International Journal of Cardiology 170 (2013) 140–145

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Bergamot polyphenolic fraction enhances rosuvastatin-induced effect on LDL-cholesterol, LOX-1 expression and protein kinase B phosphorylation in patients with hyperlipidemia

Micaela Gliozzi [a,1], Ross Walker [a,1], Saverio Muscoli [b,1], Cristiana Vitale [c,1], Santo Gratteri [a,1], Cristina Carresi [a,1], Vincenzo Musolino [a,1], Vanessa Russo [a,1], Elzbieta Janda [a,1], Salvatore Ragusa [a,1], Antonio Aloe [a,1], Ernesto Palma [a,1], Carolina Muscoli [a,1], Franco Romeo [b,1], Vincenzo Mollace [a,c,*,1]

[a] Research Centre for Food Safety & Health (IRC-FSH), University "Magna Graecia", Catanzaro, Italy
[b] Department of Medicine, Chair of Cardiology, University of Rome Tor Vergata, Rome, Italy
[c] IRCCS San Raffaele, Rome, Italy

ARTICLE INFO

Article history:
Received 2 April 2013
Received in revised form 3 August 2013
Accepted 30 August 2013
Available online 8 September 2013

Keywords:
Hyperlipidemia
Cardiometabolic risk
Bergamot polyphenolic fraction
Rosuvastatin

ABSTRACT

Background: Statins are the most commonly prescribed drugs to reduce cardiometabolic risk. Besides the well-known efficacy of such compounds in both preventing and treating cardiometabolic disorders, some patients experience statin-induced side effects. We hypothesize that the use of natural bergamot-derived polyphenols may allow patients undergoing statin treatment to reduce effective doses while achieving target lipid values. The aim of the present study is to investigate the occurrence of an enhanced effect of bergamot-derived polyphenolic fraction (BPF) on rosuvastatin-induced hypolipidemic and vasoprotective response in patients with mixed hyperlipidemia.

Methods: A prospective, open-label, parallel group, placebo-controlled study on 77 patients with elevated serum LDL-C and triglycerides was designed. Patients were randomly assigned to a control group receiving placebo ($n = 15$), two groups receiving orally administered rosuvastatin (10 and 20 mg/daily for 30 days; $n = 16$ for each group), a group receiving BPF alone orally (1000 mg/daily for 30 days; $n = 15$) and a group receiving BPF (1000 mg/daily given orally) plus rosuvastatin (10 mg/daily for 30 days; $n = 15$).

Results: Both doses of rosuvastatin and BPF reduced total cholesterol, LDL-C, the LDL-C/HDL-C ratio and urinary mevalonate in hyperlipidemic patients, compared to control group. The cholesterol lowering effect was accompanied by reductions of malondialdehyde, oxyLDL receptor LOX-1 and phosphoPKB, which are all biomarkers of oxidative vascular damage, in peripheral polymorphonuclear cells.

Conclusions: Addition of BPF to rosuvastatin significantly enhanced rosuvastatin-induced effect on serum lipemic profile compared to rosuvastatin alone. This lipid-lowering effect was associated with significant reductions of biomarkers used for detecting oxidative vascular damage, suggesting a multi-action enhanced potential for BPF in patients on statin therapy.

1. Introduction

Dyslipidemia (or hypercholesterolemia) is an important risk factor for the development of atherosclerosis and coronary artery disease, as shown in several lipid intervention trials and prospective studies [1]. Increased concentrations of low-density lipoprotein cholesterol (LDL-C), total blood cholesterol (TC) and triglycerides (TG) comprise the main pathogenic risk profile. Conditions of insulin resistance such as impaired glucose tolerance or "prediabetes" are also associated with a high risk of cardiovascular disease (CVD) and are often accompanied by low levels of high-density lipoprotein cholesterol (HDL-C) [2]. The majority of therapeutic protocols rely on drugs that belong to the "statin" family. Statins inhibit the activity of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, which catalyzes the rate-limiting step in mevalonate biosynthesis, a key intermediate in cholesterol metabolism. A meta-analysis of placebo-controlled "standard dose" statin trials shows a reduction in cardiovascular mortality averaging 20% and a decrease in major cardiovascular events by approximately 25% [3]. Treatment with high-dose statins was shown to reduce the morbidity by 36%, and a reduction of cardiovascular

* Corresponding author at: Faculty of Pharmacy, Department of Health Sciences, University "Magna Graecia", Complesso Ninì Barbieri, Roccelletta di Borgia, 88100 Catanzaro, Italy. Tel.: +39 0961 369 4128, +39 0961 369 5732; fax: +39 0961 369 4237.
E-mail address: mollace@unicz.it (V. Mollace).
[1] Each author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

0167-5273/$ – see front matter © 2013 Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.ijcard.2013.08.125

events up to 40% [4]. Despite the significant clinical benefits provided by statins, many patients, in particular those with diabetes or metabolic syndrome, do not achieve their recommended low-density and high-density lipoprotein (LDL-C, HDL-C) cholesterol target goals with statins alone [5]. Moreover, statins have been reported to cause dose-related side effects, the more serious including liver disease or severe myopathy, in up to 22% of patients eligible for this therapeutic approach [6,7]. This limits the use of statins and suggests the need for alternative and/or supplementary therapeutic approaches.

