Effect of pravastatin on endothelial function and endothelial progenitor cells in healthy postmenopausal women

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Summary

Purpose: Coronary heart disease is the leading cause of morbidity and mortality in postmenopausal women. Among statins, pravastatin has been shown to significantly reduce fatal and non-fatal cardiovascular events in primary and secondary prevention trials. The aim of the present research was to investigate whether treatment with pravastatin can modify some indices of cardiovascular risk in healthy postmenopausal women such as significant reductions in total and LDL cholesterol and triglyceride levels. *Methods:* 20 patients were randomized in double-blind fashion to treatment for eight weeks with either pravastatin 40 mg/day or placebo, and subsequently, after one-week wash-out, crossed-over to the alternative treatment (placebo or pravastatin) for the following eight weeks. We performed clinical and laboratory investigations, before and at the end of each treatment period, to evaluate patient response to the treatment with pravastatin. *Results:* After eight weeks pravastatin therapy reduced the median low density lipoprotein (LDL) and total cholesterol ($p < 0.01$ in both cases). In contrast, insulin level and insulin sensitivity did not show any difference with regard to values observed after placebo treatment. The absolute number of endothelial progenitor cells-colony forming unit (EPC-CFU) was significantly increased by pravastatin treatment (30.6% increase, $p < 0.05$) and the number of senescent cells was significantly decreased. However pravastatin did not increase tube-like formation by EPC and did not improve endothelial function. *Conclusions:* Despite beneficial effect on lipids and EPC, short term pravastatin does not seem to improve other cardiovascular risk factors, at least in healthy postmenopausal women.

Key words: Menopause; Endothelial function; Pravastatin; Endothelial progenitor cells; Cardiovascular risk factors; Insulin resistance.

Introduction

Coronary heart disease (CHD) represents one of the main causes of death in Western countries [1]. In particular CHD is the leading cause of morbidity and mortality among postmenopausal women [2]. Women have much less coronary atherosclerosis than men, especially those in younger age groups. The lower coronary atherosclerosis is likely a function of both lower premenopausal risk factors and the effects of estrogen on the arterial wall [3]. The beneficial effects of statins in treating hypercholesterolemic subjects have been well established. Among statins, pravastatin has been shown to significantly reduce fatal and non-fatal cardiovascular events in primary and secondary prevention trials [4, 5]. Accumulating evidence indicates that the cardiovascular protection of pravastatin therapy is not confined solely to its anti-lipemic effects. Other potential effects such as inhibition of inflammation and improvement of insulin sensitivity have also been suggested [6]. It is well known that long-term therapy with pravastatin might reduce levels of CRP and prevent cardiovascular risk in high-risk subjects [7]. In contrast, the effect of statins on insulin resistance is controversial and poorly studied in non diabetic subjects [8]. Pravastatin not only reduces serum lipids, but also improves the glucose metabolism, including insulin resistance, in dyslipidemic patients [9]. Pravastatin use may be an effective approach in the treatment of metabolic syndrome with impaired glucose tolerance (IGT) by its advantageous effects on insulin resistance [10]. The question of whether therapy with statins decreases cardiovascular-related mortality rates along with a better quality of life in postmenopausal women remains to be investigated [11]. According to recent data [12, 13], cardiovascular risk factors as age, sex, hypertension, diabetes, smoking, positive family history of CHD and LDL cholesterol levels, are inversely related to the number of circulating endothelial progenitor cells (EPCs). EPCs may contribute to repair areas of initial vascular damage caused by these risk factors [12, 13]. There is evidence that chronic exposure to increased plasma cholesterol levels might also oppose the repair of lipoprotein-mediated endothelial injury, possibly by reducing the availability and function of circulating endothelial progenitors [14]. The number of circulating EPC can be assessed in vitro, either as number of colony formations [13] or by functional assays [15]. Statins are known to increase circulating EPC in patients with stable coronary artery disease [16], as well as restore endothelial function [17], and prevent ischemic vascular disease [18, 19], and diabetes [20]. Statins, therefore, could be useful in the primary prevention of vascular disease in postmenopausal women [21], a population at increased cardiovascular risk. The aim of the

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present study was to investigate whether treatment with pravastatin could modify some indices of cardiovascular risk in healthy postmenopausal women. To this end we evaluated, in a double-blind, placebo-controlled, randomized, cross-over study, the effect of pravastatin on endothelial function, insulin-resistance, lipid profile, inflammatory response and number and function of circulating EPCs.

