

## Original immunophenotype of blood endothelial progenitor cells and microparticles in patients with isolated arterial erectile dysfunction and late onset hypogonadism: effects of androgen replacement therapy

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### Abstract

**Objective.** Blood endothelial progenitor cells (EPCs) and endothelial microparticles (EMPs) have been proposed as markers of endothelial dysfunction. Aim of this study was to evaluate an original immunophenotype of EPCs and EMPs in patients with isolated arterial erectile dysfunction (ED) and late onset hypogonadism (LOH) before and after androgen replacement therapy.

**Materials and methods.** Fifty patients (50–64 years) with ED and LOH were selected. EPC (CD45<sub>neg</sub>/CD34<sub>pos</sub>/CD144<sub>pos</sub>) and EMP (CD45<sub>neg</sub>/CD34<sub>neg</sub>/CD144<sub>pos</sub>) blood concentrations were evaluated by flow cytometry. Thirty patients received androgen replacement therapy (Tostrex<sup>®</sup> ProStrakan) for 6 months (group A), other 20 patients not received androgen therapy for the contraindications in their clinical history (group B).

**Results.** After 6 months, group B showed IIEF-5 score, peak systolic velocity and acceleration time significantly worse than group A; in addition EPCs and EMPs were significantly higher in group B compared to group A.

**Conclusions.** Patients with isolated arterial ED and LOH not treated with androgen therapy showed worst vascular parameters measured by penile Doppler and higher EPCs and EMPs compared to treated hypogonadal patients, hence, LOH appears to be an additional vascular risk factor, and these markers may be considered as predictors of cavernous artery disease. Finally, androgen therapy improves endothelial dysfunction.

**Keywords:** Endothelial dysfunction, erectile dysfunction, late onset hypogonadism, androgen replacement therapy

### Introduction

Cardiovascular disease (CVD) as coronary artery disease (CAD), cerebrovascular disease and peripheral artery disease (PAD) are the most important causes of morbidity and mortality in the developed and developing world. Particularly, CAD is the commonest cause of death worldwide. Testosterone (T) is an anabolic hormone with putative beneficial effects on men's health and restoration of normal T levels in deficient men represents an important key-point of male well-being. In the past years, it has emerged a possible linkage between androgen deficiency and CVD. Many studies noted that T deficiency might contribute to increased risk of CAD. Furthermore, adult's androgen deficiency defined that late onset hypogonadism (LOH) is frequently associated with increased levels of glucose, total cholesterol, low-density lipoprotein, increased production of pro-inflammatory cytokines and increased thickness of the arterial

wall that all contribute to worsen endothelial function. The linkage between androgen deficiency and men's vascular health has a great impact in the modern approach to the ageing male, and should be further investigated to determine the therapeutic potential of androgens in men with vascular disease [1].

All these conditions share an endothelial dysfunction which has a well established role in the pathogenesis of both atherosclerosis and plaque instability [2,3]. 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 generalised CVD [4–7]. Therefore, LOH may also favour the onset of ED [8,9].

Endothelial dysfunction may be evaluated in many ways and recently this has been done by estimating the number of circulating endothelial precursor cells (EPCs) and endothelial microparticles (EMPs). Hypotestosteronemia is associated with a low number of circulating PCs and EPCs in young hypogonadal subjects [10], on