Experimental and epidemiological evidence suggests that dietary polyphenols, in particular flavonoids, may play a role in ameliorating atherosclerosis, due to a pleiotropic anti-oxidative and anti-inflammatory effect proposed as underlying mechanisms [8–11]. In support of this hypothesis, it has been shown that certain plant polyphenols, such as nobiletin and poncirin, inhibit monocyte activation *in vitro* [9,12–17], and the enhancing effects of such components may play a central role in this beneficial effect. This observation provides the rationale for the optimal use of appropriate, evidence-based phytochemicals as an adjunct to treat hyperlipidemia with the aim of preventing cardiovascular events.

Bergamot (*Citrus bergamia*) is an endemic plant growing in the Calabrian region of Southern Italy with a unique profile of flavonoid and glycosides present in its juice and albedo, such as neoeriocitrin, neohesperidin, naringin, rutin, neodesmin, rhoifolin and poncirin. Bergamot differs from other citrus fruits, not only for the composition, but also for the particularly high content of flavonoids [18,19]. Some of these flavonoids, such as naringin (which is also present in grapefruit), have already been shown to be active in animal models of atherosclerosis [20,21], while neoeriocitrin and rutin have been found to exhibit a strong capacity to inhibit LDL oxidation [22]. Importantly, bergamot juice is rich in 3-hydroxy-3-methylglutaryl neohesperidosides of hesperetin (brutieridine) and naringenin (melitidine) with an ability to inhibit HMG-CoA reductase [23]. These compounds are likely to contribute to the substantial hypolipidemic effects of bergamot juice and to the vasoprotective effects of bergamot oil derivatives in rats [24,25]. However, no clinical study has been performed to date to evaluate the fruit's potential dyslipidemia-correcting properties.

Recently, we found that bergamot-derived polyphenolic fraction (BPF), given orally both in animal models of diet-induced hyperlipidemia and in patients suffering from metabolic syndrome, produces a significant and sustained reduction of serum cholesterol, triglycerides and glycaemia [26]. This effect was accompanied by a significant improvement in vascular reactivity in patients with both hyperlipidemia and elevated serum glucose, thus suggesting a potential protective role for the use of BPF in patients with metabolic syndrome and cardiometabolic risk.

Apart from these results, no evidence has been collected to date, on the mechanism of action of bergamot-derived extract and on its potential benefit when used in comparison with statins in patients with metabolic syndrome.

To address these issues, we conducted a prospective, open-label, parallel group, placebo-controlled study on patients with elevated serum LDL-C and triglycerides, to investigate the effect of BPF in modulating serum lipidemic profile in patients suffering from mixed hyperlipidemia, either untreated or treated with rosuvastatin. The effect of rosuvastatin with BPF on the expression of biomarkers of vascular oxidative stress and circulating cell viability was also studied in these patients. In particular, we examined the accumulation of malondialdehyde (MDA), a marker of lipid peroxidation, the expression of LOX-1 receptor for oxidized LDL and protein kinase B (PKB) phosphorylation in peripheral mononuclear cells (PMC) in patients taking BPF alone or in combination with rosuvastatin treatment.

2. Materials and methods

2.1. Plant material

C. bergamia Risso & Poiteau fruits were collected from plants located in a range of 90 km from Bianco to Reggio Calabria, Italy.

2.2. Preparation of BPF

Bergamot juice was obtained from peeled-off fruits by squeezing. The juice was oil fraction-depleted by stripping, clarified by ultra-filtration and loaded on to a suitable polystyrene resin column able to absorb polyphenol compounds of molecular weight between 300 and 600 Da (Mitsubishi). Polyphenol fractions were eluted by a 1 mM KOH solution. The basic eluate was incubated at a rocking platform to reduce the fumocumarin content. The shaking time was adjusted proportionally to the amount of fumocumarin contaminants. Next, the phytocomplex derived from the defurocoumarinization process was neutralized by filtration on cationic resin at acidic pH. Finally it was vacuum dried and minced to the desired particle size to obtain BPF powder. BPF powder was analyzed for flavonoid, furocumarin and other polyphenol content. In addition, all toxicological analyses were performed, including heavy metal, pesticide, phthalate and sinephrine content which revealed the absence of known toxic compounds at significant levels (data not shown). Standard microbiological test showed that the final BPF was free of mycotoxins and contaminating bacteria.

Finally, 500 mg aliquots of the BPF powder supplemented with 50 mg of ascorbic acid as antioxidant were encapsulated into suitable gelatin capsules by a semi-automatic gelatin encapsulation device employing an authorized pharmaceutical manufacturer (Plants, Messina, Italy). All procedures have been performed according to Good Manufacture Practice (GMP) headlines of European Legislation.

The main flavonoids identified in BPF were neoeriocitrin (370 ppm), naringin (520 ppm), and neohesperidin (310 ppm). Tablets containing 1000 mg of maltodestrin supplemented with 50 mg ascorbic acid were used as placebo.

2.3. Patient selection and study design

A prospective, open-label, parallel group, placebo-controlled study was designed. We enrolled 77 patients suffering from mixed hypercholesterolemia (LDL-C levels of >160 mg/dl, and triglycerides levels >225 mg/dl) from a primary care setting at the Department of Cardiology of the IRCCS San Raffaele– Rome and at the Atherosclerosis Prevention Ambulatory centre at the University of Rome "Tor Vergata" facility. Age, gender, and body mass index were matched among all subjects. Patients were randomly assigned to five groups, all following a low fat diet: a control group receiving placebo ($n = 15$), two groups receiving oral rosuvastatin (10 and 20 mg/daily for 30 days; $n = 16$ for each group), a group receiving BPF alone orally (1000 mg/daily for 30 days; $n = 15$) and a group receiving BPF (1000 mg/daily given orally) plus rosuvastatin (10 mg/daily for 30 days; $n = 15$). In all groups, we determined the efficacy of each treatment by monitoring serum lipid profiles and urinary mevalonic acid concentration (expressing inhibition of HMG-CoA reductase).

