

## RETHINKING CARDIOVASCULAR THERAPY - THE EFFECT OF IRBESARTAN ON CIRCULATING MICROPARTICLES AND ENDOTHELIAL PROGENITOR CELLS IN PATIENTS WITH HYPERTENSION AND DYSLIPIDEMIA

ELISABETA BĂDILA<sup>1,2</sup>, ANA MARIA DARABAN<sup>1</sup>, SILVIU  
GHIORGHE<sup>1</sup>, ADRIANA GEORGESCU<sup>3</sup>, NICOLETA ALEXANDRU<sup>3</sup>,  
DANIELA BARTOȘ<sup>1,2</sup>, CRISTINA TÎRZIU<sup>1\*</sup>

<sup>1</sup> *Clinical Emergency Hospital, Bucharest, Romania*

<sup>2</sup> *"Carol Davila" University of Medicine and Pharmacy, Romania*

<sup>3</sup> *"Nicolae Simionescu" Institute of Cellular Biology and Pathology,  
Bucharest, Romania*

*\*corresponding author: tirziuc@yahoo.com*

### Abstract

Endothelial progenitor cells (EPCs) and circulating microparticles (MPs) have been proposed as markers of endothelial function. In turn, the protective role of irbesartan, an angiotensin receptor blocker, on the endothelium has been demonstrated in the recent years but the mechanism has not been yet fully elucidated. In this context, we investigated whether irbesartan can influence these two new biomarkers in patients with cardiovascular (CV) risk factors. We compared the levels of EPCs and MPs in patients with hypertension and dyslipidemia which received irbesartan with the levels in patients with the same CV risk factors but receiving other antihypertensive drugs and in healthy individuals. Using this methodology we showed the positive effect of angiotensin receptor blockade on the two proposed markers of endothelial function, reflecting both endothelial injury and repair, MPs and EPC respectively, in the same subjects with cardiovascular risk factors. Moreover, we found that patients with hypertension and dyslipidemia display an altered balance between the levels of endothelial regenerative cells like EPCs and markers of endothelial injury like circulating MPs.

### Rezumat

Celulele endoteliale progenitoare (EPC) și microparticulele circulante (MP) au fost propuse ca markeri ai funcției endoteliale. Pe de altă parte, în ultimii ani a fost demonstrat rolul protector al irbesartanului, un blocant al receptorilor de angiotensină, asupra endoteliului, dar mecanismul implicat nu a fost complet elucidat. În acest context am studiat potențialul irbesartanului de a influența acești noi biomarkeri la pacienții cu factori de risc cardiovasculari (CV). Am comparat nivelurile circulante de EPC și MP la pacienți cu hipertensiune arterială și dislipidemie ce au primit tratament cu irbesartan, cu cele măsurate la pacienți cu aceiași factori de risc CV, dar tratați cu altă clasă de medicamente antihipertensive și la indivizi sănătoși. Cu ajutorul acestei metodologii am demonstrat efectul benefic al blocadei receptorilor de angiotensină asupra markerilor de disfuncție endotelială ce reflectă atât afectarea cât și procesul reparator endotelial, MP și EPC

respectiv, la același grup de pacienți cu factori de risc CV. Mai mult, am arătat că pacienții cu HTA și dislipidemie prezintă un raport alterat între nivelul de celule endoteliale regenerative, precum EPC și markeri de afectare endotelială precum MP.

**Keywords:** endothelial dysfunction, endothelial progenitor cells, circulating microparticles, irbesartan.

### **Introduction**

The endothelium, once considered inert, has now been established as a strategically-located multifunctional organ, playing many pivotal roles in the regulation of vascular tone and endothelial integrity as well as in the maintenance of blood fluidity and homeostasis [8]. Atherosclerosis, in turn, is an inflammatory disease of the vascular wall characterized by leucocytes infiltration, smooth muscle cells accumulation, and neointima formation. Along with becoming dysfunctional, endothelial cells can also lose integrity, progress to senescence, and detach into circulation [27]. Circulating markers of such endothelial cell damage include endothelial microparticles (EMPs) [22]. Microparticles (MPs) are vesicles (100 nm to 1  $\mu$ m diameter) shed from plasma membranes following cell activation or apoptosis. In the blood flow, circulating MPs originate mainly from platelets (PMP), leukocytes (LMP), red blood cells, endothelial cells, smooth muscle cells and macrophages [19]. MPs harbour at their surface, a panel of phospholipids and proteins specific from the parent cells which confer them a potential role in many physiological processes, including thrombosis, inflammation, endothelial dysfunction and angiogenesis [7]. Consequently, MPs appear as a new key player in atherothrombosis. The relevance of MPs in various pathological conditions as end-stage renal disease, acute coronary syndromes, diabetes mellitus or others, has been studied but still a complete understanding of the formation, mechanisms of action and ways of quantification of these MPs is still needed as it can be of importance in the development of novel therapeutic strategies to combat atherothrombosis [20].

