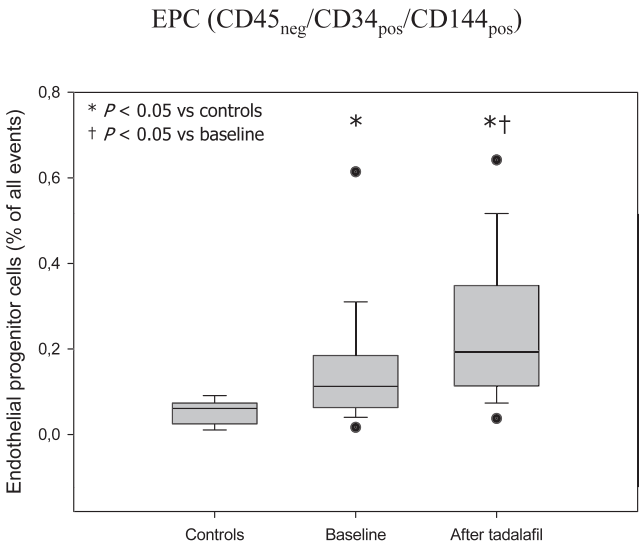

Figure 2. Endothelial progenitor cells ($CD45_{neg}/CD34_{pos}/CD144_{pos}$) in healthy men (controls) (n = 17) and in 100 patients with erectile dysfunction of arterial origin and metabolic syndrome before (baseline) and after tadalafil administration. The dots represent minimum and maximum.

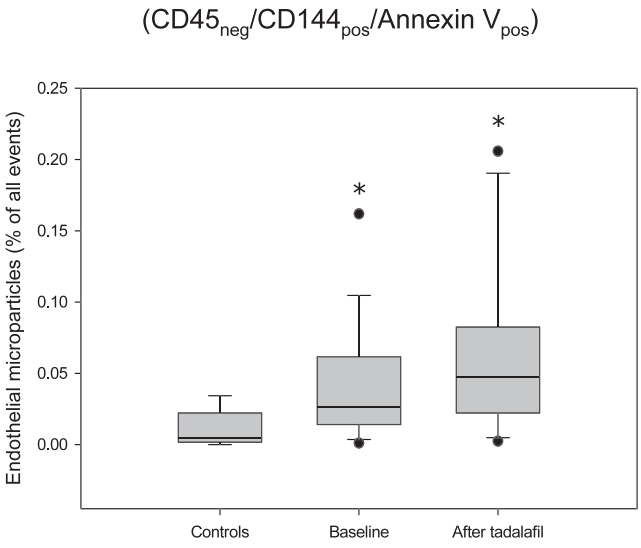

Figure 3. Endothelial microparticles ($CD45_{neg}/CD144_{pos}$/Annexin V_{pos}) in healthy men (controls) (n = 17) and in 100 patients with erectile dysfunction of arterial origin and metabolic syndrome before (baseline) and after tadalafil administration. The dots represent minimum and maximum. *$P < 0.05$ vs controls.

We found that patients with arterial ED and MetS had higher circulating EPCs and EMPs compared with healthy men, adding further evidence that these parameters are markers of endothelial dysfunction markers. This higher endothelial repair commitment suggests a severe vascular abnormality in these patients. EMPs are released as a consequence of endothelial dysfunction, atherogenesis, and endothelial apoptotic processes. The significant positive correlation between EPCs and EMPs at baseline suggests a more severe vascular damage in these men. In response to tadalafil, the endothelial repair response appears to be compensatory, because a significant increase in EPCs was found. The increase of these more endothelial regenerating EPCs may be able to rebuild the endothelial monolayer of the corpus cavernosum with amelioration of erectile function and

Table 2. *Correlation between circulating endothelial precursor cells ($CD45_{neg}/CD34_{pos}/CD144_{pos}$) or endothelial microparticles ($CD45_{neg}/CD144_{pos}$/Annexin V_{pos}) and age, body mass index (BMI), parameters of dynamic penile echo color Doppler or cavernosal artery intima-media thickness (IMT) in 100 patients with arterial erectile dysfunction and metabolic syndrome before (baseline) and after tadalafil administration*

Parameter	Endothelial Progenitor Cells	Endothelial Microparticles
At baseline		
Age, y	$r = 0.26$, P = NS	$r = 0.11$, P = NS
BMI, kg/m^2	$r = 0.65$, $P < .001$	$r = 0.41$, $P < .05$
IIEF-5 score	$r = -0.10$, P = NS	$r = -0.06$, P = NS
Peak systolic velocity, cm/s	$r = 0.43$, $P < .05$	$r = 0.39$, $P < .05$
End diastolic velocity, cm/s	$r = -0.03$, P = NS	$r = -0.10$, P = NS
Acceleration time, ms	$r = 0.67$, $P < .001$	$r = 0.64$, $P < .001$
Resistance index	$r = -0.03$, P = NS	$r = 0.10$, P = NS
IMT, mm	$r = 0.77$, $P < .001$	$r = 0.53$, $P < .05$
After tadalafil administration		
Age, y	$r = 0.35$, $P < .06$	$r = 0.20$, P = NS
BMI, kg/m^2	$r = 0.81$, $P < .001$	$r = 0.49$, $P < .01$
IIEF-5 score	$r = -0.40$, $P < .05$	$r = -0.18$, P = NS
Peak systolic velocity, cm/s	$r = -0.47$, $P < .01$	$r = -0.21$, P = NS
End diastolic velocity, cm/s	$r = 0.38$, $P < .05$	$r = 0.25$, P = NS
Acceleration time, ms	$r = 0.74$, $P < .001$	$r = 0.58$, $P = 0.005$
Resistance index	$r = -0.41$, $P < .05$	$r = -0.27$, P = NS
IMT, mm	$r = 0.71$, $P < .001$	$r = 0.50$, $P < .01$

Abbreviations: IIEF-5, International Index of Erectile Dysfunction; NS, not significant.

protection from the disease progression in these patients. Although at present this hypothesis remains speculative, the increased circulating EPC levels following tadalafil administration suggest that this treatment may activate EPCs. Future studies need to evaluate the response of these cells after tadalafil, comparing the findings of patients with severe vascular injury to patients without apparent vascular damage.

