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Review

Molecular mechanisms of lipid- and glucose-lowering activities of bergamot flavonoids

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ABSTRACT

Bergamot (*Citrus bergamia* Risso et Poiteau) juice has a particularly high content and a unique composition of flavonoids. Neoeriocitrin, neohesperidin, naringin, melitidin and brutieridin represent more than 95% of Bergamot Polyphenol Fraction (BPF), while rhoifolin, diosmin, poncirin and others can be found in the remaining 5%. The brilliant performance of BPF in clinical practice against as a treatment for hyperlipidemia and moderate hyperglycemia in metabolic syndrome, awaits a plausible mechanistic explanation. Considering the overwhelming scientific evidence, it is likely that flavonoid components of BPF are responsible for majority of pharmacological effects. Here, we will review the scientific evidence showing that flavonoids, in particular citrus flavonoids present in bergamot fruits, influence lipid and sugar metabolism at the molecular level. Anti-diabetic and dyslipidemia-correcting effects of bergamot polyphenols may be explained by their ability to activate AMP kinase (AMPK), which is a central regulator of glucose and fatty acids metabolism and inhibit cAMP phosphodiesterases (PDE), involved in regulation of lipolysis in adipocytes and liver. Importantly, certain polyphenols can act as 3-hydroxy-3-methlglutaryl coenzyme A (HMG-CoA) reductase inhibitors, thereby mimicking statins action. In addition, flavonoids bind and act as natural inhibitors of quinone oxidoreductase 2 (QR2/NQO2) and other enzymes with potential roles in metabolic regulation. Finally, pleiotropic and possible synergistic effects may account for enhanced nutraceutical effects of natural flavonoid mixtures, such as BPF as compared to purified flavonoids.

Contents

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1. Introduction

Metabolic syndrome (MS) is a cluster of common cardiovascular risk factors, including, atherogenic dyslipidemia, insulin resistance or glucose intolerance, visceral obesity, hypertention and endothelial dysfunction. 10–30% of individuals in industrialized countries, including Italy, suffer from this condition. MS is associated with an increased risk of accelerated atherosclerosis and cardiovascular events [1]. Cardiovascular risk factors are also represented by different dyslipidemias, such as increased cholesterol levels (hypercholesterolemia) or increased level of tryglicerides (hypertryglicedemia), that occur separately or by diabetes. Experimental and epidemiological evidence suggest that dietary polyphenols, such as flavonoids may prevent atherosclerosis by counteracting its risk factors [2–4]. Accordingly, we should expect better clinical results by increasing the dosage and quality of consumed flavonoids. Indeed, a concentrated mixture of bergamot (*Citrus bergamia* Risso et Poiteau) flavonoids, so called Bergamot Polyphenol Fraction (BPF), shows brilliant results in clinical practice. BPF derived from bergamot juice has particularly high (40%) and unique composition of polyphenols, as discussed in Section 2.

Bergamot, as the endemic plant of Calabria, occupied and continues to occupy and important place in Calabrian economy as the main source for the production essential oil used in the cosmetic industry. However, the medicinal use of bergamot derivatives, forgotten for decades now is being rediscovered. For example, bergamot juice was considered by local population of Calabria, as a remedy for "fatty arteries" and heart diseases. This inspired Mollace and co-workers to address the efficacy of bergamot juice and then its derivative BPF in experimental and clinical settings [5]. Mollace et al. showed in animal models and human studies that BPF is effective in combating several symptoms of metabolic syndrome such as increased total cholesterol (totChol), LDL-cholesterol (cLDL) and trygliceridemia (TG), increased blood glucose levels and endothelial dysfunction in a group of metabolic syndrome patients. The effects on mean cholesterol parameters were compara

ble with a moderate dose of simavastatin (20 mg daily), including a marked increase in cHDL levels. In addition, 1000 mg BPF taken for 30 days reduced moderate hyperglycemia by more than 20% [5].