We excluded patients with overt liver disease, chronic renal failure, hypothyroidism, myopathy, uncontrolled diabetes, severe hypertension, stroke, acute coronary events within the preceding 30 days, coronary revascularization within the preceding 3 months, or alcohol abuse. No patient was taking hormone replacement therapy or antioxidant vitamin supplements during the 2 months preceding our study.

All the patients were given placebo, rosuvastatin, BPF alone or BPF plus rosuvastatin before meals daily during a 30 day treatment period. A research nurse counted pills at the end of treatment to monitor compliance. The patients were seen at least weekly during the study. To minimize side effects, we measured serum asparate aminotransferase, alanine aminotransferase, creatine kinase, blood urea nitrogen, creatinine and blood cell counts before and after therapy. Seven (7) patients taking placebo and 16 patients taking BPF and/or rosuvastatin were smokers. Two (2) patients taking placebo, and 13 patients taking BPF alone and/or rosuvastatin were also taking ACE inhibitors, angiotensin AT1 receptor blockers or beta adrenergic blockers to control blood pressure. No additional medications including aspirin or nonsteroidal anti-inflammatory drugs were allowed during the study period to avoid confounding effects of other drugs. Anti-hypertensive drugs were withheld for ≥48 h before starting the study. Blood samples for assessing lipid profile and 24 h urinary collection were performed at day 0 and after 30 day treatment in placebo, rosuvastatin and rosuvastatin/BPF-treated patients. At the same time, blood samples were collected for assessing baseline values for blood cell counts, transaminases, creatinine blood levels and PMN separation and culturing.

All patients participating in the study signed an informed consent according to the European Legislation and the protocol was previously submitted and approved by the Regional Ethical Committee. In addition, the study protocol was performed according to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research committee.

2.4. Serum lipids

Total cholesterol, LDL-C, HDL-C and triglycerides in serum samples of patients were determined colorimetrically and enzymatically using commercial assay kits (BioSystems S.A., Barcelona, Spain).

2.5. Urinary mevalonic acid detection

Twenty-four-hour urine samples were collected from each patient before and after treatment with placebo, supplement and/or drugs. Total volume was recorded, and aliquots were frozen at −20 °C. Frozen urine samples were thawed and centrifuged at 1200 *g* for 30 min to remove solids. Urinary mevalonic acid (MVA) concentrations were determined by means of a modification of the radioenzymatic isotope-dilution method of Popjak (26). The intra-subject coefficient of variation was evaluated; 90% of all values were found to lie within 35% of a single 24-h determination, whereas for two determinations, the variation was 25%. The intra-assay coefficient of variation in each run was less than 5%, whereas the inter-assay variation, based on the repeated analysis of the same standard over a 2-year period, was 3.9% (25). The completeness of urine samples was determined on the basis of subjects' records and their careful questioning; if these were deemed incomplete, a repeated collection was obtained.

2.6. Mononuclear cell isolation

Twenty milliliter of heparinised blood was diluted with the same volume of PBS and carefully layered over 6 ml of Ficoll-Paque Plus (Amersham Biosciences, USA). The mixture was centrifuged at 400 ×*g* for 40 min at 18–20 °C. The interface containing peripheral blood mononuclear cells (PMNs) was collected and washed two times with PBS. This preparation contained approximately 25% monocytes and 75% lymphocytes. Further purification of the monocytes from the PMNs fraction was accomplished by allowing the monocytes to adhere to plastic. Using 25 cm^2 culture dishes, PMNs were cultured for 45 min in RPMI 1640 medium (with glutamax-I, Dutch modification, Gibco BRL) containing 10% pooled human serum. The nonadhering lymphocytes were removed by washing the culture dishes with PBS, after which the adherent monocytes were harvested. The reagents used for the isolation of monocytes contained no detectable endotoxins, as determined by the E-Toxate assay (Sigma, St. Louis, USA).

2.7. Cell extracts and SDS-PAGE Western blotting

In order to study phosphor-PKB expression, PMNs were lysed with ice-cold lysis buffer (20 mM Tris–HCl, pH 7.5; 150 mM NaCl; 2 mM EDTA; 2 mM EGTA; 1% Triton X-100; 1 nM okadaic acid, protease inhibitor cocktail - Sigma; phosphatase inhibitor cocktail — Calbiochem), centrifuged at 10,000 ×*g* for 15 min at +4 °C. Alternatively, for LOX-1 detection, PMNs were lysed with a different ice-cold lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1% Nonidet P-40; 2 mM Pefablock, 1 microM ml^{-1} Aprotinin, 10 microM ml^{-1} Leupepeptin, 2 microM ml^{-1} Pepstatin A, trypsin inhibitor), centrifuged at 13,000 ×*g* for 30 min at +4 °C. Protein concentrations in supernatants were determined by standard Bradford assay.