Materials and Methods

Study design

Informed consent was obtained from each subject before the study. The subjects were randomized in double-blind fashion to treatment for eight weeks with either pravastatin 40 mg/day or placebo, and after one-week wash-out, crossed-over to the alternative treatment (placebo or pravastatin) for eight weeks. Clinical and laboratory investigations were performed before and at the end of each treatment period. At each time-point a 50 ml blood sample was used for the biochemical and functional evaluations outlined below. The study was conducted at the Department of Obstetrics and Gynecology at the Catholic University in Rome.

Subjects

We selected 25 patients for the study in our Divisional outpatient menopause center. Of these, 20 patients that fulfilled the following inclusion criteria: spontaneous menopause for a period of ≥ 1 year, body mass index ≤ 30 kg/m^2, and serum LDL cholesterol < 190 mg/dl participated to the study. None of these patients had serum LDL cholesterol > 160 mg/dl or > 2 risk factors (as defined by NCEP III guidelines) [22]; neoplastic diseases, surgical menopause, past or current hormone replacement therapy, ischemic vascular disease, diabetes mellitus, liver, renal or respiratory insufficiency, treatment with antidepressant drugs, ACE-inhibitors, AT-receptor antagonists, beta-blockers, calcium-antagonists and non-steroidal anti-inflammatory drugs.

Analytical methods

Measurement of endothelial function

Ultrasound evaluation of endothelium-dependent and endothelium-independent arterial dilatation was performed as follows [23, 24]. Brachial artery diameter was measured by B mode ultrasound image, by the use of a 7.5 Mhz linear array transducer and a standard ESAOTE AU 570 A system. In all patients, scans were obtained with the subject at rest, after inflation of a pneumatic tourniquet placed around the forearm (distal to the scanned part of the artery) to a pressure of 250 mmHg for 4-5 min, and 3-4 min after the administration of sublingual nitroglycerin spray (400 μm). The brachial-artery diameter was expressed as a percentage of the average diameter of the artery in two resting control scans (considered as 100%). The velocity of arterial flow was measured with a pulsed Doppler signal. For the reactive hyperemia scan, measurements of diameter were taken 50–60 sec after deflation of the cuff. The vessel diameter in scans obtained after reactive hyperemia [flow-mediated dilatation (FMD)] and the administration of nitroglycerin (nitrate-induced dilatation NID) was expressed as a percentage of the average diameter of the artery in the two resting (or control) scans (considered 100%). Reactive hyperemia was calculated as the maximal flow recorded in the first 15 sec after cuff deflation divided by the flow during the first resting (baseline) scan.

Measurement of body composition

The bioelectrical impedance was evaluated using a tetrapolar impedance plethysmograph (Soft Tissue Analyzer, Akern Bioresearch, Florence, Italy) to estimate body composition according to Lukaski [25]. The percentage of body fat, fat free mass, and total body water were assessed by specific software (Bodygram, Akern Bioresearch, Italy). The patients, free of any conductive materials, were asked to lie on a bed. The electrodes were placed in the middle of the dorsum of the hands and feet proximal to the metacarpal-phalangeal metatarso-phalangeal joints respectively, and also medially between the distal prominences of the radius and the ulna and between the medial and lateral malleolus at the ankle. An excitation current of 800 mA, AC, at 50 Khz was introduced at the distal electrodes and the voltage drop across the patient was detected by the proximal electrodes.