the contrary, EPC positive for osteocalcin were significantly increased [11]. EPCs are sub-populations of leucocytes that may differentiate into mature endothelial cells both *in-vitro* and *in-vivo* [12]. The relevant contribution of these cells in the processes of re-endothelialisation at sites of endothelial injury and neovascularisation has also been confirmed [13–16]. Circulating EPC repair capability of the damaged endothelium suggests that these cells play a key role in maintaining the endothelial homeostasis. As a result, the number of EPCs may reflect ‘the vascular health’ of an individual and it has been shown to be an independent predictor of CVD [17]. The first EPC phenotype was defined by the presence of the following antigens in blood cells: CD34, CD133 and VEGFR2 (or KDR) [13]. Many articles have reported statistically significant correlation between the number of this EPC subset in 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 in the biology of the phenotype used, namely to assess the identity of their clonal progeny which participates in vessel repair [18,19]. No clear evidence has been reported about the existence of specific markers which allows to prospectively isolate EPCs with a phenotype superior to the one so far described [19]. Recently, some studies reported that purified CD34<sub>pos</sub>/CD133<sub>pos</sub>/KDR<sub>pos</sub> blood EPCs are highly enriched in hematopoietic progenitor cell (HPC) activity and express the universal hematopoietic cell-surface antigen CD45. Moreover, these cells are unable to give rise to endothelial colony-forming cells (ECFC) *in-vitro*. Indeed, CD45 is not expressed in endothelial cells even at the mRNA level. ECFC were enriched by 368-fold only when CD45<sub>neg</sub>/CD34<sub>pos</sub> cells were plated and not when CD45<sub>pos</sub>/CD34<sub>pos</sub> cells were used [18,20]. Therefore, the latter phenotype is a HPC-enriched cell subpopulation and thus EPCs are restricted to the phenotype CD45<sub>neg</sub>/CD34<sub>pos</sub> [18]. A closer look at the biology of these EPCs indicate a progressive loss of CD133 and CD34 antigens and the expression of CD31, VE-cadherin (CD144) and Vwf, following their mobilisation into the general circulation [21,22]. In fact, CD34 is an adhesion molecule, expressed in haematopoietic stem cells, vascular progenitors and certain microvascular endothelium [23]. 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 organises the adherent junctions which control permeability and migration and they are partially responsible to inhibit growth by contact [24]. The concomitant presence of CD34 and CD144 antigens and the loss of the panleukocyte CD45 antigen assures that these cells are true stem/progenitor cells of endothelial nature [17,18,20,22]. Furthermore, CD144 or VEGFR-2 mRNA were not detected in CD45<sub>pos</sub>/CD34<sub>pos</sub>, but only in all sorted bone marrow CD34<sub>pos</sub>/

CD45<sub>neg</sub> cells [19]. Thus, the CD45<sub>neg</sub>/CD34<sub>pos</sub>/CD144<sub>pos</sub> phenotype defines a more endothelial committed cells [18]. These EPCs have been shown able to form discrete colonies of endothelial cells in culture [17]. On this account, we chose to evaluate this EPCs phenotype.

In addition to EPCs, microparticles of endothelial origin (EMPs) may be found in the general circulation. EMPs are membrane fragments which increase in several pathological conditions, including atherosclerosis, sepsis and diabetes mellitus [25]. The main characteristics of these elements are the small dimension (< 1.5  $\mu$ m) and the externalisation of phosphatidylserine (PS). EMPs are regarded as markers of cardiovascular dysfunction with a pro-thrombotic role associated with risk factors such as those that LOH promotes [26–30]. The percentage of EMPs is higher in patients with ED and inversely correlated with the IIEF-5 score [31]. The presence of diabetes mellitus seems to be associated with a greater elevation of EMPs [32]. Many studies have evaluated the correlation between EPCs or EMPs with the severity of CVD. Only few have assessed simultaneously these biomarkers and correlated them with the arterial status. There are no studies that have evaluated directly the serum concentration of EMP in hypogonadal patients.

On this basis, the present study was undertaken to evaluate the number of original immunophenotype both of EPCs and EMPs in patients with LOH and ED of isolated arterial origin before and after androgen replacement therapy. 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, 50 patients with isolated arterial dysfunctional-based ED and LOH, established by criteria of major scientific societies as International Society for the Study of the Aging Male (ISSAM), International Society of Andrology (ISA), European Association of Urology (EAU), European Academy of Andrology (EAA) and American Society of Andrology (ASA) [33], were selected and the number of circulating EPCs and EMPs (CD45<sub>neg</sub>/CD34<sub>neg</sub>/CD144<sub>pos</sub>) was determined. Within this group, 20 patients untreated for the presence of clinical contraindications, represented the controls.