On the other hand, the restoration and reconstitution of the damaged endothelium may be a prerequisite for the prevention of endothelial dysfunction. A growing body of evidence suggest that circulating endothelial progenitor cells (EPC) play an important role in endothelial cell regeneration thus being an alternative mechanism for maintenance and repair of the endothelium [19]. EPCs could be released from bone marrow, fat tissue and vessel wall, especially adventitia, and possibly spleen, liver into blood [28]. Mobilization of these cells is in part NO dependent, and may thus be impaired in patients with cardiovascular risk factors [18].

Strategies to reverse endothelial dysfunction and thus preventing atherosclerosis and adverse cardiovascular events have been intensely studied. Angiotensin receptor blockers (ARBs) have been shown to positively affect the endothelial function beyond the antihypertensive action but the exact mechanism has not been fully elucidated [23]. To date, only a few studies have investigated the levels of both MPs and EPCs in patients with atherosclerosis. Furthermore, there are no data regarding the effect of irbesartan on MPs and EPCs in the same population of cardiovascular patients. Bringing new insights into mechanisms of vascular dysfunction, such as a better understanding of the inverse relationship between circulating MPs and EPCs, may lead to new therapeutic strategies having the potential to improve the prognosis of atherosclerotic disease. Thus, measurement not only of EMPs, but also of less studied PMPs and LMPs, and of EPCs, may provide novel and exciting means to follow the determinants of endothelial injury and repair. Moreover, the treatment with irbesartan could prevent the appearance of vascular endothelial dysfunction by decreasing MP levels and increasing EPC levels.

### **Materials and Methods**

The study enrolled 40 hypertensive – hypercholesterolemic patients that were randomly divided in 2 groups. Group HH included 20 hypertensive – hypercholesterolemic previously untreated patients (12 male, age:  $47.3 \pm 4$  years, body mass index (BMI):  $22.9 \pm 0.9$  kg/m<sup>2</sup>) that were assigned to receive other antihypertensive drugs than angiotensin converting enzyme (ACE) inhibitors / angiotensin receptor blockers (ARB) for 2 months. Blood pressure (BP) control was obtained with beta blockers or diuretics in this first group. The second group (HH-I), the hypertensive–hypercholesterolemic patients, included also 20 patients (12 male, age:  $48.5 \pm 6$  years, BMI :  $23.4 \pm 0.8$  kg/m<sup>2</sup>) that were assigned to receive treatment with irbesartan (150 mg or 300 mg once daily, in a dose targeted to control BP) for 2 months. Another group, the C group, was used as a control group and included 20 (11 male, age:  $46.6 \pm 2$  years, BMI :  $22.8 \pm 0.6$  kg/m<sup>2</sup>) age and sex matched, normotensive and normocholesterolemic controls. None of the patients received cholesterol lowering agents during the study period. Subjects with severe hypertension, diabetes mellitus, heart failure, recent cardiovascular events or ongoing inflammation (cancer, infectious disease, etc) were excluded from the study. Hypercholesterolemia was defined by the NCEP/ATP III recommendation (total cholesterol above 190 mg/dl) and the ESH/ESC guideline recommendation was used in order to define arterial

hypertension (systolic BP  $\geq$  140 mmHg and/or diastolic BP  $\geq$  90 mmHg). (26)

Clinical and biochemical characterization of patients in each group consisted in demographic data, clinical data (BP, ankle – brahial index), echographic data [left ventricular mass index - LVMI, intima-media thickness - IMT) and biochemical parameters [lipidic profile, glycemia, serum creatinine, brain natriuretic peptide (BNP), homocysteine, uric acid, C reactive protein (CRP), microalbuminuria].