Biochemical and functional evaluation

• *Serum:* glucose, insulin, total HDL and LDL-cholesterol, triglycerides, creatinine, blood urea nitrogen, AST, ALT, CK, total bilirubin, C-reactive protein. *Whole blood:* full blood count [26]. Plasma glucose levels were measured by the glucose oxidase method (Beckam, USA). Total cholesterol and triglyceride concentrations were determined by an enzymatic assay, high-density lipoprotein cholesterol (HDL-C) concentrations were determined after precipitation of chylomicrons, very-low-density lipoprotein cholesterol (VLDL-C), and low-density lipoprotein cholesterol (LDL-C) (Boehringer, Germany). All blood tests were performed in fasting subjects.

HOMA insulin resistance

$$\text{Insulin resistance (HOMA IR)} = \frac{\text{fasting insulin (μU/ml) x fasting glucose (mmol/l)}}{22.5}$$

Evaluation of circulating EPCs by colony formation

EPCs were isolated from 12 ml of citrate/dextran anticoagulated peripheral blood as follows: peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation (FICOLL, Sigma-Aldrich, USA). Recovered cells were washed twice with phosphate buffered saline (PBS, Sigma) and plated (5 x 10^6 cells/well) on dishes coated with human fibronectin (Sigma) in EndoCult medium (Stem Cell Technologies). After 48h hours, non adherent cells were harvested and replated (1x 106 cells/well) in fibronectin-coated 24-well plates in EndoCult medium. This step removes monocytes and mature endothelial cells. Growth medium was changed every three days. EPCs were characterized by the appearance of a spindle-shaped morphology and their ability to develop CFUs (colony forming units). EPC-CFU were composed of a central core of predominantly spherical cells surrounded by sprouting elongated cells. Numbers of colonies were counted seven days after plating.

Measurement of senescence

In each colony, β-galactosidase activity was evaluated by a commercial Kit (Cell Signalling) designed to histochemically detect β-galactosidase activity at pH 6, a known characteristic of senescent cells. Briefly, the cells were fixed in fixative solu-

Fig. 1

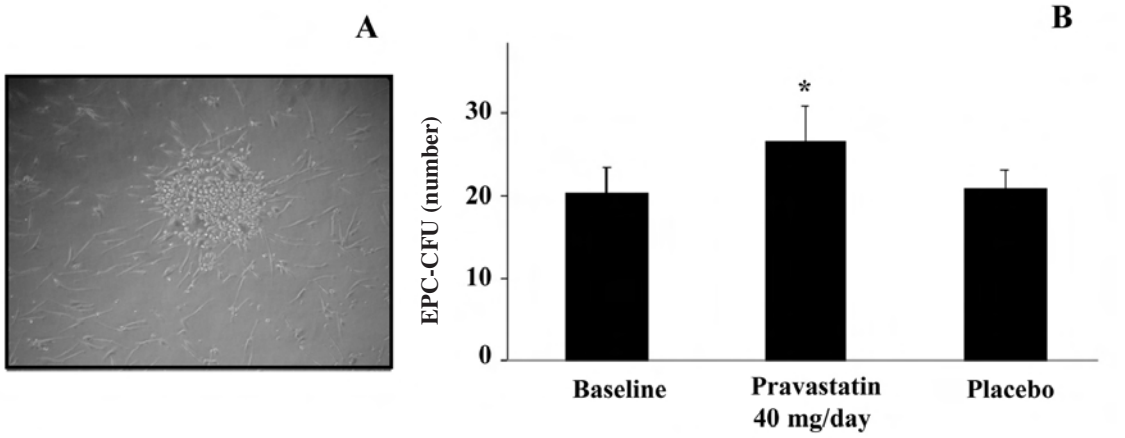

Figure 1. — Effect of treatment with pravastatin on the number, the cellular senescence and in vitro angiogenesis ability of isolated EPCs from healthy postmenopausal women. At day 7 in endothelial specific culture medium, PBMCs developed an elongated spindle-shaped morphology and produced colonies **(A)**. The absolute number of EPC-CFU was significantly affected by treatment with pravastatin (40 mg/day) as compared to baseline or placebo group patients **(B)**.