## Patients and methods

### Patient selection

Fifty patients with arterial ED and LOH (group A) were enrolled, mean age: 59.3  $\pm$  0.5 years (range: 50–64). The diagnosis of arterial ED was made when all the following criteria were fulfilled: (a) international index of erectile function (IIEF-5) score < 21 [34]; (b) cavernousal artery peak systolic velocity (PSV) < 30 cm/sec, 10 and 20 min after the intracavernousal

injection of alprostadil (20  $\mu\text{g}$ ) by echo colour Doppler [35] and (c) acceleration time >110 msec [36]. The diagnosis of LOH was made according to the criteria of major scientific societies as ISSAM, ISA, EAU, EAA and ASA [33].

Exclusion criteria were the following: CAD, carotid and lower limbs atherosclerosis, smokers and diabetics.

By initial 50 patients, 30 patients underwent transdermal androgen replacement therapy (group A) for 6 months. Tostrex was administered with initial dose of 60 mg and subsequent adjustment of the dose after 14 days according to the serum concentrations of total testosterone.

Twenty patients were excluded from androgen treatment (group B) for the presence of clinical contraindications: abnormal digital rectal examination ( $n=7$ ), reported sleep apnea patients ( $n=5$ ), high hematocrit value ( $n=4$ ) and high values of transaminases  $n=4$ .

All patients (group A and group B) underwent blood withdrawal (10 ml), for EPC and EMP measurements by flow cytometry. The protocol was approved by the

internal Institutional Review Board and an informed written consent was obtained from each patient.

#### Blood EPC and EMP determination

EPCs and EMPs evaluation was performed in blood following incubation in erythrocyte lysing solution (Versalyse, IL, Milan, Italy) for 1 min. The suspension was then washed twice with phosphate buffer solution (PBS), centrifuged and the pellet was rapidly incubated in PBS containing the following monoclonal antibodies: anti-CD45 (10  $\mu\text{l}$ ), anti-CD34 (20  $\mu\text{l}$ ) and anti-CD144 (20  $\mu\text{l}$ ), respectively, labelled with r-phycoerythrin covalently bound to Texas red (ECD), fluorescein isothiocyanate (FITC) and r-phycoerythrin (PE), at room temperature for 20 min.

Each sample was analysed by flow cytometry (EPICS XL, Coulter Electronics, IL, Milan, Italy) using the following gating strategy (Figure 1). Histograms 1 report the forward *versus* side scatter dot plot: three different cell populations can be identified; gate F: lymphocytes, gate I: monocytes, gate L: polymorpho-