Lysates from each treatment (30 micrograms/lane) were resolved by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After incubation in blocking solution (4% dry nonfat milk, Sigma–Aldrich, St. Louis, MO), membranes were incubated with anti-LOX-1 (1:10,000; gift from Professor T. Sawamura, Osaka, Japan); anti-phospho-PKB (Ser 473) (1:2000; Cell Signaling Technology, USA). Membranes were rinsed and then incubated with anti-mouse/anti-rabbit IgG antibody (GE Healthcare, Buckinghamshire, England) for 1 h at room temperature. The specific complex was detected by an enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire, England) and relative intensities of protein bands were analyzed by MSF-300G scanner (Microtek International, Inc., Hsinchu, Taiwan). Membranes were stripped with restore western blot stripping reagent following the manufacturer's instructions (Pierce Chemical, Rockford, IL) and then blocked (1 h at room temperature) with blocking solution and incubated with mouse monoclonal anti-alpha-actin (1:1000 dilution; Sigma) or anti-PKB (1:2000; Cell Signaling Technology, USA). After rinsing, the membranes were incubated with antimouse horseradish peroxidase-conjugated secondary antibody (1:20,000 dilution; GE Healthcare, Buckinghamshire, England) and the specific complex was detected by the enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire, England). The relative intensities of protein bands were analyzed by MSF-300G scanner.

2.8. Malondialdehyde determinations

Malondialdehyde (MDA), a reliable biomarker used for studying lipid peroxidation, was measured in PMNs of hyperlipemic patients either treated or not with BPF, rosuvastatin or their combination. Levels of MDA were measured in PMNs isolated from blood samples collected at the day 0 and 30 of treatment. Briefly, cells were homogenized in potassium chloride (1.15%) and frozen in liquid nitrogen. Chloroform (2 ml) was then added to each homogenate and then spun for 30 min.

The organic layer of the sample was removed and dried under nitrogen gas and reconstituted with 100 μl of saline. MDA generation was evaluated by the assay of thiobarbituric acid (TBA)-reacting compounds. The addition of a solution of 20 μl of sodium dodecyl sulphate (SDS; 8.1%), 150 μl of 20% acetic acid solution (pH3.5), 150 μl l of 0.8% TBA and 400 μl l of distilled water, produced a chromogenic product which was extracted in n-butanol and pyridine. Then, the organic layer was removed and MDA levels read at 532 nm and expressed as nmol MDA/g prot.

2.9. Statistical analyses

Bartlett's test was performed on each set of data to ensure that variance of the sets was homogenous. In case of homogenous set of data, ANOVA was performed to determine the treatment effects, and Dunnett's test was employed as appropriate. In case of heterogenous data, F test was carried out to determine which pairs of groups were heterogenous. This was followed by Student's *t* tests.

3. Results

3.1. The effect of BPF and/or plus rosuvastatin on serum lipids of patients with mixed hyperlipidemia

Oral administration of BPF (1000 mg/daily for 30 consecutive days; $n = 15$) in patients suffering from mixed hyperlipidemia significantly reduced total cholesterol, LDL-C, triglyceride and enhanced HDL-C levels (Table 1). No change in serum total cholesterol, LDL-C, HDL-C and triglycerides was found in the group of patients receiving placebo compared to basal lipidemic profile (Table 1).

Neither symptoms nor hematochemical signs of toxicity were found in any patients undergoing BPF and/or placebo administration (not shown). In 2 patients treated with BPF/daily, a moderate gastric pyrosis was observed. However, none of the patients taking BPF interrupted the treatment.

Administration of rosuvastatin (10 and 20 mg/daily; $n = 16$ for each group; Table 1) produced a significant ($P < 0.05$) reduction of total cholesterol and LDL-C compared to placebo. Minimal and insignificant changes in triglycerides occurred after treating patients with rosuvastatin at both doses daily. Furthermore, an elevation of HDL-C was found.

Association of BPF (1000 mg/daily) with the lower dose of rosuvastatin (10 mg) for 30 consecutive days produced a significant enhancement of the hypolipidemic effect of rosuvastatin compared with the effect of rosuvastatin alone (Table 1). Indeed, the lower dose of rosuvastatin (10 mg/daily) when given in combination with a single oral dose of BPF (1000 mg/daily) produced a significant decrease on total cholesterol and LDL-C serum levels in hyperlipidemic patients when compared to rosuvastatin alone, an effect which was nearly the same than the one produced by 20 mg of rosuvastatin. In addition, triglycerides were reduced by 36 ± 5% and HDL-C was increased by 37 ± 2%, an effect which was significantly higher while compared to the use of rosuvastatin alone.

3.2. The effect of BPF and/or rosuvastatin on urinary mevalonate (MVA)

Twenty-four-hour urinary MVA excretion in hyperlipidemic patients undergoing BPF (1000 mg/daily for 30 days), rosuvastatin (10 and 20 mg/daily for 30 days) or combination of BPF (1000 mg) plus rosuvastatin was significantly decreased ($P > 0.05$) in all groups compared to placebo (Fig. 1). Interestingly, the group of patients receiving both BPF and the low concentration of rosuvastatin, showed that BPF produced a supplementary urinary excretion of MVA compared to

Table 1
Data on lipidemic serum profile of 77 patients undergoing 30 consecutive day treatment with a daily dose of placebo, rosuvastatin (10 or 20 mg), BPF 1000 mg and BPF 1000 mg + rosuvastatin 10 mg, respectively. Data are expressed in mg/dl.