Each study participant underwent vascular examination by means of carotid ultrasound and ankle-brachial index measurement and echocardiography for LVMI and function assessment. The same day, venous blood samples were taken after an overnight fast, between 07.30 and 08.30 am, in order to measure blood glucose, serum lipids, homocysteine, uric acid, circulating MPs and EPCs.

All these parameters were evaluated at inclusion. After 2 months of treatment, either with irbesartan in the HH-I group or non-ACE inhibitor/ARB antihypertensive drugs, vascular examination was repeated and again blood samples were collected from each patient to assess levels of circulating MPs and EPCs. Moreover, at this time point, endothelium-dependent vasodilatation test was performed in the treatment groups.

All the protocols were approved by the Ethics Committee of our hospital and written informed consent was obtained from each patient.

#### ***Vascular assessment***

A single operator unaware of the clinical status of the subject under investigation performed all vascular recordings and readings. Measurements were performed in a quiet room, with a stable temperature of 22°C, starting with carotid scanning, after resting comfortably for at least 20 minutes in supine position.

*Carotid scanning* was performed on the right carotid artery using an ultrasound scanner equipped with a linear 10 MHz probe (Siemens Sonoline G 40) and intima-media thickness (IMT) was measured.

*Ankle-brahial index* was assessed using a Doppler ultrasonic instrument and measuring systolic BP readings in the right and left brachial arteries, right and left *dorsalis pedis* arteries, and right and left posterior tibial arteries.

*Endothelium-dependent vasodilatory* capacity was estimated assessing a reactive hyperemia index (RHI) by means of an EndoPAT system (Itamar Medical Ltd, Cesarea, Israel) [14].

*Echocardiography* was done using a Phillips HD7XE machine. Global systolic function of the LV was assessed in order to exclude patients with

depressed function and LVMI was derived using the Devereux modified ASE cube formula  $LVM = LV \text{ mass (g)} = 0.8 (1.04 (IVSd + LVIDd + PWTd)^3 - (LVIDd)^3) + 0.6$  and then divided by body weight, with IVSd – interventricular septum thickness in diastole, LVIDd – LV diastolic diameter, PWTd - LV posterior wall thickness [9].

#### ***Quantification of MPs and EPCs***

Both quantifications of EPCs and MPs were done by the method previously reported by Georgescu *et al.* [11].

#### ***Preparation of platelet free plasma, the source for circulating MPs***

Plasma EMPs, PMPs and LMPs were separated according to the method reported by Boulanger *et al.* 2001 [5]. Briefly, the procedure consisted in collection of blood, centrifugation at 1,000 g for 15 min at 15°C, and separation of platelet rich plasma (PRP). The latter was further centrifuged at 2,500 g for 15 min at 15°C, and the platelet free plasma (PFP) was obtained. Centrifugation of PFP at 13,000g for 5 min at 15°C allowed collection of MPs in the supernatant.

#### ***Sorting of EMPs, PMPs and of LMPs by flow cytometry***

MPs were characterized as EMPs using specific antibodies to VE-cadherin (C-19)PE (for CD144), and Annexin V-FITC (for phosphatidylserine, PS). PMPs were assayed using specific antibodies to Integrin  $\alpha$ IIb (M 148) PE (for CD41) and Annexin V FITC (for PS) and LMPs using specific antibodies to Integrin  $\beta$ 2 (3H1058) PE (for CD18), and Annexin V FITC (for PS). As negative control human IgG1-PE was used.

The reaction consisted in: 20  $\mu$ L PFP and 10  $\mu$ L Annexin V-FITC and 10  $\mu$ L VE-cadherin-PE or Integrin  $\alpha$ IIb-PE or Integrin  $\beta$ 2-PE, mixing and incubation in the dark for 40 minutes at room temperature. Freshly filtered PBS buffer (200  $\mu$ L) was added right before detection.

Cell sorting involves physical separation of MPs (EMPs, or PMPs, or LMPs) from suspension stained with specific fluorescent dyes (as above). MPs were gated as events with a 0.1 $\mu$ m to 1.0  $\mu$ m diameter identified in forward-scatter and side-scatter intensity dot-plot representation using standard synthetic beads of 10  $\mu$ m in diameter. MPs concentration was assessed by comparison with calibrator Flowcount beads (10  $\mu$ m in diameter) with a predetermined concentration.

