

The effect of bergamot-derived polyphenolic fraction on LDL small dense particles and non alcoholic fatty liver disease in patients with metabolic syndrome

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ABSTRACT

The occurrence of Metabolic Syndrome (MS) represents an independent risk factor for developing cardiovascular disease states in patients suffering from type 2 Diabetes Mellitus. Moreover, both the size of LDL particles and liver dysfunction identified as non alcoholic fatty liver disease (NAFLD) represent important biomarkers for the development of cardiometabolic risk in patients with MS.

Here we studied the effect of bergamot polyphenolic fraction (BPF) in patients with MS and NAFLD. 107 patients were enrolled at the San Raffaele IRCCS (Rome). All of them showed ultrasonographic evidences of NAFLD and at least three out of five previous identified criteria for the diagnosis of MS. Patients were divided into two groups one receiving placebo and the second receiving BPF 650mg twice a day for 120 consecutive days. In the group receiving BPF 650 mg twice a day, a significant reduction of fasting plasma glucose, serum LDL cholesterol and triglycerides alongside with an increase of HDL cholesterol was found. This effect was accompanied by significant reduction of both ultrasonographic and metabolic biomarkers of NAFLD. Moreover, a significant reduction of small dense LDL particles, as detected via proton NMR Spectroscopy, was found after BPF treatment.

In conclusion, our data confirm the beneficial effect of bergamot-extract in patients with MS an effect highlighted by significant reduction of small dense LDL particles and by improvement of NAFLD biomarkers. This suggests a potential preventive role of bergamot derivatives in reducing cardiometabolic risk.

Keywords: Metabolic Syndrome, Non Alcoholic Fatty Liver Disease, cardiometabolic risk, bergamot polyphenolic fraction

1. INTRODUCTION

Evidence has been collected showing that the occurrence of metabolic syndrome (MS) is associated to increased risk of developing cardiovascular system diseases and type 2 diabetes [1]. In particular, MS has been found to double the cardiometabolic risk and to enhance the risk of development of type 2 diabetes by five times [2].

Besides imbalanced glycemic control has been associated with parameters of vascular damage [3], increasing hyperglycemia in type 2 diabetes does not contribute to the cardiovascular risk to the same extent as it does in type 1 diabetes [4], pointing to the importance of nonglycemic related risk factors belonging to the MS. Numerous studies demonstrated an increased cardiovascular risk in patients with MS prior to the development of overt hyperglycemia [5,6]. Likewise, patients with type 2 diabetes or MS have an increased cardiovascular risk despite optimal control of other risk factors as low-density lipoprotein cholesterol (LDL-C) [7].

In the context of the shortcomings of commonly assessed risk factors in individuals with features of the MS, the characterization and sub-classification of LDL and high-density lipoprotein HDL particles emerged as a tool that may offer a better risk prediction. A decrease of HDL associated with an increase in small, dense LDL particles (sdLDL), class III and IV, is

closely associated with an increased cardiovascular risk, independently of the traditional risk factors both in patients with [8-10] and without [11-13] diabetes or MS. Individual LDL particle profiles normally cluster into two patterns of LDL size distribution: the majority of profiles demonstrates a predominance of large or medium sized LDL particles (LDL pattern A), whereas a substantial minority exhibits the LDL pattern B with a higher proportion of smaller LDL particles [14]. Several aspects must be taken into account when evaluating the potential role of sdLDL in patients with MS. In particular, evidence has been accumulated suggesting that the formation of sdLDL particles seems to be favoured in the presence of insulin resistance and elevated triglycerides [15,16]. Although the mechanism underlying this effect is still unknown, it is likely also to involve an increased hepatic uptake of LDL particles, possibly due to the upregulation of hepatic LDL receptor activity and/or increased clearance of LDL particles due to their altered composition and liver dysfunction.