	Total cholesterol	LDL-C	HDL-C	Triglycerides
Basal levels	278 ± 4	191 ± 3	38 ± 2	238 ± 5
Placebo	275 ± 4	190 ± 2	38 ± 3	235 ± 5
Rosuvastatin 10 mg	195 ± 3*	115 ± 4*	42 ± 3*	200 ± 4*
Rosuvastatin 20 mg	174 ± 4*	87 ± 3*	48 ± 3*	202 ± 5*
BPF 1000 mg	191 ± 5*	113 ± 4*	45 ± 4*	165 ± 3*
Rosuvastatin 10 mg + BPF 1000 mg	172 ± 3*	90 ± 4*	52 ± 4*	152 ± 5*

* $P < 0.05$ vs placebo.

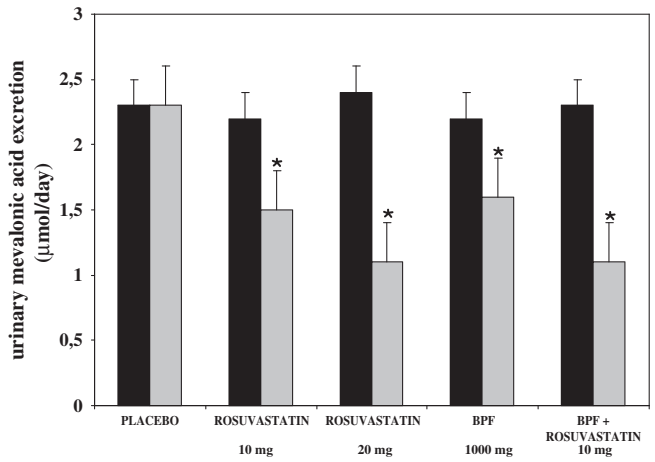

Fig. 1. The graph shows mean values for 24 h urinary mevalonic acid excretion in patients at 0 time (black columns) and after 30 days (grey columns) of oral treatment with placebo, BPF (1000 mg/daily), rosuvastatin (10 and 20 mg/daily) and rosuvastatin (10 mg/daily plus BPF 1000 mg/daily). Error bars show the standard deviation (S.D.). * - indicates a statistically significant change compared to the placebo group at $P < 0.05$.

rosuvastatin alone, confirming that BPF contributed to inhibiting HMG-CoA reductase.

3.3. The effect of BPF and/or rosuvastatin on MDA, phosphoPKB and LOX-1 levels in peripheral PMNs of hyperlipemic patients

Peripheral PMNs were isolated from the peripheral blood of each patient entering the study and re-suspended into Krebs buffer. The levels of MDA (a marker of lipid peroxidation) and the expression of LOX-1 receptor for oxyLDL were detected in peripheral PMNs before and at the end of the treatment with BPF and rosuvastatin given alone or in combination.

The basal levels of MDA and the expression of LOX-1 have been previously found elevated in patients with hyperlipidemia compared to a group of normolipidemic patients ($n = 15$; not shown). Administration of BPF (1000 mg/daily for 30 days), rosuvastatin (10 and 20 mg/daily for 30 days) or a combination of both (1000 mg plus 10 mg, respectively) significantly decreased both MDA levels and LOX-1 expression in PMNs of patients with mixed hyperlipidemia (Figs. 2 and 3). In particular, the effect of BPF produced a further enhancement of rosuvastatin antioxidant effect (as shown by decreased MDA levels) and a significant increment of rosuvastatin-induced activity in LOX-1 expression. Both

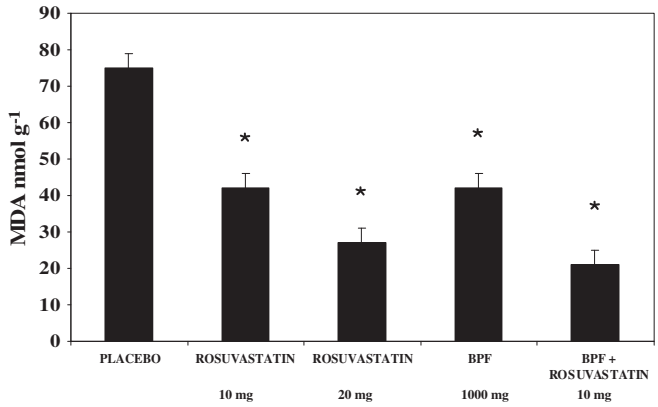

Fig. 2. The graph shows mean values for malondialdehyde (lipid peroxidation) in PMNs of patients after 30 days of oral treatment with placebo, BPF (1000 mg/daily), rosuvastatin (10 and 20 mg/daily) and rosuvastatin (10 mg/daily plus BPF 1000 mg/daily). Error bars show the standard deviation (S.D.). * - indicates a statistically significant change compared to the placebo group at $P < 0.05$.

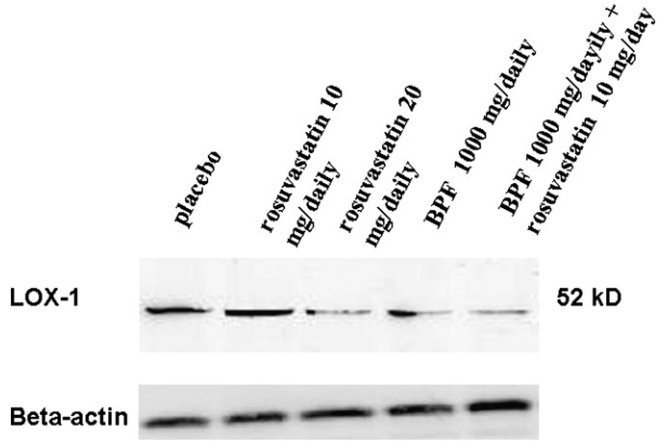

Fig. 3. Representative western blotting analysis showing the expression of oxyLDL receptor LOX-1 in peripheral PMNs of patients with mixed hyperlipidemia patients after 30 days of oral treatment with placebo, BPF (1000 mg/daily), rosuvastatin (10 and 20 mg/daily) and rosuvastatin (10 mg/daily plus BPF 1000 mg/daily).

effects significantly correlated with vasoprotective properties of statins in patients with cardiometabolic risk. The effect of BPF in combination with rosuvastatin also resulted in an enhanced expression of PKB levels in PMNs of hyperlipemic patients (Fig. 4). In particular, we found an enhanced phosphorylation of PKB, an effect which accounts for a better vasoprotection against factors supporting smooth muscle cell proliferation and vascular damage.