#### ***Preparation of viable mononuclear cells from blood***

The whole blood was collected from each patient considered in this study and mononuclear cells (MNCs) were isolated by density centrifugation. Briefly and carefully, we layered 1 mL whole blood onto 3 mL Histopaque-1077, centrifuge at 400xg for 30 min, aspirated the upper layer to within 0.5 cm of the opaque interface containing MNCs. We

discarded the upper layer and transferred the opaque interface into clean tube. We added 10 mL isotonic Phosphate Buffered Saline Solution (PBS containing 2% fetal ser), mixed, centrifuged at 256xg for 10 min, aspirated supernat and discard, repeat three times the washing and afterwards resuspended the MNCs pellet in 10 mL PBS [16].

#### *Sorting of EPCs by flow cytometry*

EPCs were characterized by the expression of specific surface markers: CD34, CD133, and vascular endothelial growth factor receptor (VEGFR) 2 (KDR or Flk-1). The reaction consisted in: 100  $\mu$ L containing 10000 freshly isolated MNCs in PBS with 2% serum and 10  $\mu$ L antibodies against KDR-FITC, CD133-APC and CD34-PE, mixing and incubation in the dark for 40 min at room temperature. Freshly filtered PBS (200  $\mu$ L) was added right before detection, and the samples were quantified and sorted. The separation of EPCs was made from MNC suspension stained with specific fluorescent dyes (as above). As negative control the dual colour control reagent IgG1-FITC/IG2a-PE was used.

For flow cytometry technique, the sorting speed was around 10000–12000 MPs, or EPCs per second. The experiments were conducted using the Flow cytometer MoFlo (Dako, USA) equipped with high-speed cell sorter [10].

#### *Data analysis*

For flow cytometry experiments, a software based on auto and manual compensation was used (Summit 4.0 b2060 Software, DakoCytomation, USA).

All values were expressed as mean  $\pm$  S.E.M. One-Way Analysis of Variance (Anova) was employed to quantify the results. The data were considered significant when  $p < 0.05$ .

## **Results and Discussion**

The clinical data from the 3 groups that were enrolled are presented in Table I. At baseline there were no significant differences between treatment groups (HH and HH-I) in terms of BMI, systolic BP, serum cholesterol, triglycerides, uric acid, creatinine clearance, BNP levels or homocysteine levels. Compared to control group, the HH and HH-I patients presented significantly higher systolic BP values as well as higher levels of cholesterol and triglycerides as defined by inclusion criteria but with no differences in uric acid levels, creatinine clearance, homocysteinemia or plasmatic BNP levels. When looking at endothelial dysfunction parameters (IMT and ABI),

there were no differences between treatment groups, but they were significantly higher when compared with the C group.

**Table I**  
Clinical characteristics of the 2 treated groups and the control group.

Clinical Parameters	HH-I Group (n=20)	HH Group (n=20)	Control Group (n=20)
Gender (male)	12	12	11
Age (years)	48.5±6	47.3±4	46.6±2
BP (mmHg)	148± 4*	147± 2*	121± 2
BMI (kg/m <sup>2</sup> )	23.4± 0.8	22.9 ± 0.9	22.8± 0.6
Serum Cholesterol (mg/dL)	262,5± 17.7*	295±18.95*	165±10.2
Triglyceride (mg/dL)	200.62 ± 19.88*	220 ± 20.5*	130 ± 10.22
Glycemia (mg/dL)	97.5 ± 6.26	99 ± 7.2	95 ± 4.5
Uric acid (mg/dL)	6.2 ± 0.64*	6.65 ± 0.7*	4.4 ± 0.5
Creatinine Clearance (mL/min)	104.55 ± 4.7	105 ± 3.2	110 ± 2.4
BNP (pg/mL)	10 ± 4.24*	13 ± 2.3*	5 ± 1.6
Homocysteine (µmol/L)	12.45 ± 0.21	15 ± 1.3	10 ± 0.9
IMT (mm)	0.9 ± 0.1*	1 ± 0.2*	0.5 ± 0.1
LVMI (g/mp)	100.28 ± 19*	104 ± 15*	85 ± 5.6

\* p<0.05 compared with the control group.

HH – hypertensive-hypercholesterolemic; HH-I – HH receiving treatment with irbesartan; BP – blood pressure; BMI – Body mass index; BNP – brain natriuretic peptide; IMT – intima-media thickness; LVMI - Left ventricular mass index.