Recently, it has been shown that MS is associated with non alcoholic fatty liver disease state (NAFLD). In particular, NAFLD is one of the most common causes of liver-related mortality worldwide [17]. NAFLD encompasses a morphological spectrum from simple fatty liver (SFL), non-alcoholic steatohepatitis (NASH) to hepatic cirrhosis [18]. NAFLD has been identified as the hepatic manifestation of MS [19] and the association between NAFLD and MS has been reported in earlier observational studies [20-22]. There are a number of common mechanisms underlying the development of NAFLD and MS. For example, they may have the same pathophysiological basis of insulin resistance [23]. A systematic review suggested a potential predictive effect of liver fat on the presence of MS [23]. However, no evidence was provided regarding the causal association between NAFLD and MS. Moreover, many compounds used for counteracting the detrimental effect of MS on cardiovascular system, such as statins, have not been found to produce beneficial effect on liver dysfunction or enhanced fat accumulation in the liver [24], as well as asymptomatic elevation of serum aminotransferases alanine amino-transferase (ALT) and aspartate aminotransferase (AST) [25].

Recently, we found that bergamot-derived polyphenolic fraction (BPF) produces significant reduction of serum cholesterol, triglycerides and fasting glucose in hyperlipemic patients [26,27], an effect which occurs at multilevels including an enhanced metabolism of lipoproteins at the hepatic level [28]. In addition, the administration of bergamot juice in rats fed with an hypercholesterolemic rats significantly improved histopathological pattern of hepatic tissues, though the mechanism is still unclear (26).

The present experiments have been performed to study the effect of BPF on lipoprotein subfraction profile and NAFLD in patients with MS.

Study design. The study has been carried out in 107 patients enrolled at the San Raffaele IRCCS, Rome and at the University of Catanzaro, Italy suffering from MS and NAFLD. Diagnosis of MS was based, according to the NCEP-ATP III criteria, on the occurrence of at least three out of five components of the syndrome, such as: elevated fasting plasma glucose (or previously diagnosed type 2 diabetes), abdominal obesity measured by waist circumference, elevated triglycerides, decreased HDL-cholesterol and increased arterial blood pressure. The occurrence of concomitant NAFLD was detected by ultrasonographic examination of patients. Only patients with an hepato-renal index (see below) in the range of 2,5-3,5 were admitted to the study. After randomization, patients were taking BPF 650 mg twice a day before meals for 120 consecutive days.

All participants provided written informed consent prior to participation. The study was approved by the local Medical Ethics Committee.

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

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 to 600 Da (Mitsubishi). Polyphenol fractions were eluted by a 1mM KOH solution. The basic eluate was incubated at a rocking platform to reduce the furocoumarin content. The shaking time was adjusted proportionally to the amount of furocoumarin contaminants. Next, the phytocomplex derived from the process performed to remove furocoumarins 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 analysed for flavonoid, furocoumarin 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 the final BPF was free of mycotoxins and contaminating bacteria.

Finally, 650 mg aliquots of the BPF powder 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 neohesperidin (370 ppm), naringin (520 ppm), and neohesperidin (310 ppm). Tablets containing 1000 mg of maltodextrin supplemented with 50 mg ascorbic acid were used as placebo.

2. MATERIALS AND METHODS

Data collection and measurements. At baseline, all participants were asked to come in the morning after fasting for >10 h. Fasting blood samples were collected for measuring conventional risk factors of liver or cardiovascular disease (CVD), including lipids, glucose, transaminases and inflammatory markers. Face-to-face interviews and physical examinations were performed by well-trained nurses or physicians. Demographic and lifestyle information was collected by a standardized questionnaire. Alcohol consumption was classified into never, occasional, moderate and excessive based on the usual frequency of intake and the usual amount per occasion. Those who did not drink any alcohol throughout their life were classified as non-drinkers. Those who drank <1 occasion per week or drank only on special occasions in the past one year were classified as occasional drinkers. Moderate drinkers were regular drinkers (≥ 1 occasion per week) who drank <20 g alcohol per day in males or <10 g alcohol per day in females, while excessive drinkers with alcohol consumption of ≥ 20 g per day in males or ≥ 10 g per day in females were not included in this study.

Diagnostic criteria of NAFLD. Following exclusion of subjects with excessive alcohol consumption and viral or autoimmune liver disease, NAFLD was diagnosed by abdominal ultrasound, which is a widely accessible imaging technique with high diagnostic accuracy and reliability for the detection of fatty liver. An ultrasonographic examination was performed by an experienced radiologist using a real-time scanner (3.5 MHz; Mod. Aplio, Toshiba, Japan) equipped with a convex-array probe. All subjects were evaluated in the left lateral recumbent position of 15°-20° to see the liver parenchyma and the right kidney cortex was seen contemporaneously.