4. Discussion

Our data show that bergamot extract rich in polyphenols (BPF), given orally for 30 consecutive days allows the reduction of daily dose of rosuvastatin while achieving target lipid values in patients with mixed dyslipidemia (elevated serum LDL-C with hypertriglyceridemia). This effect was characterized by a reduction of both total cholesterol and LDL-C and amplified the increase of HDL-C produced by rosuvastatin alone. This enhancement of statin-induced effects suggests a potential benefit in the reduction of cardiometabolic risk. Furthermore, the reduction of serum cholesterol in patients taking both BPF and rosuvastatin is accompanied by a significant reduction in triglyceride levels, an effect which has not been found with rosuvastatin alone, thus suggesting an additive role of BPF in statin-induced hypolipidemic response.

These data are in accordance with previous evidence showing that bergamot derivatives possess antilipidemic and vasoprotective effects both in rat models of hyperlipidemia and vascular disease and in hyperlipidemic patients [24–26]. In addition, there is a significant additive

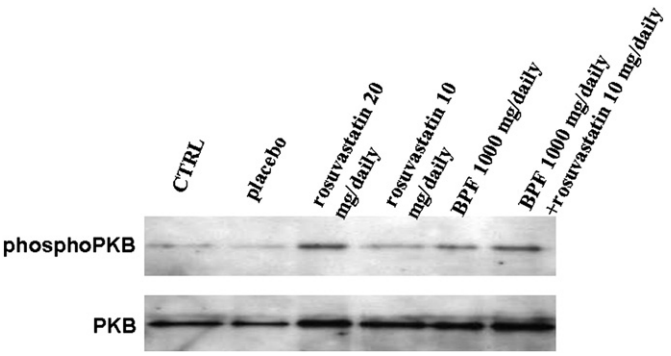

Fig. 4. Representative western blotting analysis showing the state of phosphorylation of PKB (phosphoPKB) in peripheral PMNs of patients with mixed hyperlipidemia patients either untreated (CTRL) as well as after 30 days of oral treatment with placebo, BPF (1000 mg/daily), rosuvastatin (10 and 20 mg/daily) and rosuvastatin (10 mg/daily plus BPF 1000 mg/daily).

effect of BPF with rosuvastatin (compared with either alone), an effect consistent with the further reduction seen in urinary MVA as detected in patients after treatment with both BPF and lower doses of rosuvastatin.

Beyond the clear antilipemic/hypoglycemic response which follows 30 day treatment with BPF in patients, the mechanism of action of this bergamot derivative remains to be elucidated. Previous data showed that non-nutritive constituents of *Citrus* family fruits, such as pectin and flavonoids found in peel extracts, cause lowering of serum and/or tissue cholesterol levels by modulating hepatic HMG-CoA levels, possibly by binding bile acids and increasing the turnover rate of blood and liver cholesterol [27–31]. Since BPF showed enhancement in the excretion of fecal sterols in rats [26], such a mechanism may contribute to both the hypolipidemic and hypoglycemic response of bergamot derivatives. However, a major contribution to the hypolipidemic response found in patients undergoing BPF treatment seems to be related to the modulatory properties in the flavonone glycoside component of the bergamot juice extract, in particular naringin and neo-hesperidin. Indeed, evidence exists that dietary hesperetin reduces hepatic TG accumulation associated with a reduced activity of TG synthetic enzyme, PAP [32]. In addition, *in vitro* studies suggest that hesperetin may, in part, reduce apoB levels [33]. Together with an enhanced expression of the LDL receptor, these mechanisms may explain, at least in part, the hypocholesterolemic properties of the citrus flavonoids [34] and of BPF, as found in our experiments.

Furthermore, naringenin has been shown to act at multiple levels in regulating lipid metabolism in patients [35]. Moreover, naringin was found to reduce both VLDL-derived and endogenously synthesized fatty acids, preventing muscle triglyceride accumulation and, finally, improving overall insulin sensitivity and glucose tolerance [36].

The effect of both naringin and hesperidin seems also to involve direct inhibition of the HMG-CoA reductase enzyme system [37]. This is confirmed by our data in which MVA, the end product of HMG-CoA reductase activity, was reduced by treatment with BPF. Furthermore, ACAT activities as a result of naringin supplementation could account for the decrease of fecal neutral sterols [37]. In addition, BPF seems to contain high concentration of buteridine and melitidine [38], two flavonone derivatives shown to selectively inhibit HMG-CoA reductase, and this may well explain the potency of BPF in reducing cholesterol levels [23].