After 2 months of antihypertensive treatment, systolic BP decreased significantly ( $p < 0.05$ ) and comparably in both treated groups, i.e, from 148±4 to 130±5 mmHg in the irbesartan treated group, and from 147±3 to 133±4 mm Hg in the comparative treatment group.

#### *Vascular assessment*

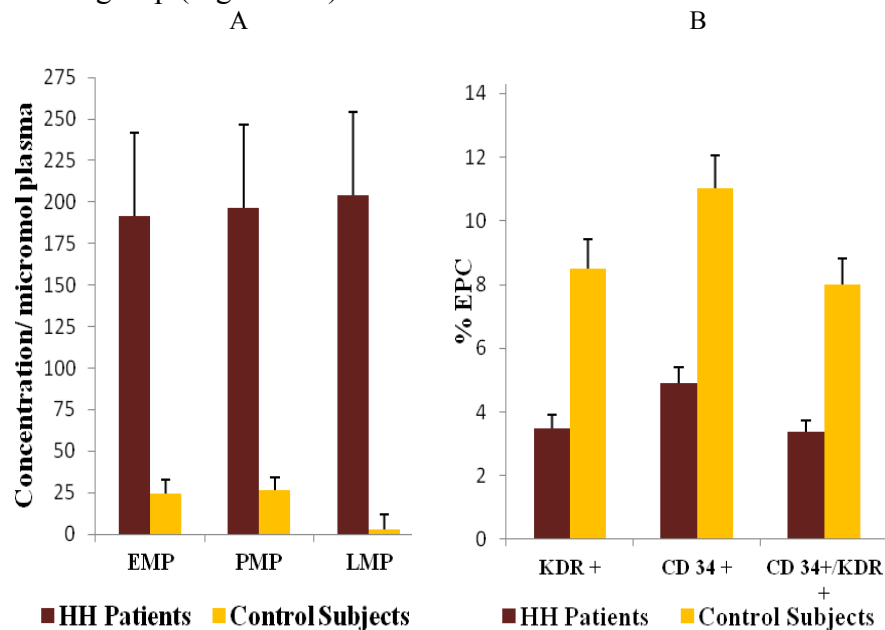
At the time of enrolment we found no significant differences between HH and HH-I group in the parameters assessing vascular health (IMT and ABI) but when compared with the healthy subjects, there were pathological changes (IMT: 0.87±0.09 in the treatment group vs 0.66±0.11 in the C group,  $p < 0.05$ ; ABI: 0.85±0.12 in the treatment group vs. 0.94±0.09 in the C group,  $p = \text{not significant - NS}$ ). After 2 months of antihypertensive treatment, there was a decreasing tendency in IMT (HH: 0.89±0.08 at baseline vs. 0.85 ±0.06 at follow-up,  $p = \text{NS}$ ; HH-I: 0.85±0.1 at baseline vs 0.78±0.08 at follow-up,  $p = \text{NS}$ ) and a increasing tendency for ABI (HH: 0.83±0.9 at baseline vs. 0.84±0.1 at follow-up,  $p = \text{NS}$ ; HH-I: 0.87±0.08 at

baseline vs  $0.90 \pm 0.07$  at follow-up,  $p = \text{NS}$ ), with a more pronounced change in the HH-I group, however not statistically significant.

RHI index was found to be significantly higher in the hypertensive-hypercholesterolemic patients that received treatment with irbesartan for 2 months than in the HH group ( $1.78 \pm 0.3$  vs.  $1.56 \pm 0.24$ ,  $p < 0.05$ ).

#### Levels of circulating MPs

When assessing MP levels, there were no differences in the baseline values between treatment groups but the levels of MPs (EMPs, PMPs, LMPs) were significantly higher in the HH and HH-I groups than in the control group (Figure 1.A).