The brilliant performance of BPF on MS and dyslipidemia is astonishing and needs a mechanistic explanation. Unfortunately, so far there is very limited experimental evidence proving the modulation of one or another metabolic pathway by BPF itself. However, the vast scientific literature suggests that certain individual flavonoids present in BPF are implicated in the regulation of several metabolic enzymes, expressed in the liver, blood and endothelial cells. These regulation in many cases may be direct, *i.e.* mediated by the physical interaction of a receptor (specific enzyme) and the flavonoid compound and may lead to either inhibition or activation of the catalytic function of the enzymes. In addition, natural polyphenols show less specific, antioxidant properties, that depend on the free radicals scavenging ability of hydroxyl groups linked to carbon aromatic rings [6–8]. Together with antioxidant properties, dietary flavonoids and their metabolites may modulate basic signal transduction pathways of every cell leading to anti-proliferative, anti-aging and immune responses and other beneficial effects for human health, as discussed in vast literature on the subject [6,7]. Finally, besides intracellular, molecular effects, flavonoids in cooperation with pectins, can work at the level of intestine and liver to stimulate fat excretion and reduce fat absorption, which augments the direct activity on enzymes, involved in the regulation of carbohydrate and lipid metabolism [9–11].

Here, we will review the scientific evidence showing that citrus flavonoids, present in bergamot juice and albedo may influence lipid and sugar metabolism at the molecular level *via* AMPK, PDE and other enzymes modulation. In this regard we will focus on direct molecular receptors of flavonoids, identified by crystallography and computational chemistry studies. We will also discuss a possible contribution of flavonoids to intestine and liver physiology. At the end we propose that pleiotropic and synergistic effects in natural mixtures of polyphenols defined here as "fitocomplex" may account for their superior performance *in vivo* compared to purified flavonoids.

2. Bergamot flavonoids and their metabolism

Natural phenols are vast group of compounds produced by biological sources, that contain at least one hydroxylated aromatic ring [12]. For the purpose of this review we will define polyphenols as natural compounds with at least aromatic rings. They will include: flavonoids, stilbenes, coumarins, quinones, phenolic acids and others. Bergamot juice is particularly rich in flavanones and flavones belonging to flavonoids group and is characterized by a unique profile of flavonoids, showing partial similarity to *Citrus* × *myrtifolia* Raf. (chinotto) [13] and *Citrus aurantium* L. (sour orange)[14].

It contains relatively rare neoeriocitrin and neohesperidin and, as recently discovered, rare brutieridin and melitidin [15]. Careful analysis of flavonoid content in 42 *citrus* species and cultivars, reported by Nogata et al. [16], leads to the conclusion that the amount of the flavonoids per volume unit of juice, or per mass unit of albedo (peels), is absolutely the highest in bergamot compared to other *Citrus* fruits. Bergamot shows the highest concentrations of flavanones: neoeriocitrin, neohesperidin, naringin, melitidin and brutieridin, and the highest content of certain flavones: rhoifolin, neodiosmin, poncirin and rutin among all 42 different *Citrus* species [16]. Bergamot juice can be further concentrated by a patented method, involving a preparative size exclusion chromatography based on polystyrene gel filtration and the eluate exsiccation to give rise to a polyphenol-enriched powder, BPF [5]. BPF contains over 40% flavonoids, carbohydrates, pectins, and other compounds, in contrast to bergamot juice powders obtained by spry-drier method that rich maximum 1% polyphenols concentration. (D. Malara, personal communication). The main polyphenol components of BPF are flavonoids and their composition basically mirrors the bergamot juice polyphenol profile (Fig. 1), with the only difference that flavonoids are over 200 times more concentrated in BPF.

95% of flavonoids present in BPF (and in bergamot juice) are flavanones, while flavone can be found in the remaining 5%. Up to date there are no published bioavailability and pharmacokinetic studies for BPF. However, absorption, metabolism and excretion parameters have been described for several individual flavonoids present in bergamot juice. It is well known that flavonoid glycosides are hydrolysed to aglycones by bacterial flora of the gut. Gut microflora hydrolysis is thought to favor flavonoid glycoside bioavailability [17]. When sugar unit is removed, the resulting aglycone can be absorbed more readily [12]. Indeed, flavonoid aglycones of diosmin, hesperidin and naringin, which are diosmetin, hesperitin and naringenin, respectively, diffuse usually easily through the plasma membrane of Caco-2 cells, in contrast to the respective glycosides. Moreover, naringin, hesperidin, rutin and poncirin are hydrolyzed to their respective aglycones by human intestinal microflora, and the resulting aglycones are absorbed better [18]. However, the bioavilability of flavonoids is low and it is estimated that only 10% of total consumed polyphenols are absorbed, although these numbers vary between species and individuals [12]. When inside the enterocytes part of