Our data also show an additive vaso-protective effect of BPF when given associated with rosuvastatin, an effect probably due to the antioxidant properties of bergamot polyphenols. This is shown by the enhanced reduction of both LOX-1 and phosphorylation of PKB in PMNs of patients undergoing rosuvastatin treatment in combination with BPF and by simultaneous reduction of MDA, a biomarker of lipid peroxidation. This confirms previous studies showing that BPF given in hyperlipemic patients, improves reactive vasodilatation [26]. Furthermore, we recently showed that expression of LOX-1 receptor is associated with smooth cell proliferation in injured blood vessel, an effect counteracted by bergamot extract [25] as well as by exogenous antioxidants [39]. Finally, BPF contributed to the statin-induced increase of PKB phosphorylation. This may be relevant due to the evidence that PKB is a member of the second-messenger family that regulates the sub-family of protein kinases implicated in signaling downstream of tyrosine kinases and PI3-kinase. When phopshorylated, PKB leads to vasoprotection, an effect which is confirmed by evidence that statins are known to produce protection against vascular atherogenic injury also *via* PKB phosphorylation in response to mitogens and survival factors [40]. Thus, exogenous supply of natural antioxidants, such as bergamot-derived polyphenols, highlighted the modulation of peripheral biomarkers of cardiometabolic risk induced by rosuvastatin showing an additive vasoprotective potential for this natural extract in patients with hyperlipemia.

In conclusion, our data show that BPF enhances the effect of rosuvastatin in normalizing the serum lipemic profile. Clinically, this may allow a reduction in daily rosuvastatin doses for maintaining patients to target levels of cholesterol. In addition, BPF contributes to the reduction in oxidative processes found in hyperlipemic patients, thus suggesting a potential vasoprotective benefit of using such natural polyphenols in hyperlipemic patients undergoing statin treatment.

Acknowledgments

This paper has been supported by PON MIUR a3_00359. We would like to thank Prof. James Ehlrich (University of Denver, Colorado, USA) for the contribution in discussing data and revising the manuscript.

References

[1] Gielen S, Sandri M, Schuler G, Teupser D. Risk factor management: antiatherogenic therapies. Eur J Cardiovasc Prev Rehabil 2009;16:S29–36.
[2] Lorenzo C, Wagenknecht LE, D'Agostino Jr RB, Rewers MJ, Karter AJ, Haffner SM. Insulin resistance, beta-cell dysfunction, and conversion to type 2 diabetes in a multiethnic population: the Insulin Resistance Atherosclerosis Study. Diabetes Care 2010;33:67–72.
[3] Baigent C, Keech A, Kearney PM, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 2005;366:1267–78.
[4] Cannon CP, Steinberg BA, Murphy SA, Mega JL, Braunwald E. Meta-analysis of cardiovascular outcomes trials comparing intensive *versus* moderate statin therapy. J Am Coll Cardiol 2006;48:438–45.
[5] Jones PH. Expert perspective: reducing cardiovascular risk in metabolic syndrome and type 2 diabetes mellitus beyond low-density lipoprotein cholesterol lowering. Am J Cardiol 2008;102:41L–7L.
[6] Alsheikh-Ali AA, Karas RH. The relationship of statins to rhabdomyolysis, malignancy, and hepatic toxicity: evidence from clinical trials. Curr Atheroscler Rep 2009;11:100–4.
[7] Joy TR, Hegele RA. Narrative review: statin-related myopathy. Ann Intern Med 2009;150:858–68.
[8] Dohadwala MM, Vita JA. Grapes and cardiovascular disease. J Nutr 2009;139:1788S–93S.
[9] Gorinstein S, Caspi A, Libman I, et al. Red grapefruit positively influences serum triglyceride level in patients suffering from coronary atherosclerosis: studies *in vitro* and in humans. J Agric Food Chem 2006;54:1887–92.
[10] Jeon SM, Bok SH, Jang MK, et al. Antioxidative activity of naringin and lovastatin in high cholesterol-fed rabbits. Life Sci 2001;69:2855–66.
[11] Whitman SC, Kurowska EM, Manthey JA, Daugherty A. Nobiletin, a citrus flavonoid isolated from tangerines, selectively inhibits class A scavenger receptor-mediated metabolism of acetylated LDL by mouse macrophages. Atherosclerosis 2005;178:25–32.
[12] Eguchi A, Murakami A, Ohigashi H. Nobiletin, a citrus flavonoid, suppresses phorbol ester-induced expression of multiple scavenger receptor genes in THP-1 human monocytic cells. FEBS Lett 2006;580:3321–8.
[13] Kim JB, Han AR, Park EY, et al. Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF-kappaB inactivation in RAW 264.7 macrophage cells. Biol Pharm Bull 2007;30:2345–51.
[14] Gouedard C, Barouki R, Morel Y. Dietary polyphenols increase paraoxonase 1 gene expression by an aryl hydrocarbon receptor-dependent mechanism. Mol Cell Biol 2004;24:5209–22.
[15] Vinson JA, Liang X, Proch J, Hontz BA, Dancel J, Sandone N. Polyphenol antioxidants in citrus juices: *in vitro* and *in vivo* studies relevant to heart disease. Adv Exp Med Biol 2002;505:113–22.
[16] Devaraj S, Jialal I, Vega-Lopez S. Plant sterol-fortified orange juice effectively lowers cholesterol levels in mildly hypercholesterolemic healthy individuals. Arterioscler Thromb Vasc Biol 2004;24:e25–8.
[17] Kaplan M, Hayek T, Raz A, et al. Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis. J Nutr 2001;131:2082–9.
[18] Dugo P, Presti ML, Ohman M, Fazio A, Dugo G, Mondello L. Determination of flavonoids in citrus juices by micro-HPLC-ESI/MS. J Sep Sci 2005;28:1149–56.
[19] Nogata Y, Sakamoto K, Shiratsuchi H, Ishii T, Yano M, Ohta H. Flavonoid composition of fruit tissues of citrus species. Biosci Biotechnol Biochem 2006;70:178–92.
[20] Choe SC, Kim HS, Jeong TS, Bok SH, Park YB. Naringin has an antiatherogenic effect with the inhibition of intercellular adhesion molecule-1 in hypercholesterolemic rabbits. J Cardiovasc Pharmacol 2001;38:947–55.
[21] Jeong YJ, Choi YJ, Choi JS, et al. Attenuation of monocyte adhesion and oxidised LDL uptake in luteolin-treated human endothelial cells exposed to oxidised LDL. Br J Nutr 2007;97:447–57.
[22] Yu J, Wang L, Walzem RL, Miller EG, Pike LM, Patil BS. Antioxidant activity of citrus limonoids, flavonoids, and coumarins. J Agric Food Chem 2005;53:2009–14.
[23] Di Donna L, De Luca G, Mazzotti F, et al. Statin-like principles of bergamot fruit (*Citrus bergamia*): isolation of 3-hydroxymethylglutaryl flavonoid glycosides. J Nat Prod 2009;72:1352–4.
[24] Miceli N, Mondello MR, Monforte MT, et al. Hypolipidemic effects of *Citrus bergamia* Risso et Poiteau juice in rats fed a hypercholesterolemic diet. J Agric Food Chem 2007;55:10671–7.