The brightness of both zones was examined. The liver was recorded from the intercostal space, posing the region of interest (of 1.5 cm \times 1.5 cm) in the mid or anterior axillary line (seventh or eighth intercostal space).

The right kidney was evaluated, posing the region of interest (0.5 cm \times 0.5 cm) in the cortical zone. Hepato-Renal Index Difference (HRI-diff: Echo Levels in the Liver–Echo Levels in the Kidney) was estimated using built-in software on the scanner enabling local measurement of attenuation in dB. Mild steatosis was diagnosed for hyperechogenic liver tissue (compared with the kidney cortex) when the sonographic index results were between 1 and 2. Values between 2 and 2.5 were indicative of moderate liver steatosis. Finally, hepatic steatosis was judged as severe when the hepatorenal ratio was > 2.5. In each case, the calculation of the hepatorenal index was repeated at least twice.

Laboratory measurements. Plasma samples were collected in EDTA-containing vials after a 12 h overnight fast on day 0 and after 120 days of treatment with BPF (650 mg twice a day before each meal).

Total cholesterol (in mg/dL), high density lipoprotein cholesterol (HDL-C), LDL-C, triglycerides (TG) and fasting plasma glucose, steato test, ALT, AST and γ -glutamyl peptidase (γ -GT) were evaluated at baseline and after four months of treatment with BPF. Under the same treatment schedule with BPF, lipoprotein particles were detected by means of proton NMR spectroscopy technique which simultaneously measures the particle concentrations of lipoprotein subclasses of different sizes. Each of the lipoprotein subclasses emits a distinctive NMR signal, the amplitude of which is directly proportional to the number of subclass particles emitting the signal. Importantly, variation in lipoprotein particle lipid composition does not alter the relationship between the NMR signal and the particle size. The NMR also provides calculated values for mean very-low-density lipoprotein (VLDL), LDL, and HDL particle sizes plus estimates of total and VLDL, TG and HDL cholesterol. NMR-based estimates of TG and HDL cholesterol were calculated using conversion factors that assume normal lipid content of the various subclasses.

Circulating TNF- α levels was measured by ELISA using kits from R&D Systems and Biosource and, finally, C-reactive protein (CRP) was measured using nephelometry and reagents from Beckman Coulter.

Statistical analysis. For categorical variables, differences between BPF and baseline levels were assessed using Pearson's chi-square test or Fisher's exact test (if Cochran's rules were not satisfied). For continuous variables, baseline differences between the BPF and baseline levels were assessed using Student's t test for independent samples. To assess the response to intervention, change variables (post-treatment value minus pretreatment value) were computed for all continuous variables, and between-group differences in changes were assessed using Student's t test for independent samples. Data analyses were conducted using SPSS software (version 18.0).

3. RESULTS

Demographics and glyco-lipemic serum profile in patients with MS and NAFLD at baseline as well as following 120 consecutive days of treatment with BPF (650 mg given orally twice a day before meals) are shown in Table 1. Data on biomarkers of hepatic function, steato test and inflammatory biomarkers such as TNF- α and CRP are also displayed.

Basal levels showed an elevated BMI combined with a mixed hyperlipemia (elevated total cholesterol plus hypertriglyceridemia). High LDL cholesterol was associated with reduced HDL cholesterol and elevated fasting serum glucose, suggesting the occurrence of MS. The elevated lipemic and glycemic biomarkers were accompanied by changes in transaminases and steato test, suggesting that MS was accompanied by NAFLD. Moreover, inflammatory biomarkers were found elevated in patients at baseline thus suggesting

that MS with hepatic dysfunction was accompanied by an inflammatory state.

In patients treated with BPF (650 mg given orally twice a day before meals) for 120 consecutive days a significant reduction of serum total cholesterol, LDL-C and triglycerides was found (Table 1). This effect was accompanied by significant reduction of serum glucose, transaminases, gamma-glutamyl-transferase, steato test and inflammatory biomarkers such as TNF- α and CRP.

Moreover, a substantial re-arrangement of lipoprotein particles was found compared to the baseline profile (Table 2). Indeed, as measured using NMR, data detected in patients following 120 days of BPF treatment showed relevant changes in mean particle diameters for VLDL, LDL, and HDL ($P < 0.05$ for all) compared to the baseline levels (Table 2).