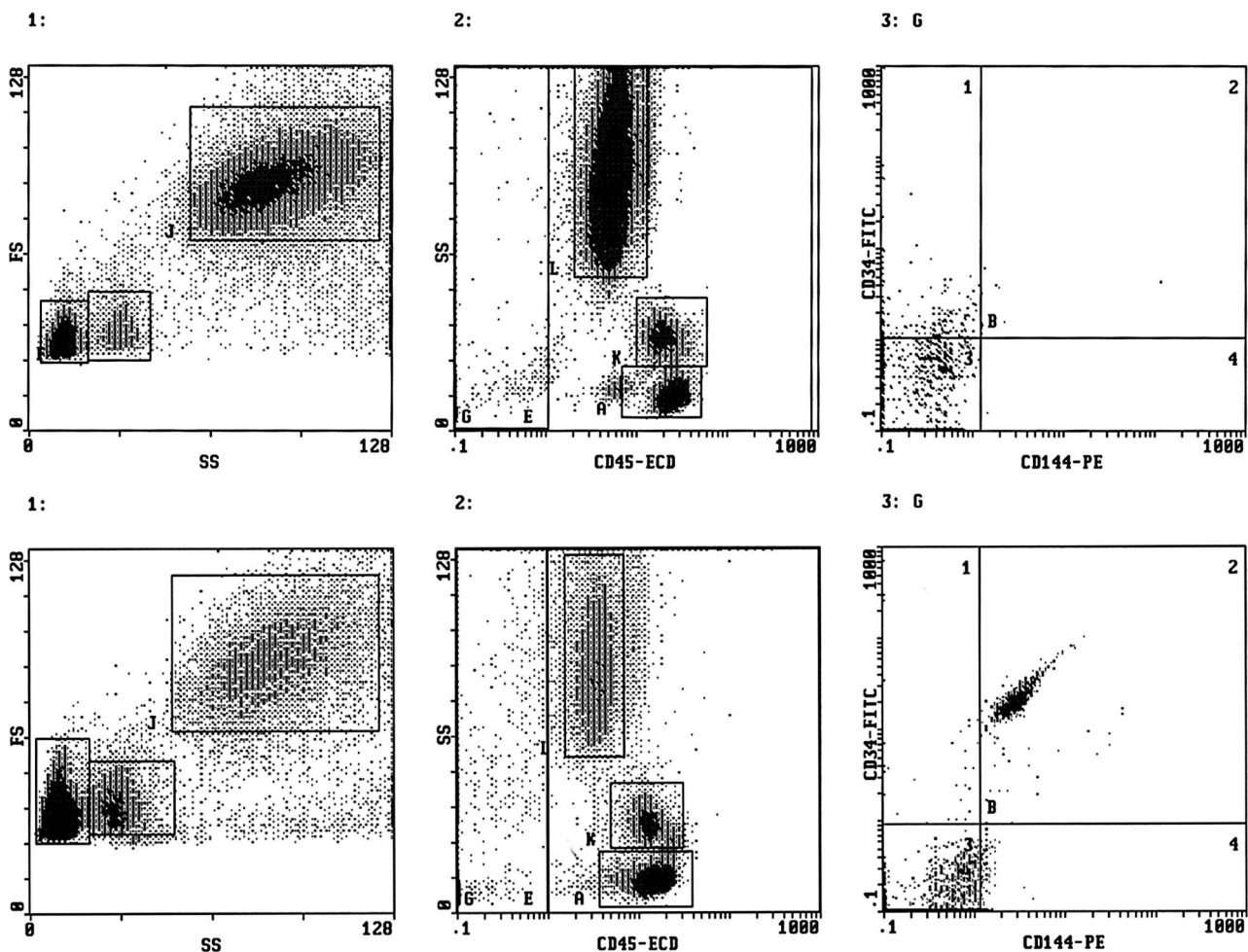


Figure 1. Representative flow cytometric scattergrams showing the gating strategy used in a normal healthy men (upper panels) and in a patient with late onset hypogonadism and erectile dysfunction (lower panels). Both histograms 1 report the forward *versus* side scatter dot plot: three different cell populations can be identified (gate F: lymphocytes; gate I: monocytes; gate J: polymorphonuclear cells). Both histograms 2 report the CD45<sub>pos</sub> (E) and CD45<sub>neg</sub> (G) cells. Both histograms 3 report only the CD45<sub>neg</sub> cells (gate G) subdivided according to the expression of CD34 and CD144 antigen staining. EPCs were defined as CD45<sub>neg</sub>/CD34<sub>pos</sub>/CD144<sub>pos</sub>, whereas EMP CD45<sub>neg</sub>/CD34<sub>neg</sub>/CD144<sub>pos</sub>.

nuclear cells, Histograms 2 report the CD45<sub>pos</sub> (E) and CD45<sub>neg</sub> (G) cells; Histograms 3 report only the CD45<sub>neg</sub> cells (gate G) subdivided according to the staining with CD34 and CD144 antigens. EPCs were defined as CD45<sub>neg</sub>/CD34<sub>pos</sub>/CD144<sub>pos</sub>, whereas EMPs as CD45<sub>neg</sub>/CD34<sub>neg</sub>/CD144<sub>pos</sub> events. Fluocytometric analysis was conducted for 600 sec or 100,000 events (cells) whichever occurred first.

### Statistical analysis

Results are shown as mean  $\pm$  SEM. EPC and EMP values were log-transformed before being analysed. Statistical analysis was conducted by one-way analysis of variance (ANOVA) followed by the Duncan Multiple range test or unpaired Student's *t* test, as appropriate. Correlation analysis was carried out by Pearson test. Statistical analysis was conducted using SPSS 10.0 for Windows. A *p* value lower than 0.05 was accepted as statistically significant.

### Results

At baseline, no statistically significant difference was observed between the two groups, relatively to the following parameters: IIEF-5 score, serum testosterone concentration, peak systolic velocity, acceleration time and relatively to others vascular parameters as: end diastolic velocity, resistance index, intimal cavernous thickness (Table I).