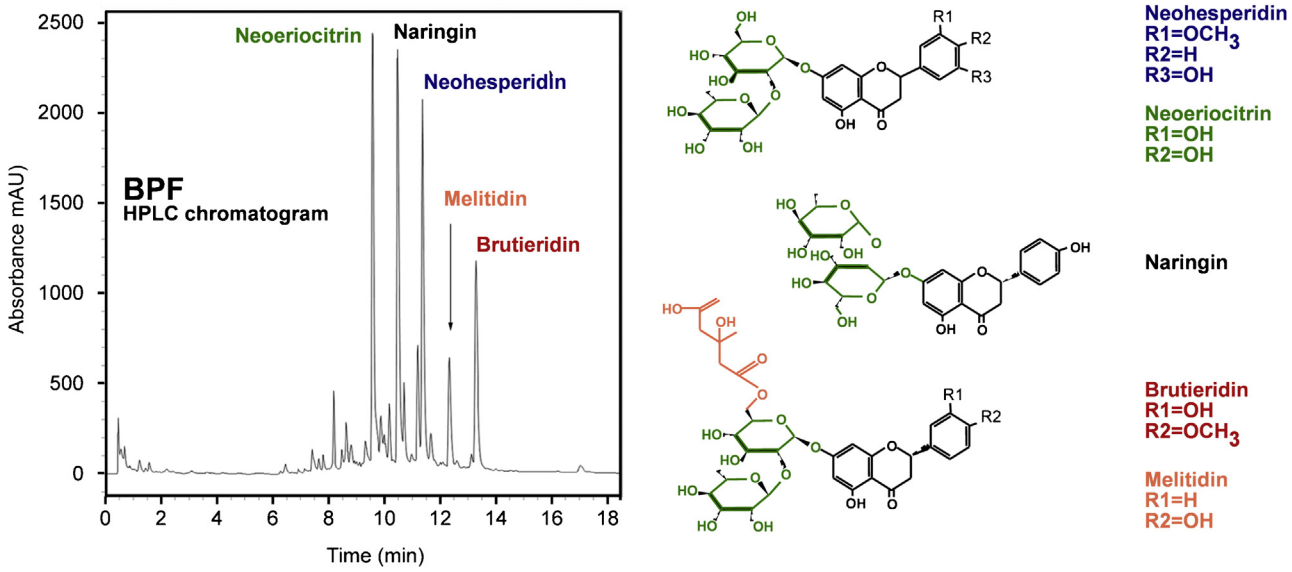

Fig 1. Typical HPLC (High Pressure Liquid Chromatography) profile of Bergamot Polyphenol Fraction (BPF). The names of the most abundant flavonoids are indicated on the corresponding peaks in the chromatogram. The remaining peaks are indicated by the retention times. Structures of the main flavanones present in BPF are displayed on the right.

aglycones are subjected to intestinal metabolism, such as glucuronidation and sulfation. Phase II metabolism is the main route of metabolism for polyphenols. Conjugation of free phenolic groups *via* glucuronidation and/or sulfation will increase their polarity and water solubility, enabling their elimination from the body [12]. Generally citrus and other flavonoids have a short lifetime and it is unclear if their metabolites retain some biological activity. Since sulfonated or methylated metabolites of resveratrol retain full or partial activity of the parent compound [19], it is likely, that *Citrus* flavonoid metabolites behave in a similar way.

Therefore although not properly investigated, it is likely both aglycones of citrus flavonoids and their metabolites contribute to the modulation of the intracellular targets and the subsequent biological response. In such a case, the concentration of these compounds achieved in the plasma would suggest that they elicit *in vivo* majority of the molecular effects discovered *in vitro*, even though bioavailability of citrus flavonoids is low and they have short half-life.

3. Molecular targets and antidiabetic/antilipemic effects of flavonoids

Several mechanisms have been proposed to explain anti-diabetic and anti-lipemic effects of flavonoids, but AMP kinase (AMPK) activation, discussed in the Section 3.1, seems the most plausible. AMPK is a crucial regulator of glucose and fatty acids metabolism in all tissues. AMPK is also an important target of metformin, a well-known anti-diabetic drug. The evidence discussed in Section 3.1. suggest, that undoubtedly certain flavonoids, including naringin, present in bergamot can activate AMPK, but it is not clear what is the exact molecular mechanism, since neither metformin nor flavonoids bind directly to AMPK [20].