[25] Mollace V, Ragusa S, Sacco I, et al. The protective effect of bergamot oil extract on lecitine-like oxyLDL receptor-1 expression in balloon injury-related neointima formation. J Cardiovasc Pharmacol Ther 2008;13:120–9.
[26] Mollace V, Sacco I, Janda E, et al. Hypolipemic and hypoglycaemic activity of bergamot polyphenols: from animal models to human studies. Fitoterapia 2011;82:309–16.
[27] Bok SH, Lee SH, Park YB, et al. Plasma and hepatic cholesterol and hepatic activities of 3-hydroxy-3-methyl-glutaryl-CoA reductase and acyl CoA: cholesterol transferase are lower in rats fed citrus peel extract or a mixture of citrus bioflavonoids. J Nutr 1999;129:1182–5.
[28] Marounek M, Volek Z, Synytsya A, Copikova J. Effect of pectin and amidated pectin on cholesterol homeostasis and cecal metabolism in rats fed a high-cholesterol diet. Physiol Res 2007;56:433–42.
[29] Terpstra AH, Lapre JA, de Vries HT, Beynen AC. Dietary pectin with high viscosity lowers plasma and liver cholesterol concentration and plasma cholesteryl ester transfer protein activity in hamsters. J Nutr 1998;128:1944–9.
[30] Anonymous. The hypocholesterolemic effect of lemon peels, lemon pectin, and the waste stream material of lemon peels in hybrid F1B hamsters. Eur J Nutr 2002;41:19–26.
[31] Garcia-Diez F, Garcia-Mediavilla V, Bayon JE, Gonzalez-Gallego J. Pectin feeding influences fecal bile acid excretion, hepatic bile acid and cholesterol synthesis and serum cholesterol in rats. J Nutr 1996;126:1766–71.
[32] Cha JY, Cho YS, Kim I, Anno T, Rahman SM, Yanagita T. Effect of hesperetin, a citrus flavonoid, on the liver triacylglycerol content and phosphatidate phosphohydrolase activity in orotic acid-fed rats. Plant Foods Hum Nutr 2001;56:349–58.
[33] Borradaile NM, Carroll KK, Kurowska EM. Regulation of HepG2 cell apolipoprotein B metabolism by the citrus flavanones hesperetin and naringenin. Lipids 1999;34:591–8.
[34] Wilcox LJ, Borradaile NM, de Dreu LE, Huff MW. Secretion of hepatocyte apoB is inhibited by the flavonoids, naringenin and hesperetin, *via* reduced activity and expression of ACAT2 and MTP. J Lipid Res 2001;42:725–34.
[35] Huong DT, Takahashi Y, Ide T. Activity and mRNA levels of enzymes involved in hepatic fatty acid oxidation in mice fed citrus flavonoids. Nutrition 2006;22:546–52.
[36] Li JM, Che CT, Lau CB, Leung PS, Cheng CH. Inhibition of intestinal and renal Na + -glucose cotransporter by naringenin. Int J Biochem Cell Biol 2006;38:985–95.
[37] Shin YW, Bok SH, Jeong TS, et al. Hypocholesterolemic effect of naringin associated with hepatic cholesterol regulating enzyme changes in rats. Int J Vitam Nutr Res 1999;69:341–7.
[38] Leopoldini M, Malaj N, Toscano M, Sindona G, Russo N. On the inhibitor effects of bergamot juice flavonoids binding to the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) enzyme. J Agric Food Chem 2010;58:10768–73.
[39] Muscoli C, Sacco I, Alecce W, et al. The protective effect of superoxide dismutase mimetic M40401 on balloon injury-related neointima formation: role of the lectin-like oxidized low-density lipoprotein receptor-1. J Pharmacol Exp Ther 2004;311(1):44–50.
[40] Li DY, Chen HJ, Mehta JL. Statins inhibit oxidized-LDL-mediated LOX-1 expression, uptake of oxidized-LDL and reduction in PKB phosphorylation. Cardiovasc Res 2001;52(1):130–5.