In addition, no statistically significant difference was observed between the two groups for the following metabolic characteristics: body mass index, total cholesterol, HDL, triglycerides, blood pressure detected during the enrolment visit (Table I). At baseline, no statistically significant difference was observed between the two groups, relatively to serum EPCs ( $14.2 \pm 1.2\%$  vs  $14.3 \pm 2.3\%$ ) and EMP concentration ( $6.1 \pm 0.4\%$  vs  $6.1 \pm 1.1\%$ ) (Figure 2).

During the therapy, after 14 days, at first clinical control, no patient showed supraphysiologic levels of serum testosterone, therefore no patients changed the initial dose. No patient discontinued the study for the occurrence of complications (pathological digital rectal examination, high hematocrit values, sleep apnea, gynecomastia and increased liver enzymes).

After six months, group A showed mean serum T concentration significantly higher compared to baseline (Table I) and showed statistically significant difference relatively to all parameters investigated at baseline (IIEF-5 score, peak systolic velocity, acceleration time, body mass index, total cholesterol, HDL, triglycerides, blood pressure detected during 2nd visit), compared to group B (Table I).

Finally, group A showed mean serum concentrations of EPCs ( $6.3 \pm 1.2\%$  vs  $14.4 \pm 2.8\%$ ) and EMP ( $2.2 \pm 0.46\%$  vs  $6.2 \pm 0.9\%$ ) significantly lower compared to group B (Figure 2).

### Discussion

This study evaluated the number of circulating original immunophenotype of EPCs and EMPs in patients with isolated arterial dysfunctional-based ED and late onset hypogonadism before and after replacement transdermal androgen therapy, since no data are available in these patients relative to these circulating phenotypes.

It is now well documented that the simultaneous measurement of both EPCs and EMPs may be proposed as biomarker of CVD and atherosclerotic complication and progression. We found that patients with arterial ED and LOH not treated with androgen therapy had worse penile vascular damage and metabolic features compared to treated patients and higher circulating original immunophenotype of EPCs and EMPs, suggesting that LOH improves endothelial dysfunction and androgen therapy improves reparative endothelial function, finally that these markers indicate a more severe vascular damage.

Table I. Penile dynamic echo-color doppler parameters, IIEF-5 score, T serum concentration and metabolic features (mean  $\pm$  SEM) of the 50 patients (group A and group B) with arterial erectile dysfunction and late onset hypogonadism at baseline and after six months of transdermal androgen replacement therapy for group A.

Parameters	Group A baseline	Group B baseline	Group A after six months	Group B after six months
Peak systolic velocity (cm/sec)	23.0 $\pm$ 0.5	24.0 $\pm$ 1.2	32.3 $\pm$ 1.0* <sup>†</sup>	22.0 $\pm$ 1.0
EDV (cm/sec)	2.0 $\pm$ 0.2	2.0 $\pm$ 0.4	2.0 $\pm$ 0.1	2.0 $\pm$ 0.6
Acceleration time (msec)	134.2 $\pm$ 1.6	133.3 $\pm$ 1.2	112.1 $\pm$ 1.2* <sup>†</sup>	136.3 $\pm$ 1.2
Resistance index	0.91 $\pm$ 0.03	0.92 $\pm$ 0.01	0.93 $\pm$ 0.08	0.91 $\pm$ 0.01
Intima-media thickness (mm)	0.33 $\pm$ 0.02	0.34 $\pm$ 0.04	0.32 $\pm$ 0.02	0.35 $\pm$ 0.04
IIEF-5	9.0 $\pm$ 1.0	9.5 $\pm$ 0.7	18.0 $\pm$ 1.0* <sup>†</sup>	8.0 $\pm$ 0.7
Serum T concentration (ng/dl)	176 $\pm$ 10.2	180 $\pm$ 15.0	420 $\pm$ 17.2* <sup>†</sup>	176 $\pm$ 20.0
Body mass index (kg/m <sup>2</sup> )	27.7 $\pm$ 1.0	27.9 $\pm$ 1.2	25.7 $\pm$ 1.0* <sup>†</sup>	28.2 $\pm$ 1.2
Total cholesterol (mg/dl)	213.1 $\pm$ 13.1	218.0 $\pm$ 11.2	114.1 $\pm$ 10.1* <sup>†</sup>	228.0 $\pm$ 11.2
HDL cholesterol (mg/dl)	43.0 $\pm$ 3.3	42.0 $\pm$ 0.7	38.0 $\pm$ 2.2* <sup>†</sup>	41.0 $\pm$ 1.7
Triglycerides (mg/dl)	189.1 $\pm$ 10.1	194.0 $\pm$ 17.2	119.1 $\pm$ 10.2* <sup>†</sup>	198.0 $\pm$ 17.2
Systolic blood pressure (mmHg)	143.4 $\pm$ 7.1	142.4 $\pm$ 11.2	134.4 $\pm$ 5.1* <sup>†</sup>	148.4 $\pm$ 11.2
Diastolic blood pressure (mmHg)	94.1 $\pm$ 7.4	92.5 $\pm$ 6.2	86.1 $\pm$ 8.4* <sup>†</sup>	92.5 $\pm$ 6.2