Bergamot polyphenols can act as HMG-CoA reductase inhibitors, thus mimicking statins action. This has been first suggested for naringin and more recently for melitidin and brutieridin. Structural characteristics of latter compounds allow them to mimic the natural substrates of HMG-CoA and block the rate-limiting step in cholesterol synthesis. In Section 3.2, we will critically review the scientific evidence for the inhibition of cholesterol pathway by these flavonoids.

In the Section 3.3. we will discuss a direct flavonoid target identified by computational chemistry studies which is cAMP phosphodiesterase (PDE). PDE inhibition by certain citrus flavonoids has been shown to stimulate lipolysis in liver cells, therby preventing hepatic steatosis [4].

Crystallographic studies provide a more powerful evidence of a direct physical interaction between a compound and the target enzyme. Such studies are available for polyphenol resveratrol and quinone oxidoreductase 2 (QR2/NQO2) [21]. As discussed in Section 3.4, functional studies indicate that resveratrol and other flavonoids, including those present in bergamot potently inhibit QR2 [22]. The problem is that biological function of the direct interaction QR2-flavonoid, is still unkown, although QR2 may play a role in the metabolism of steroids, including cholesterol, as discussed later.

3.1. AMPK activation

AMP (adenosine monophosphate)-activated protein kinase (AMPK) is a master regulator of the metabolic pathways involved in ATP production in mammalian cells. AMPK works as a sensor of AMP/ATP levels. The intracellular AMP increases, when energy status is low and binds to AMPK to allow its activation. AMPK is a serine/threonine protein kinase, consisting of three subunits – α, β, and γ – that are necessary for full activity of the enzyme. The kinase activity resides in the α (catalytic) subunit and it is regulated by the phosphorylation of the threonine residue 172 by the upstream LKB1 kinase, which is crucial for AMPK activation under ATP-depleted conditions. The role of the γ subunit is to bind AMP and to sense adenine nucleotide levels [23,24]. The β subunit of AMPK functions as a scaffold between the α and γ subunits and determines the target specificity of the kinase. Different isoforms have been described for each subunit and their expression can be ubiquitous or tissue specific (myocardium and skeletal muscle) [25,26]. In addition, AMPK regulates cell growth and proliferation as well as apoptosis as amply discussed elsewhere [27] (see Fig. 2) and these effects underline a tumor suppressor function of AMPK.

AMPK can be activated by a serine/threonine kinase also known as LKB1 or inhibited by Protein Kinase A (PKA). LKB1 was first identified as a tumor suppressor protein and germline mutations