Fig. 2

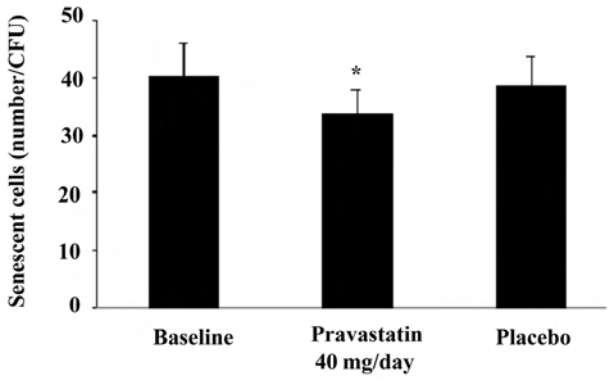

Figure 2. — Senescence evaluation of EPCs by acidic β-galactosidase staining. The number of senescent cells was significantly decreased in EPCs obtained from patients treated with pravastatin with respect to baseline or placebo groups.

tion for 10-15 min at room temperature and incubated with staining solution overnight at 37°C. Senescent cells were checked under an optical microscope (200 x total magnification) for development of blue color. The absolute numbers of β-galactosidase-positive cells were counted out of 100 cells.

Functional evaluation of EPC by tubule assay

An in vitro angiogenesis assay was used to examine the ability of EPCs to differentiate into capillary-like tube structures.

Early EPCs (cells obtained from colonies after 1 week of culture) were labelled with fluorescent Dil-Ac-LDL (2x 104) and co-plated with human umbilical vein cells (HUVEC, 4x 104) in matrigel coated wells (96 well plates) with endothelial cell culture medium (EGM-2) at 37°C for 24 hours. Incorporation of fluorescence-labeled EPC into the tube-like structure formed by HUVEC was examined under a fluorescence microscope (Carl Zeiss).

Late EPCs (cells obtained from colonies cultured for 3 weeks that show a better differentiation capability) [27] were added into matrigel coated wells (96 wells plates) with endothelial cell culture medium (EGM-2, Cambrex), while HUVECs were placed in other wells as controls, and incubated at 37°C for 48-72 hours. The tube formation was observed using an inverted phase optical microscope (Olimpus IX50).

Capillary-like tube structure was defined as a structure exhibiting a length 4 times its width. Images were acquired with a digital camera (Nikon) and quantified by Photoshop software measuring the number and the total length of the tubules in each well.

Statistical analysis

Sample size computation

Sample size was computed in relation to total cholesterol and flow-mediated vasodilatation (FMD). Power was set at 80% and alpha at 0.05. The difference in means was assumed approximately 60 mg/dl and standard deviation (SD) was set at 35 mg/dl for cholesterol level while it was considered respectively 5 and 3% for FMD [24]. In both cases minimal sample size was five pairs.

Statistical analysis

Descriptive statistics were used to evaluate median and interquartile range (IQR: 75th percentile - 25th percentile) of each parameter. Univariate analysis was carried out applying a non parametric test for paired samples (Wilcoxon signed rank test) because of the limited number of patients in each group. The purpose of univariate analysis was to find out differences between the eight weeks of pravastatin treatment and the eight weeks of placebo. The significance level was set at $p \leq 0.05$ and statistical analysis was performed using SPSS version 12.00 for Windows.

Results

Baseline characteristics

As expected by study design, all patients had normal characteristics without any risk factors (Table 1).