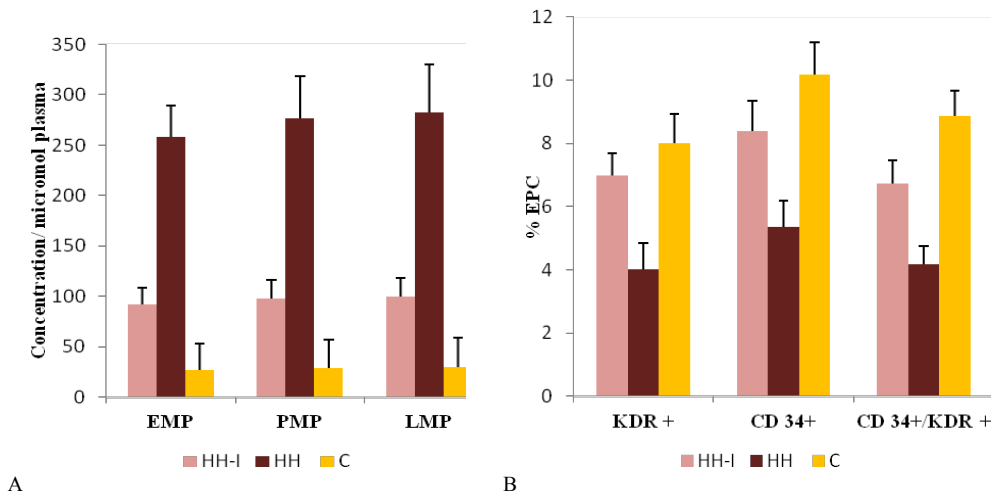


**Figure 1**

**A.** Levels of endothelial (EMP), platelet (PMP) and lymphocyte (LMP) circulating microparticles (MPs) and **B.** Levels of endothelial progenitor cells (EPCs) (CD 34+, KDR+, CD 34+/KDR+) in 40 hypertensive hypercholesterolemic patients and in 20 age- and gender-matched healthy subjects.

After 2 months of treatment, circulating levels of EMPs, PMPs and LMPs, were significantly lower in the HH-I group than in the comparator, HH group as shown in figure 2.A. Even though significantly reduced, MP levels were still higher than in normal subjects.





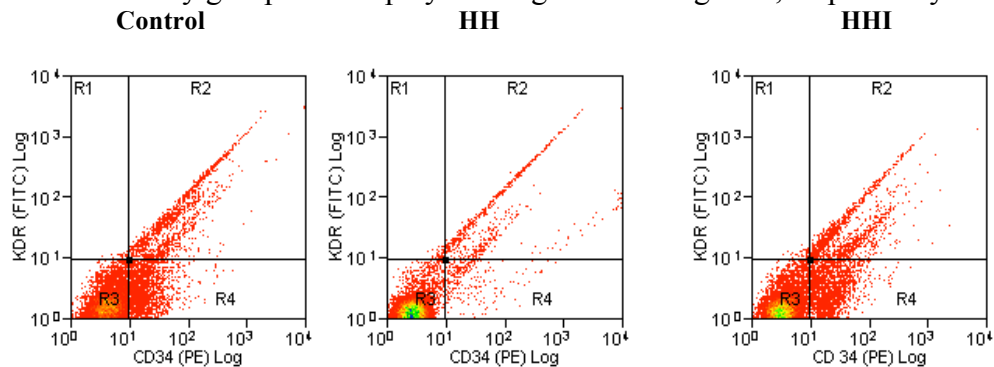
**Figure 2**

**A.** Comparative display of endothelial (EMP), platelet (PMP) and lymphocyte (LMP) levels of circulating microparticles (MPs) and **B.** Levels of endothelial progenitor cells (EPCs) (CD 34+, KDR+, CD 34+/KDR +) after 2 months of either irbesartan treatment (HH-I) or other antihypertensive treatment (HH) and healthy subjects – control subjects (C).