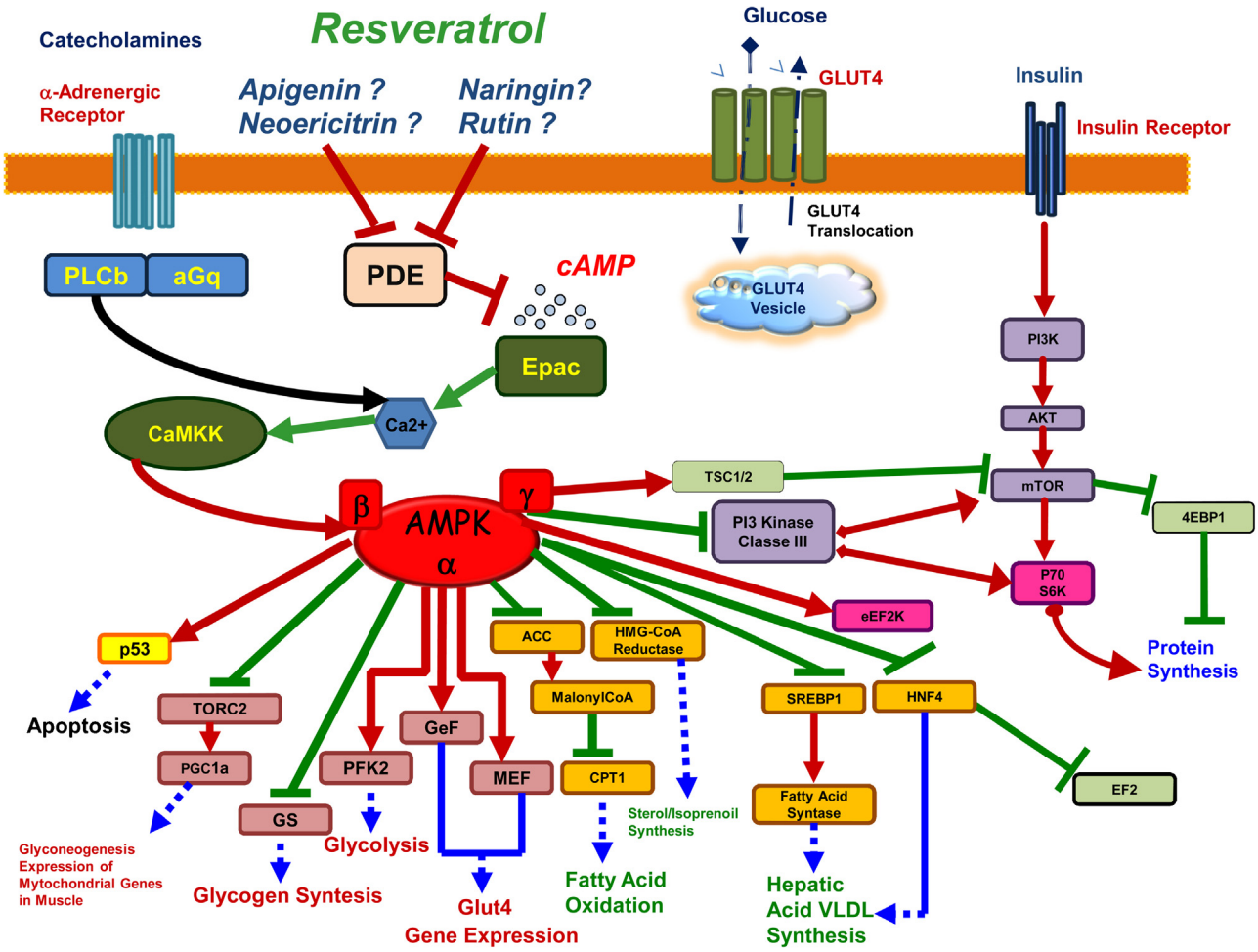

Fig. 2. Metabolic pathways and molecular players regulated by AMPK.
Cascade of events following PDE inhibition by flavonoids leads to AMPK activation which is the central regulator of lipid and glucose metabolism, as well as a negative regulator of oncogenic pathways. This is thanks to AMPK ability to inhibit or activate several enzymes involved in these processes indicated in the figure: ACC (Acetyl-CoA carboxylase), PFK2 (Phosphofructokinase 2), PGC1a (Peroxisome Proliferator—Activated Receptor Gamma Coactivator 1 α), GS (Glycogen synthase), GeF (Glut4 enhancer factor), MEF (myocyte enhancer factor), CPT1 (Carnitine palmitoyltransferase 1), SREBP1 (Sterol regulatory element-binding transcription factor 1), HNF4 (Hepatocyte nuclear factor 4). Other abbreviations depicted in the figure, but not explained in the text: Glut4—Glucose transporter type 4, CaMKK—Calcium/calmodulin-dependent protein kinase, aGq—guanine nucleotide binding protein (G protein), alpha subunit, PLCb phospholipase beta, Epac—Exchange Protein Activated by Cyclic AMP, p70 S6K- p70 S6 kinase, eEF2K—Eukaryotic elongation factor-2 kinase, EF2—elongation factor 2, 4EBP1—Eukaryotic translation initiation factor 4E-binding protein 1.

in LKB1 predispose to cancer (Peutz-Jeghers syndrome). The inhibitory effects of LKB1 on cell growth and proliferation are likely mediated by AMPK, that suppresses mTOR (mammalian target of rapamycin), Akt, and p70S6kinase, *via* stimulation of another tumor suppressor TSC 1/2 (tuberous sclerosis complex 1/2) [28–30] (see Fig. 2). For this reason AMPK has been recently recognized as a target in treating or preventing cancer. More importantly, AMPK is an established target in the treatment of diabetes type II. Metformin, a first line anti-diabetic drug, currently prescribed to more than 120 million people with type II diabetes world wide, activates AMPK and this activation is required for blood glucose lowering effects of the drug.

As shown in Fig. 2, AMPK plays a central role in regulating glucose and lipid metabolism and energy production in several different organs [31]. To ensure the energy production, AMPK, not only facilitates glucose uptake, but it triggers several catabolic and blocks anabolic pathways. First off all, AMPK prevents the accumulation of fat by modulating downstream-signaling components like acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (Fig. 2).