Table 1. — *Patient characteristics (n = 20).*

	Median	25th-75th percentile	IQR
Age (years)	57.50	52 - 62	10
Height (cm)	161.00	156 - 168	12
Weight (kg)	69.50	61 - 76	15
BMI	26.65	24 - 30	6
Years from menopause	6.00	3 - 11	8
Systolic pressure (mmHg)	130.00	110 - 140	30
Diastolic pressure (mmHg)	80.00	70 - 90	20
Glycemia (mg/dl)	77.00	69 - 88	9
Insulinemia (mU/l)	6.35	5 - 9	4
HOMA	1.22	1 - 2	1
HDL cholesterol (mg/dl)	66.50	54 - 76	12
LDL cholesterol (mg/dl)	137.00	119 - 163	44
Total cholesterol (mg/dl)	213.50	202 - 252	50
Triglycerides (mg/dl)	104.00	67 - 120	53
Creatinine (mg/dl)	0.90	1 -1	0
Aspartate aminotransferase (AST) (U/l)	21.00	17 - 24	7
Alanine aminotransferase (ALT) (U/l)	21.50	18 - 29	9
Creatine kinase (CK) (U/l)	85.00	62 - 121	39
Total bilirubin (mg/dl)	0.70	1 - 1	0
C-reactive protein (PCR) (mg/dl)	3.00	3 - 4	1
Hemoglobin (Hb) (mg/dl)	13.75	13 - 15	2
Platelets (PLT) (N/ml)	248.00	203 - 274	71
White blood cells (WBC) (N/ml)	6.01	5 - 7	2
Fat mass (FM) (%)	33.15	30 - 40	10
Baseline diameter (mm)	3.35	3 - 4	1
Baseline velocity (cm/s)	4.80	0 - 8	8

Table 2. — *Pravastatin (after 8 weeks) versus placebo (after 8 weeks). Median, interquartile range (IQR), p-value.*

	Placebo		Pravastatin		
	Median	IQR	Median	IQR	*p*
Systolic pressure (mmHg)	120	27	120	20	0.34
Diastolic pressure (mmHg)	80	14	78	15	0.42
Glycemia (mg/dl)	77.00	24.50	81.00	14.25	0.11
Insulinemia (mU/l)	6.35	3.37	7.21	5.28	0.07
HOMA	1.28	0.90	1.51	0.78	0.10
HDL cholesterol (mg/dl)	66.00	17.85	67.00	19.25	0.90
LDL cholesterol (mg/dl)	137.00	51.35	103.50	55.25	**< 0.01**
Total cholesterol (mg/dl)	215.00	64.85	189.00	56.25	**< 0.01**
Triglycerides (mg/dl)	101.00	71.75	94.00	49.25	0.30
Creatinine (mg/dl)	0.85	0.18	0.80	0.10	0.48
AST (U/l)	21.50	6.00	20.50	6.75	0.66
ALT (U/l)	18.00	15.50	21.00	4.75	0.28
CK (U/l)	83.00	60.25	84.00	43.75	0.26
Total bilirubin (mg/dl)	0.60	0.35	0.60	0.20	0.34
PCR (mg/dl)	3.00	1.23	3.00	0.93	0.87
HB (mg/dl)	13.75	1.50	13.50	1.55	0.25
PLT (N/ml)	239.50	59.50	231.50	47.50	0.38
WBC (N/ml)	5.63	1.50	5.95	2.15	0.84
FM (%)	33.00	9.60	36.10	13.90	0.39
Basal diameter (mm)	3.45	0.77	3.45	0.58	0.29
Basal velocity (cm/s)	4.95	7.28	6.20	8.87	0.21
FMD	8.16	8.42	5.33	8.17	0.46
NID	17.95	13.82	15.79	6.06	0.64
Reactive hyperemia	144.28	75.66	152.90	179.51	0.60

FMD: flow-mediated dilatation (see Table 1 for the other abbreviations); NID: nitrate-induced vasodilatation.

Metabolic profile

The results of univariate analysis (Table 2) showed significant differences only in relation to LDL and total cholesterol. In fact in the placebo group, the median LDL and total cholesterol were respectively 137 mg/dl (IQR: 51.35 mg/dl) and 215 mg/dl (IQR: 64.85 mg/dl) while they were 103.50 mg/dl (IQR: 55.25 mg/dl) and 189 mg/dl (IQR: 56.25 mg/dl) after the eight weeks of pravastatin treatment ($p < 0.01$ in both cases). Pravastatin was not responsible for adverse metabolic events in that AST, ALT, and CK did not significantly differ between the two groups. It is interesting to observe that insulinemia and HOMA both showed higher values in patients after treatment: they were 7.21 mU/l (IQR: 5.28 mU/l) and 1.51 (IQR: 0.78) respectively after eight weeks of pravastatin while they were 6.35 mU/l (IQR: 3.37 mU/l) and 1.28 (IQR: 0.90) in the placebo group. However, the differences were not statistically significant.