In particular, BPF was found able to decrease the mean concentration of IDL particles by 51%, to increase large LDL by 38%, and to decrease small LDL by 35%. Moreover, 120 day treatment with BPF lead to 20% increase of total HDL particles, mainly due to the increase of large HDL ($P = 0.05$ vs baseline levels).

The beneficial effect of treating patients with MS associated with NAFLD was confirmed by data obtained when studying ultrasonographic pattern of NAFLD. Indeed, hepatorenal index was significantly ($P < 0.05$) reduced from 2.8 ± 0.4 to 1.5 ± 0.5 by treatment with BPF showing a reduction of liver brightness. This suggests that treating patients suffering from mild to severe NAFLD associated with MS with BPF (650 mg twice a day for 120 consecutive days) leads to reduction of hepatic ultrasonographic pattern of steatosis.

No side effects related to the treatment with BPF have been described thus confirming data on safety profile of bergamot extract previously shown by our and other groups.

4. DISCUSSION

The present data confirmed previous results showing that BPF, the extract of bergamot juice rich in polyphenols, reduces both cholesterol, triglycerides and glucose in patients suffering from MS [26, 27]. This effect is accompanied by reduction of LDL-C and elevation of HDL-C, thus suggesting a beneficial effect in the lipemic profile of patients undergoing MS. The added value and novelty of data reported in this study using BPF in such a subgroup of patients with elevated cardiometabolic risk, is also displayed by prominent re-arrangement of lipoprotein particle profile found following 120 day BPF treatment. Indeed, BPF reduced LDL small-size, atherogenic particles and enhanced large-size anti-atherogenic HDL lipoprotein particles. This effect, combined with reduction of inflammatory biomarkers, suggests that BPF leads to an attenuation of atherogenic risk in patients with MS.

The mechanism of such an effect in lipoprotein particle size is not clear to date. The combined effect of BPF in reducing both cholesterol and triglycerides may well explain lipoprotein re-arrangement due to prolonged BPF treatment. Indeed, an increased clearance of TG-

rich lipoprotein particles makes these particles became better substrates for lipoprotein lipase. This would be expected to result in decreased levels of large and medium-sized VLDL and perhaps even intermediate density lipoprotein (IDL), which contains roughly equal amounts of TG and cholesterol.

The increased cascade of VLDL to IDL to LDL would result in increased numbers of large LDL particles and provide surface constituents for the formation of large HDL. The formation of small LDL is mainly due to cholesteryl ester transfer protein-mediated exchange of VLDL-TG for LDL cholesterol ester and the subsequent hydrolysis of LDL-TG. The decrease in large and medium VLDL diminishes the cholesteryl ester transfer protein-mediated exchange, decreasing the formation and number of small LDL particles.

The improvement of hepatocyte functionality found in patients with MS and associated NAFLD after taking BPF might also contribute in the amelioration in lipoprotein profile thereby attenuating cardiometabolic risk.

Previous studies demonstrated that insulin resistance almost universally induces NAFLD [29,30]. It is known that this condition may precede the development of cardiovascular disease [31,32]. To confirm the connection between NAFLD and atherosclerosis, carotid atherosclerosis has recently been detected in patients with NAFLD [33]. Pathogenetic mechanisms responsible for that include an increased lipolysis and increased delivery of free fatty acids to the liver [34]. Other abnormalities that can contribute to fat accumulation in the liver include decreased synthesis of apolipoproteins and microsomal transfer protein gene polymorphism, both conditions that lead to decreased export of triglycerides out of the liver [35]. The improvement of steato test and hepatorenal index in patients with MS and NAFLD following BPF treatment gives a quantitative estimation of steatosis and leads to the conclusion that BPF improves both liver function and signs of chronic liver inflammation, as confirmed by reduction of TNF- α and CRP. Mild to moderate elevation of serum aminotransferases (ALT and AST) found in our subjects at baseline represents the most common abnormality found in patients with NAFLD. Their serum levels were significantly reduced after BPF, thereby confirming data obtained with steato test and hepatorenal index. The mechanism of hepato-protective effect of BPF still remains to be elucidated.