AMPK phosphorylates and inhibits ACC1 activity, which blocks the rate-limiting step in fatty acid synthesis [32,33]. On the other hand, phosphorylation of ACC2 by AMPK augments fatty acid oxidation, which is a catabolic pathway for fat deposits (Fig. 2).

According to the studies on the "fatty mice model", a high-fat diet reduces the basal activity levels of AMPK, suggesting that a certain level of AMPK basal activity may be necessary to prevent fat accumulation [34].

Apparently, a similar mechanisms plays a role in reducing cholesterol synthesis. AMPK phosphorylates 17 HMG-CoA reductase at Ser-871, thereby lowering its catalytic activity [35]. However this mechanism does not play a role in end-product feedback regulation of HMG-CoA reductase, but rather it comes into play when cellular ATP levels are depleted, thereby lowering the rate of cholesterol synthesis and preserving the energy stores of the cell [35,36].

Critical for therapeutic effects of AMPK activation is augmentation of intracellular glucose uptake. This occurs *via* different effectors, and leads to the activation of the glucose transporter GLUT1 in all cells or upregulation and translocation of GLUT4 to the cell membrane in muscle cells [20]. At the same time AMPK

prevents glycogen synthesis to increase glucose availability for energy production and promotes glycolysis. This occurs vis direct inhibition of glycogen synthase and activation of glycolytic enzymes as 6-phosphofrukto-2-kinase (Fig. 2) [20].

There is overwhelming evidence that different flavonoids can activate AMPK, both *in vitro* and *in vivo* as a result of dietary supplementation with flavonoids in animal and human studies, as recently demonstrated [37,38]. From the historical prospective green tea epigallocatechin (ECCG) and soy genistein were the first flavonoids shown to activate AMPK. This effect was correlated with inhibition of adipocyte differentiation and therapeutic effects of ECCG and genistein against obesity [39]. Subsequently, these flavonoids were also shown to lower blood glucose in experimental mice models of diabetes and this was correlated with activation of AMPK [40,41]. However the effect of ECCG and genistein on AMPK is much weaker compared to resveratrol and apigenin, red grape and apple flavonoids respectively. Indeed resveratrol in a mice model of diabetes and dyslipidemia (LDL receptor-deficient mice) is 200 times more potent than metformin in activation of AMPK kinase and its downstream effects [42].

Resveratrol induces AMPK and ACC phosphorylation *in vitro* in hepatocytes, which can explain inhibition of fat accumulation in cells exposed to high glucose [42]. The increase in AMPK activity in response to chronic supplementation of resveratrol to animal diet is very evident in muscle cells of mice fed high-fat diet [43]. The effects of resveratrol in liver *in vivo* on AMPK are more difficult to interpret, but diabetic mice assuming resveratrol with the high-fat diet showed less lipid accumulation in the liver, less blood hyperlipidemia and consequently much less aortic lesions in long-term experiments [43]. Resveratrol beside activating AMPK, increases insulin sensitivity and peroxisome proliferator-activated receptor- gamma coactivator 1alpha (PGC-1alpha) activity, improves mitochondrial number and motor function of muscles in mice and in humans [37,38].

Similarly to other flavonoids, such as rutin [44,45], resveratrol protects the liver from non- alcoholic fatty liver disease and inhibits the expression of genes such as SREBP (sterol regulatory element binding 1protein) and FAS (fatty acid synthase); there is evidence implicating that AMPK is involved in these effects [46]. Moreover, AMPK activity has also been reported to be crucial for the prevention of alcoholic fatty liver, which involves treatment with resveratrol, *via* the regulation of SIRT1 and sterol regulatory element binding protein 1 (SREBP-1) [47].

Thus hypolipidemic and hypoglycemic properties of resveratrol and other flavonoids are associated with beneficial effects on liver metabolism and physiology and they can be well explained by pleiotropic AMPK effects.

AMPK activation is closely associated with action of several other polyphenols implicated in the prevention of various metabolic disorders, including obesity, diabetes, cardiac hypertrophy, and alcoholic and non-alcoholic fatty liver disease. Beside ECCG, quercetin and resveratrol many good activators of AMPK can be found among other groups of polyphenols: ginsenoside, berberine, curcumin and theaflavin [20]. All these compounds show anti-diabetic and hypolipidemic effects in animal models and in some cases in human studies [2,48].