Endothelial function

As far as endothelial function was concerned, no significant differences were observed in flow-mediated dilatation (FMD) and nitrate-mediated vasodilatation (NID) (Table 2).

Evaluation of circulating EPCs, senescence, and angiogenesis

At day 7 in endothelial specific culture medium, PBMCs developed an elongated spindle-shaped morphology and produced colonies (EPC-Colony Forming Unit, Figure 1A). The absolute number of EPC-CFU was significantly affected by treatment with pravastatin (40 mg/day) as compared to baseline or placebo group patients (30.6% increase, $p < 0.05$, Figure 1B).

The number of senescent cells was significantly decreased in EPCs obtained from patients treated with pravastatin with respect to baseline or placebo groups (Figure 2).

To test the endothelial function of EPCs an in vitro angiogenesis assay was carried out. Early EPCs co-cultured with differentiated endothelial cells (HUVEC) were incorporated into tube-like structures but alone failed to form tubules in matrigel three-dimensional cultures (data not shown). EPCs cultured for 21 days (late EPCs) led to successful capillary formation on matrigel. Nevertheless, there were no significant differences in the number or length of tube-like structures among cells obtained from pravastatin treated patients and the placebo group (Figure 3 A,B).

Discussion

Eight weeks of pravastatin treatment in healthy postmenopausal women was able to reduce serum lipids and increase the count of EPC colonies but did not improve the endothelial function, or insulin sensitivity.

The beneficial effect of pravastatin in preventing cardiovascular disease in peri- and postmenopausal hyperlipidemic women is described in the literature [5]. There

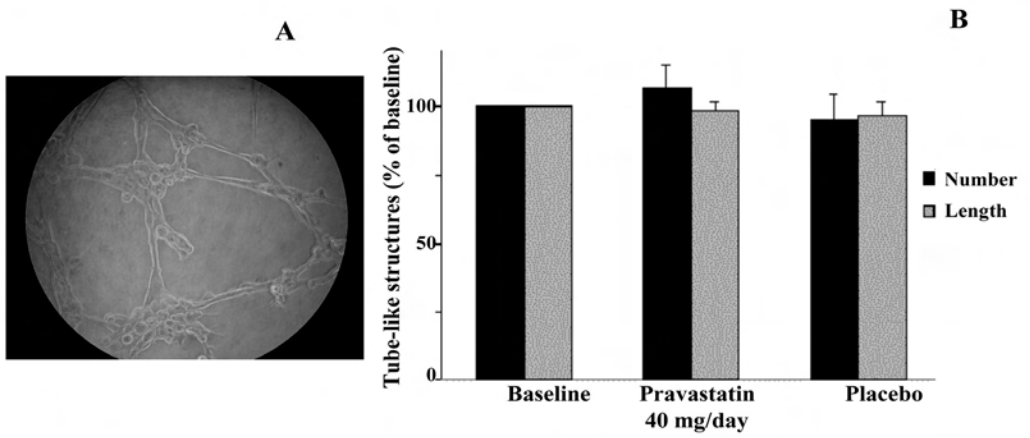

Figure 3. — EPC in vitro angiogenesis assay of EPC. EPCs cultured for 21 days (late EPCs) led to successful capillary formations on matrigel. No significant differences in the number or length of tube-like structures among cells obtained from pravastatin-treated patients and the placebo group were observed (A, B).

is now evidence that this favorable effect is present also in healthy postmenopausal women [8]. As expected, we observed that pravastatin significantly reduced total and LDL cholesterol compared to the placebo treatment.