\**p* < 0.05 compared to group A at baseline.

<sup>†</sup>*p* < 0.05 compared to group B after 6 months.

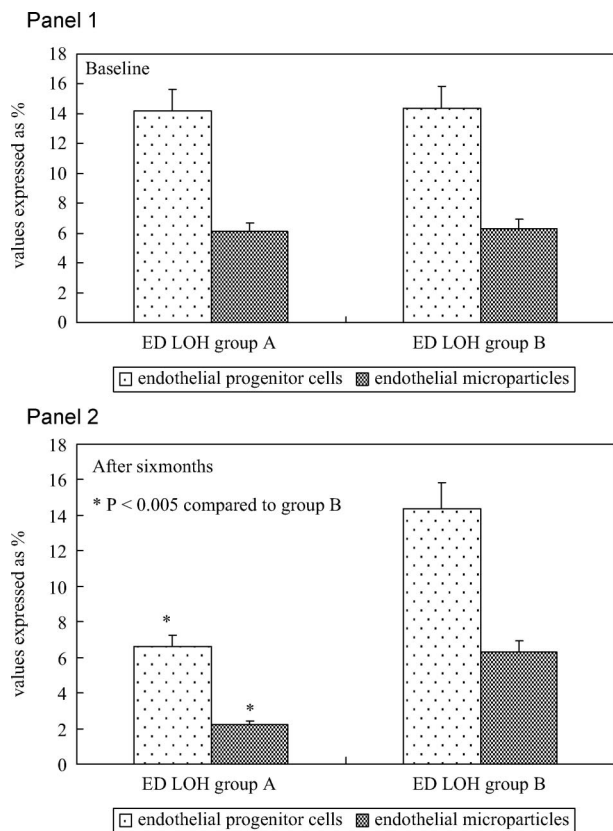


Figure 2. Panel 1: Percentage of circulating endothelial progenitor cells (immunophenotype  $CD45_{neg}/CD34_{pos}/CD144_{pos}$ ) and percentage of circulating endothelial microparticles (immunophenotype  $CD45_{neg}/CD34_{neg}/CD144_{pos}$ ) in all patients with erectile dysfunction and late onset hypogonadism (group A and group B) at baseline. Panel 2: Percentage of circulating endothelial progenitor cells (immunophenotype  $CD45_{neg}/CD34_{pos}/CD144_{pos}$ ) and percentage of circulating endothelial microparticles (immunophenotype  $CD45_{neg}/CD34_{neg}/CD144_{pos}$ ) in all patients with erectile dysfunction and late onset hypogonadism (group A and group B) after transdermal androgen replacement therapy.

It is reported that low serum levels of T in aging men are associated with increased rate of mortality from CHD and other vascular events, and that T administration has beneficial effects on the pathophysiological markers and clinical symptoms of CHD. Testosterone improves the profile of the main risk factors involved in atherosclerosis such as obesity, the metabolic syndrome (MetS), type 2 diabetes and erectile dysfunction [37].