In patients with ED, the number of EPCs with the phenotype $CD34_{pos}/CD133_{pos}/KDR_{pos}$ is reduced, and this reduction seems to be greater in the presence of cardiovascular risk factors (cigarette smoke, hypertension, diabetes mellitus, obesity, and dyslipidemia; Foresta et al, 2005; Baumhakel et al, 2006; Esposito et al, 2009). In addition, the reduced number of $CD34_{pos}/CD133_{pos}/KDR_{pos}$ EPCs is an independent risk factor for ED in patients with known CAD. These findings suggest that EPCs may represent a link between cardiovascular risk factors, endothelial dysfunction, and ED (Baumhakel et al, 2006). The administration of PDE5i or androgens has been shown to raise the number of this type of EPC (Papayannopoulou, 2004; Foresta et al, 2005, 2006, 2007, 2008; Baumhakel et al, 2006). PDE5i seems to increase blood EPCs by upregulating MMP-9 through cyclic guanosine monophosphate accumulation (Marcet-Palacios et al, 2003). The apparent discrepancy between these studies and the present one may relate to the different immunophenotypes evaluated. Indeed, more recently, an increased number of a particular phenotype of EPCs, those positive to osteocalcin, a marker of circulating osteogenic cells, has been reported in patients with ED (Foresta et al, 2009).

It is now clear that there are distinct EPC subpopulations with different immunophenotype and biologic properties (Güven et al, 2006). Cells progress from the stem cell stage to the final mature cell of each single lineage, through successive progenitor cell stages (Ingram et al, 2005). Because uniform criteria to identify these cells are lacking, we followed the proposal outlined by Case and colleagues (2007). They suggested using the $CD45_{neg}/CD34_{pos}$ phenotype because these cells form colonies of mature endothelial cells in a clonogenic in vitro assay of endothelial cells (Case et al, 2007). Therefore, these cells probably behave to a greater extent as true endothelial progenitors (Timmermans et al, 2007). Accordingly, $CD45_{neg}/CD34_{pos}/CD144_{pos}$ cells form endothelial cells in culture (Güven et al, 2006).

To our knowledge, this study is among the few available thus far that have evaluated a more endothelial-committed EPC phenotype in patients with PAD. The higher number of EPCs we found in patients with MetS—hence, at increased CVD risks and corpus cavernosum atherosclerosis—is similar to previous evidence showing that specific CVDs are associated with increased circulating EPCs levels (Adams et al, 2004; Massa et al, 2005; Sandri et al, 2005; Güven et al, 2006). In CAD patients, several mechanistic possibilities have been advanced to explain the increase of EPCs. This activation may result from a variety of proinflammatory cytokines released in patients with coronary ischemia (Cho et al, 2003; Kong et al, 2004). Moreover, exercise-induced ischemia is associated with an increased number of circulating EPCs (Adams et al, 2004; Sandri et al, 2005). Patients with unstable angina have an increased number of EPCs compared with patients with stable angina, and the stabilization of angina in these patients resulted in a 2-fold decrease in circulating EPCs (George et al, 2004).

EMPs, defined by the phenotype $CD31_{pos}/CD42_{neg}$ and $CD31_{pos}/CD42_{pos}$, are elevated in patients with ED and diabetes mellitus and independently involved in the pathogenesis of ED. EMPs inversely correlate with the IIEF score in both diabetic and nondiabetic patients, and multivariate analysis corrected for confounding factors showed that EMPs are the only independent predictor of the IIEF score (Esposito et al, 2007). In addition, ED patients with or without diabetes mellitus have significantly higher EMPs ($CD62_{pos}$) than nondiabetic men without ED. The EMP ($CD62_{pos}$)/EMP ($CD31_{pos}$) ratio, an index of endothelial activation (high ratio) or apoptosis (low ratio), was lowest in ED diabetic patients. These findings suggest that the EMP increase in ED diabetic patients is consistent with increased apoptotic activity (Esposito et al, 2008). The results of this study conducted in patients with ED and MetS imply the same conclusion. These patients share with atherosclerotic patients a common picture of endothelial damage and markers of this dysfunction. This is sustained by the significant correlation between EPCs or EMPs, both biomarkers of endothelial dysfunction, with indices of ED severity (PSV, acceleration time, and IMT).

In conclusion, this study showed that patients with arterial ED and MetS have higher blood concentrations of EPCs and EMPs compared with healthy men. Thus, these biomarkers may be considered predictors of the presence of cavernosal artery disease. ED patients responded to tadalafil administration with an EPC increase. The simultaneous evaluation of EPCs and EMPs may better monitor the biologic balance between the severity of the vascular wall damage (EMPs) and the extent of the repair mechanism (EPCs).

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