Previous studies showed that both bergamot juice and BPF act as a cytoprotectant agent in the liver of rats undergoing hypercholesterolemic diet [28,36]. This should be due to BPF activities in oxidative inflammation and changes in hepatocyte membrane permeability probably via stabilization of the hepatocyte membrane structure, thereby preventing toxins from entering the cells. In addition, it has recently been shown 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 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)

levels, possibly by binding bile acids and increasing the turnover rate of blood and liver cholesterol [37-41]. Since BPF showed enhancement in the excretion of fecal sterols in rats [42], such a mechanism may contribute to both the hypolipidemic and hepatoprotective properties of bergamot derivatives. Moreover, 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 [43]. Thus, BPF polyphenolic components, via multi-action properties, reduces liver accumulation of fat thereby producing an overall improvement of liver function.

In conclusion, our data show that bergamot-deriving polyphenolic fraction given in patients with MS and NAFLD, leads to concomitant amelioration of lipemic and glycemic profile and to substantial reduction of liver steatosis. This effect, alongside with a reduction of pro-atherogenic small dense LDL and enhancement of anti-atherogenic high dense HDL, shed new light on the potential use of bergamot-extract for reducing cardiometabolic risk in patients with MS.

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Table 1 - Demographic, haematological and ultrasonic data obtained at baseline and after treatment with BPF (650 mg twice a day for 120 consecutive days) in 107 patients suffering from metabolic syndrome and Non-alcoholic fatty liver disease (NAFLD). (Data are expressed as mean \pm SD for each value; a P value of <0.05 between values at baseline and after BPF treatment was taken as significant *).

Hematological, inflammatory

and ultrasonic biomarkers	Baseline	BPF
Patients undergoing BPF (650 x 2 daily)		
for 120 consecutive days	107	
Age (yr)	56 \pm 12	
Sex (M/F)	64/43	
BMI (kg/m ²)	29.4 \pm 2.01	28.2 \pm 1.53
Fasting plasma glucose (mg/mL)	118 \pm 1.4	98 \pm 0.8*
Total Cholesterol (mg/dL)	245 \pm 8.3	182 \pm 7.1*
LDL-C (mg/mL)	162 \pm 4.3	101 \pm 1.8*
HDL-C (mg/mL)	38 \pm 3.8	49 \pm 4*
Triglycerides (mg/mL)	232 \pm 5.1	160 \pm 4.8*
Steato test	0.74 \pm 0.12	0.44 \pm 0.09*
ALT (U/L)	54 \pm 5.4	36 \pm 5.3*
AST (U/L)	52. \pm 6.4	41 \pm 5.2*
γ -GT (IU/L)	38 \pm 5.2	29.33 \pm 1.1*
Hs-CRP (mcg/dl)	1.2 \pm 0.8	0,94 \pm 0.6*
TNF- α (pg/mL)	14.4 \pm 1.9	10.7 \pm 1.7*
Hepatorenal index	2.8 \pm 0.4	1.5 \pm 0.5*

Table 2 - Plasma lipoprotein particle size and concentration in patients with metabolic syndrome and NAFLD at the baseline and after 120 consecutive day treatment with BPF 650 mg x 2 daily. (Data are expressed as mean \pm SD for each value; a P value of <0.05 between values at baseline and after BPF treatment was taken as significant *)

Plasma lipoprotein plasma diameter, nm	Baseline	BPF
VLDL	55.3 \pm 6,4	44.5 \pm 5.2*
LDL	22.6 \pm 1.7	18.0 \pm 0.8*
HDL	7.5 \pm 0.8	9.6 \pm 0.9*
Plasma lipoprotein particles, nmol/L		
Total VLDL	83 \pm 14	54 \pm 12*
Large VLDL	4,2 \pm 2	1.8 \pm 1.3*
Medium VLDL	31 \pm 9	14 \pm 8*
Small VLDL	43 \pm 9	38 \pm 10
Total LDL	1477 \pm 75	1293 \pm 101*
IDL	77 \pm 16	38 \pm 10*
Large LDL	424 \pm 87	653 \pm 95*
Small LDL	986 \pm 105	612 \pm 98*
Total HDL	30 \pm 2	36 \pm 3*
Large HDL	5 \pm 3	15 \pm 4*
Medium HDL	7 \pm 4	7 \pm 3
Small HDL	18 \pm 5	14 \pm 4*