There are limited convincing reportes on the activation of AMPK by classical *Citrus* flavonoids, such as naringin, hesperidin and others. However, our yet unpublished results, (Lascala et al.) suggest that in the model of hepatic steatosis induced by cafeteria (CAF) diet [49], higher AMPK phosphorylation levels were observed in the liver samples from that the BPF-treated animals exposed to CAF diet for 14 weeks, suggesting that BPF supplementation enhances AMPK activity. The increased AMPK activity in rat livers correlated with potent systemic decrease in blood tryglyceride and glucose levels in CAF animals, treated with BPF [49]. In accordance, we could confirm that BPF induces AMPK phosphorylation in hepatoma cells HepG2 in a dose-dependent manner (Fig. 3), indicating that BPF directly stimulates AMPK activity. Interestingly, the analysis of AMPK expression in peripherial polymorphonuclear leukocytes (PMNs) from patients with metabolic syndrome indicated that BPF given orally at 650 mg twice a day for 120 consecutive days, leads to increased AMPK levels (Fig. 4). This suggest that hypolipemic and hypoglycemic activity of BPF is accompanied by an increased AMPK expression *in vivo*.

According to our findings [49], citrus polyphenols present in BPF, such as naringin and neohesperidin have been shown to exert potent hypolipidemic effect and amieliorate atherogenic dyslipidemia in different animal models [50–53]. In a more recent study, mice fed Western diet (high-fat, high sugar diet) for 6 months showed 5-fold increase in fasting plasma triglyceride (TG) and 8-fold increase in cholesterol levels compared with chow-fed animals [54]. These animals developed extensive atherosclerosis in the aortic sinus with 10-fold larger plaque area compared with controls. The addition of naringenin (naringin aglycon) to the Western diet significantly decreased both totChol and TG by 50% and reduced atherosclerotic lesions by more than 70% [54]. The Western diet also induced extensive hepatic steatosis, with a 10-fold increase in both TG and cholesteryl ester mass compared with chow. The addition of naringenin decreased both liver TG and cholesteryl ester mass by 80%.

Convincing results obtained with naringin derivatives is just one example of potent effects of citrus flavonoids *in vivo* animal models of metabolic syndrome induced by high calories and fat intake, revised in recent articles.

This is further confirmed by recent evidence showing that naringenin stimulated glucose uptake and AMPK phosphorylation in L6 myotubes in a dose- and time-dependent manner [55]. Maximum stimulation was seen with 75 μM naringenin for 2 h, a response comparable to maximum insulin response. The translocation of GLUT4 glucose transporters have been indicated as a mechanism underlying the increased glucose uptake. Furthermore,

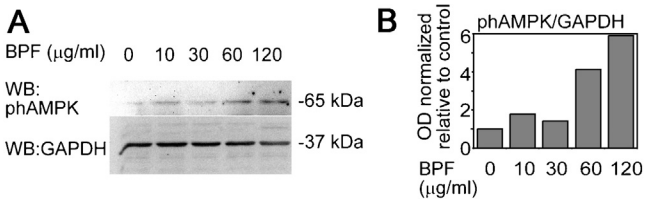

Fig. 3. Dose-dependent effect of BPF on AMPK phosphorylation in HepG2 cells. HepG2 cells were seeded (300.000 cells per 3.5 cm dish) and 48 h later treated with different concentrations of BPF. 24 h later cells were lysed and assessed by Western blotting for AMPK phosphorylation by anti-phospho-AMPK specific antibody and for equal loading by anti-GAPDH antibody (A). Densitometric analysis confirmed dose-dependent increase in phosphor-AMPK levels compared to GAPDH levels (B).

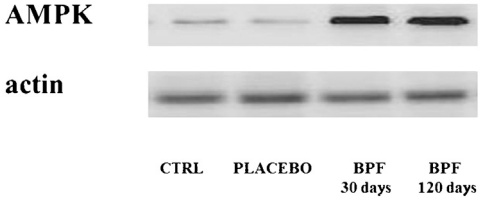

Fig. 4. The effect of BPF on AMPK expression in blood leukocytes from patients with metabolic syndrome. Polymorphonuclear cells (PMNs), collected from the blood of patients with metabolic syndrome undergoing oral treatment with BPF (650 mg twice a day for 120 consecutive days) show an increased expression of AMPK compared to placebo.

silencing of AMPK, using siRNA approach, abolished the naringenin-stimulated glucose uptake. The SIRT1 inhibitors nicotinamide and EX527 did not have an effect on naringenin-stimulated AMPK phosphorylation and glucose uptake, suggesting that naringin aglycon may show anti- diabetic effects *via* AMPK and in contrast to resveratrol, this is not mediated by SIRT1 [55].