T supplementation restores arterial vasoreactivity; reduces proinflammatory cytokines, total cholesterol and triglyceride levels; and improves endothelial function but also might reduce high-density lipoprotein levels [38].

Foresta et al., evaluated 10 patients with hypogonadotropic hypogonadism treated with testosterone (gel 50 mg daily) for 6 months. This study showed a significant increase of EPCs after androgen treatment. In this study EPCs show, however, a different phenotype from ours, indeed, these cells are positive for CD133, CD34 and KDR, and their reduced baseline serum concentrations expresses an increased cardiovascular risk [10].

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 protection from the disease progression in these patients. 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 presence of cardiovascular risk factors (cigarette smoke, hypertension, diabetes mellitus, obesity and dyslipidemia) [6,39,40].

The apparent discrepancy with above mentioned studies may relate to the different immunophenotype evaluated. Indeed, more recently, an increased number of EPCs positive to osteocalcin, a marker of circulating osteogenic cells, has been reported in ED patients [11]. In the attempt to understand the role played by EPCs on ED, 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 [6]. It is clear now that there are distinct EPC sub-populations with different immunophenotype and biological properties [17]. Cells progress from stem cell stage to the final mature cell of each single lineage, through successive progenitor cell stages [41]. Because uniform criteria to identify these cells are lacking, we followed the proposal outlined by Case et al. They suggested to use the  $CD45_{neg}/CD34_{pos}$  phenotype because these cells form colonies of mature endothelial cells in a clonogenic *in-vitro* assay of endothelial cells [18]. Therefore, probably these cells behave to a greater extent as true endothelial progenitors [20]. Accordingly,  $CD45_{neg}/CD34_{pos}/CD144_{pos}$  cells form endothelial cells in culture [17]. The higher number of EPCs we found in patients with LOH, hence at increased CVD risks and corpus cavernosum atherosclerosis, are superimposable to previous evidences showing that distinct CVDs are associated with an increased circulating EPCs levels [17,42–44]. In CAD patients, several mechanistic possibilities have been advanced to explain the increase of EPCs. This activation may result from a variety of pro-inflammatory cytokines released in patients with coronary ischemia or after intravascular radiation [15,45]. Moreover, exercise-induced ischemia is associated with an increased number of circulating EPCs [42,44]. Patients with unstable angina have an increased number of EPCs compared with patients with stable angina, and the stabilisation of angina in these patients resulted in a two-fold decrease in circulating EPCs [46]. EMPs ( $CD31_{pos}/CD42_{neg}$  and  $CD31_{pos}/CD42_{pos}$ ) are elevated in ED patients with diabetes mellitus and independently involved in the pathogenesis of ED.

EMPs are released as consequence of endothelial dysfunction, atherogenesis and endothelial apoptotic processes. There are no studies that examine directly serum concentrations of EMP in hypogonadal men, or the effects of treatment with androgens on them. EMPs inversely correlate with the IIEF score in both diabetic

and non-diabetic patients and multivariate analysis, corrected for confounding factors, showed that EMPs are the only independent predictor of the IIEF score [31]. In addition, ED patients with or without diabetes mellitus have significantly higher EMPs (CD62<sub>pos</sub>) than non-diabetic men without ED. The EMPs (CD62<sub>pos</sub>)/EMPs (CD31<sub>pos</sub>) ratio, an index of endothelial activation (high ratio) or apoptosis (low ratio), was lowest in ED diabetic patients. These findings suggest that EMP increase in ED diabetic patients is consistent with an increased apoptotic activity [32].

The results of this study conducted in patients with ED and LOH 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 bio-markers of endothelial dysfunction, with indices of ED severity (PSV, acceleration time and IMT).

In conclusion, this study showed that patients with isolated arterial ED and LOH not treated with androgen therapy showed worst vascular parameters measured by penile Doppler and higher EPCs and EMPs compared to treated hypogonadal patients with arterial ED, hence, LOH appears to be an additional vascular risk factor, and these markers may be considered predictors of cavernous artery disease. Finally, androgen therapy improves endothelial dysfunction of these patients.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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