The effects of statins administration on insulin sensivity have been previously investigated, in particular in diabetic and dyslipidemic patients; within these subjects pravastatin improved glucose metabolism, including insulin resistance. In contrast other studies performed in healthy subjects showed that pravastatin treatment did not affect glucose and insulin levels. In our research, we observed that both insulinemia and HOMA did not change with regard to pravastatin treatment. Thus, it seems that pravastatin does not give beneficial effects on carbohydrate metabolism in healthy postmenopausal women. Similarly, we assessed that blood pressure was not modified after treatment with pravastatin. This finding is consistent with a previous study, showing that pravastatin treatment significantly reduced systolic blood pressure in hypertensive patients but not in normotensive [28]. Regarding the changes in inflammatory markers (CRP and white blood cells), our results showed no significant differences between the two treatments (pravastatin vs placebo). This can be attributed to the short treatment period with pravastatin (only eight weeks). Endothelial function, evaluated in vivo by flow-mediated dilatation (FMD) and nitrate-mediated vasodilatation (NID), did not change with respect to treatment. This result is consistent with those of Davis *et al*., who observed that therapy with pravastatin did not modify FMD in postmenopausal hypercholesterolemic women [29]. In contrast, other authors have demonstrated that the pravastatin administration improved FMD within ten days and this favourable effect occurred before any significant reduction in blood lipids in patients with unstable angina [30].

Increasing evidence indicates that the integrity and functional activity of the endothelial monolayer play an important role in global cardiovascular health. The traditional view of endothelial cell repair mediated exclusively by the adjacent endothelial cells has been changed by the discovery of EPCs, bone marrow derived cells, able repair the sites of endothelial injury and ischemia, through proliferation, differentiation and integration into the endothelial layer [31].

Aging may constitute a potential limitation both to EPC mobilization from bone marrow and to EPC ability to sustain repair of ischemic tissue. In fact Scheubel *et al*. have demonstrated that aging leads to a reduction in VEGF concentration, which might limit the mobilization and survival/differentiation of EPC. After menopause, EPC reduction attributable to aging, to change in the reproductive state characterized by estrogen withdrawal and to the worsened risk profile, may cause endothelial dysfunction and predispose to cardiovascular events [32].

In our study we showed that pravastatin is able to stimulate EPC colony formation and to delay the onset of EPC senescence in healthy postmenopausal women. These two mechanisms may contribute to increase EPC bioavailability in postmenopausal women. On the other hand, through in vitro angiogenesis assay, we could not observe an increase in tube-like formations by EPCs, obtained from postmenopausal women treated with pravastatin. Presumably more than one agent might directly influence the mobilization or half-life of EPCs and the mechanism through which pravastatin acts still remains to be determined. Previous studies aimed to evaluate the effect of other statins on EPCs. Dimmeler *et al*. hypothesized that statins were able to increase EPCs by the PI 3-kinase/Akt pathway [33]. Spadaccio *et al*. in a randomized double-bind, placebo-controlled two-way cross-over trial in 50 patients undergoing elective coronary surgery, observed that three weeks of treatment with atorvastatin increased levels of EPCs in comparison with a placebo group but on the other hand this statin did not affect the levels of VEGF [34]. Matsumura *et al*. in a study, using ischemic hindlimbs of rats, showed that treatment with low-dose of atorvastatin increased signif-

icantly the regional blood flow and induced a parallel increase both in EPC colony formation and in proangiogenic factors such as VEGF, IL-8, Ang-1, Ang-2, ENOS and HO-1 [35].

A possible explanation of our data is that our study group included middle-age subjects without evidence of any vascular disease with conventional diagnostic tools; however we cannot rule out whether these subjects had any other subclinical alterations. It is presumable that reduced levels of VEGF typical of the postmenopausal period represent an important factor contributing to reduced responsiveness to pravastatin stimulus. Moreover it is possible that the duration of pravastatin treatment could be sufficient to stimulate EPC colony formation but not so effective to induce a modification in the functional capacity of EPC in tube-like structure formations and in FMD results.

In conclusion in healthy postmenopausal women eight weeks of treatment with pravastatin effectively reduced serum lipids, increased the count of EPC colonies but did not improve the endothelial function or insulin sensitivity. Thus pravastatin treatment (Pravaselect 40 mg) did not influence the latter cardiovascular risk factors in these subjects, at least after a short treatment period. Further studies are needed to better evaluate the potential beneficial effect of pravastatin as a tool to reduce cardiovascular risk in healthy postmenopausal women.

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