*Levels of circulating EPCs*

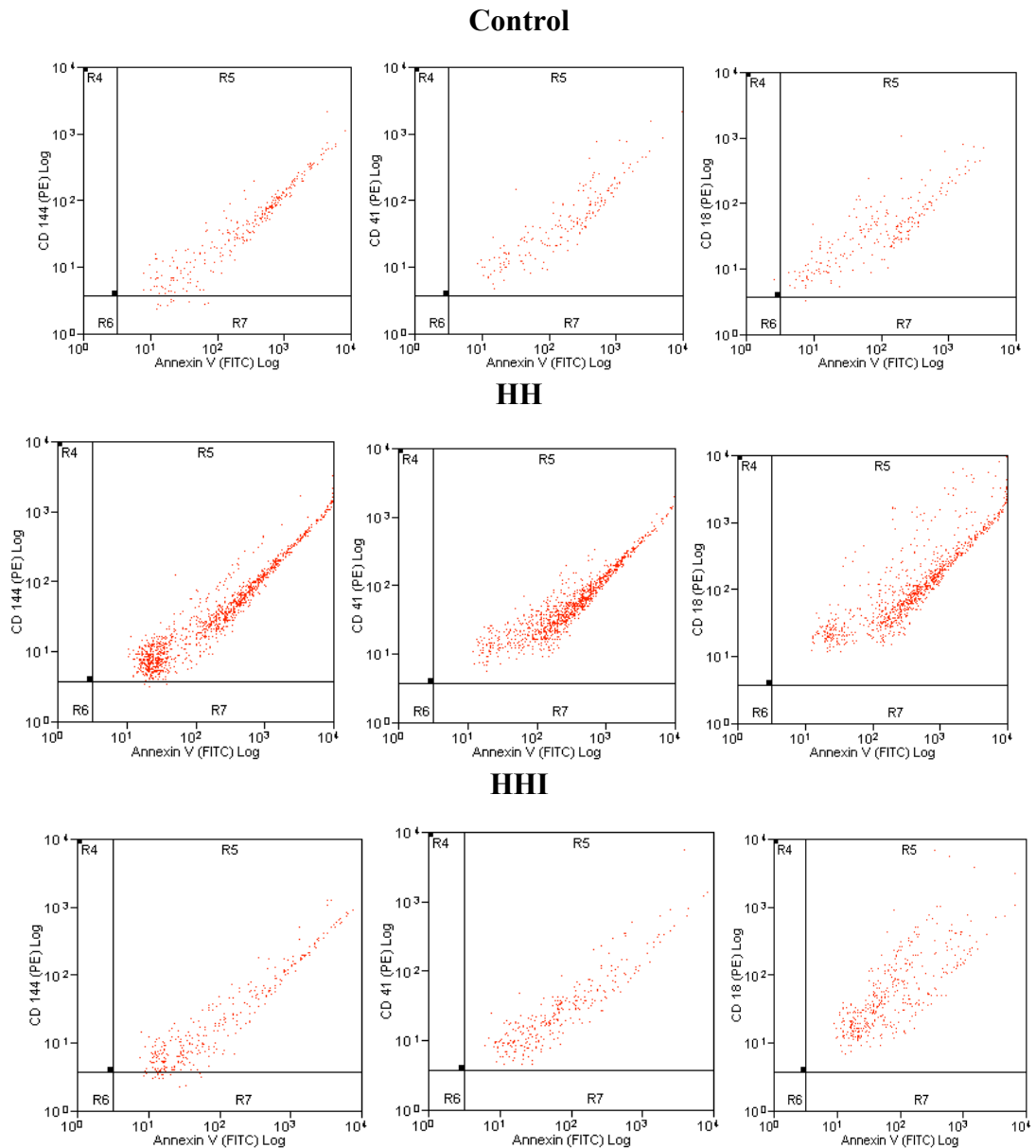
There were no significant differences at baseline in EPC levels between HH and HH-I groups but they were significantly lower when compared with the control group as shown in figure 1.B. After 2 months of treatment, EPC levels (CD34+/KDR+) were significantly higher in the HH-I group than HH-I but still lower than in the C group (Figure 2.B).

Representative measurements of EPCs and MP by flow cytometry in the three study groups are displayed in figure 3 and figure 4, respectively.



**Figure 3**

The specific identification of endothelial progenitor cells (EPCs) isolated from the three study groups.

**Figure 4**

The specific identification of microparticles (MPs) in the three study groups. Endothelial microparticles (EMPs) were labelled with CD144-PE and Annexin V-FITC, platelet MPs were labeled with CD41 and Annexin V – FITC, and leukocyte microparticles (LMPs) were labeled with CD18 and Annexin V -FITC in the dark for 40 min at room temperature. Irbesartan treatment - HH-I, hypertensive hypercholesterolemic patients - HH and healthy subjects – control subjects -C.

It has been shown that ARBs positively affect endothelial function, beyond the antihypertensive action displayed by these compounds. One of the mechanisms proposed to be involved in endothelial protection is the effect of ARBs in increasing the levels of circulating EPC. The current study shows the positive effect of angiotensin receptor blockade on two proposed markers of endothelial function, reflecting both endothelial injury and repair, MPs and EPC respectively, in the same subjects with cardiovascular (CV) risk factors. Moreover, we found that patients with hypertension and dyslipidemia display an altered balance between the levels of endothelial regenerative cells like EPC and markers of endothelial injury like circulating MPs.

Several studies have suggested the association between CV risk factors and the levels of EPC. Moreover, diabetes mellitus, one of the most important CV risk factors, has also been shown to adversely affect EPC number and function and a reduction in the number of EPCs might be useful as a surrogate marker of vascular dysfunction in diabetes as suggested by some authors [12]. Furthermore, levels of circulating MPs have been found to be increased in patients with vascular disease and are now considered markers of endothelial damage [1,2]. These findings have been confirmed by the present study, showing that, in the same patients with hypertension and dyslipidemia, the levels of EPC are significantly lower and circulating MPs are significantly higher than in healthy people. This inverse relationship between these two biomarkers reflecting endothelial injury and repair could be further exploited as a more sensitive indicator of vascular health as proposed before in animal studies [11].

Considering the balance between markers of injury and repair of the endothelium, the most interesting question is whether it can be influenced by therapeutic interventions and several studies have focused their attention on the effect of different drugs on one of the arms of the balance. For instance, it has been now shown that three classes of drugs can increase the number of EPC, namely statins [21], recombinant human erythropoietin [15] and ARBs [3]. Some ARBs have been evaluated in small studies in this regard and it has been suggested that their pleiotropic effects, not related to their main action, i.e. the BP lowering effect, could be related to the increased number of circulating EPCs after treatment. In this regard we have to point out that probably, the effect of irbesartan on both EPCs and MPs was not related to its main action as the magnitude of BP lowering was similar in both of the hypertensive hypercholesterolemic groups, regardless of the use of irbesartan or other antihypertensive drugs as ARB or ACE inhibitors.

Interestingly, as opposite to other studies, levels of EPCs after 2 months of treatment with irbesartan remained lower than those in the healthy subjects. The same pattern was observed in the levels of MPs, which remained above normal even if a significant decrease of their levels was observed after 8 weeks of irbesartan. It is possible that the shorter treatment period, only 8 weeks as compared with other similar studies, didn't allow the assessment of the full effect of irbesartan on EPC levels. Even so, it is not clear whether angiotensin receptor blockade could restore and maintain the normal levels of EPC as there is no data from longer follow up studies. Moreover, not only the duration of treatment but also the CV risk profile of the study group could have an influence on the magnitude of ARB's effect on these circulating biomarkers. Our study patients had also high serum cholesterol levels and no specific treatment was administered as it has been proven that statins have also an effect on EPC levels [21]. As such, it is possible that adding a statin to the antihypertensive therapy could normalize the levels of EPC in this type of patients [24]. Nonetheless, even if hypothetically the higher the level of progenitor cells the higher the regenerative capacity of the endothelium, no study has yet proven a clear difference in the outcome between patients with different levels of EPC and MPs.

But MPs are not merely markers of endothelial function. They are also considered to play a major biological role in inflammation, vascular injury, angiogenesis, and thrombosis as they have been reported to impair angiogenesis, promote oxidative stress, and impair vasorelaxation, and they themselves produce reactive oxygen species (ROS) [6,7]. Taking this into account and considering the known reparatory capacity of EPC, the effect of ARB on both these markers and effectors of endothelial function is of important therapeutic relevance and adds to our understanding of its pleiotropic action.

It is important to point out that as opposed to other studies that have focused their attention mainly on EMP, in this study we have assessed also the less studied PMPs and LMPs, with all three of them displaying the same response pattern to ARB therapy.

The link between EPCs, MPs and vascular function has been already proven in several studies and the ancillary effects of ARBs include a positive action on endothelial function [1,2,17,25]. In our patients we have shown that irbesartan can modify not only EPC and MP levels but also that this effect is reflected in the increase of RHI as an indirect indicator of endothelial function. Even if parameters like IMT and ABI were also

assessed in this study, no significant differences were expected as the follow up period was too short to allow morphological changes in big vessels.

### Conclusions

Standard therapy with angiotensin receptor blockers has a positive effect on two proposed markers of endothelial function, MPs and EPCs, improving both endothelial injury and repair in patients with hypertension and dyslipidemia. This effect of ARBs is yet another argument that helps tailoring with drug selection in cardiovascular preventive treatment.

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