Thus, naringenin is an example of bergamot flavanones that can activate AMPK. Another example is rutin. Rutin is a flavon, which is particularly rich in bergamot. Recently, rutin has been shown to induce AMPK in hepatocytes and pancreatic beta cells [56]. In hepatocytes, beside AMPK stimulation, rutin suppressed oleic acid-induced lipid accumulation. The expression of a critical molecule involved in lipid synthesis, SREBP-1 was also attenuated in rutin-treated cells. Moreover, long-term incubation of rutin inhibited the transcriptions of HMG-CoA reductase, ACC, fatty acid synthase (FAS), and other enzymes involved in lipogenesis. All these molecular endpoints can be explained by activation of AMPK [56].

These findings *in vitro*, correlate nicely with results of an independent study *in vivo* with a rat model of metabolic syndrome. Rutin, supplemented to western diet, reversed or prevented metabolic changes such as abdominal fat accumulation and glucose tolerance, normalized expression of liver markers and prevented hepatic steatosis, reversed oxidative stress and inflammation in the liver and heart [44]. AMPK was suggested as the main target of rutin [44].

Taken together, these results suggest that AMPK activation is a molecular target of many natural polyphenols, including naringin and rutin, rich in bergamot and AMPK may be responsible for therapeutic effects of polyphenols against various metabolic disorders, including obesity, diabetes, hyperlipidemia and non-alcoholic fatty liver disease.

3.2. HMG-CoA reductase

Bergamot juice and albedo and derivatives as BPF contains two rare flavonoids, brutieridin and melitidi that have been suggested to act as direct HMG-CoA reductase inhibitors [15,57]. Lower amounts of melitidin and brutieridin are also found in sour orange (*Citrus aurantium* L.) [14] and chinotto (*Citrus* × *myrtifolia* Raf.) [13]. Computational studies have demonstrated that both molecules bind efficiently to the catalytic site of HMG-CoA reductase [57]. This is because these two flavonoids contain a 3-hydroxy-3-methyl-glutarlyl-moiety (HMG) covalently linked to sugar residue of naringenin (melitidin) or hesperitin (brutieridin), which is identical to S-linked 3-hydroxy-3-methyl-glutarlyl residue of HMG-coenzyme A (HMG-CoA), a substrate of HMG-CoA reductase. This enzyme reduces HMG-CoA to mevalonate, which is a precursor of later steps in cholesterol synthesis. HMG-CoA reductase cannot hydrolase R-linked HMG which competes with the S-linked substrate for catalytic pocket of the enzyme. However, beside convincing theoretical consideration regarding the mechanisms of inhibition of HMG-CoA reductase by these two HMG-modified polyphenols, proposed by Sindona and co-workers [15,57], the direct demonstration of statin-like activity *in vitro* and in assays for HMG-CoA activity and their comparison to statins is still missing. It is also not clear if they exert any activity if administered to animals as purified compounds. In fact, the potent HMG-CoA inhibition shown in blood samples of patients receiving BPF in a clinical trial reported by Mollace el al. [5] can result from the activation of AMPK, as discussed in the previous section. In addition, the potent hypolipidemic effects of a citrus peel extract from tangerines, lemons or oranges, that do not contain as far as we know any brutieridin and melitidin, but mainly hesperidin, naringin and pectins, causes a significant inhibition of HMG-CoA reductase and another enzyme of cholesterol biosynthesis pathway, which is ACAT (Acyl-CoA: cholesterol acyltransferase) in animal livers [58]. This suggest that an inhibition of HMG-CoA reductase can be achieved *in vivo* by citrus polyphenol mixtures, devoid of brutieridin and melitidin. In addition, it is difficult to indicate a single active compound in these mixtures, responsible of HMG-CoA reductase inhibition. Although naringenin has been proposed to act as a direct inhibitor of cholesterol synthesis, this possibility has not been properly addressed by testing narigenin for statin-like activity *in vitro* [52,53] and in human trials pure preparations of naringin and hesperidin did not reduced cholesterol, cLDL and tryglicerides blood levels in moderately hyperlipemic patients [59]. In fact, it is possible that HMG-CoA reductase inhibition *in vivo* is a complex effect of cooperation of AMPK inhibitory properties of naringin with other properties of flavonoids, including PDE inhibition, ROS scavenging and other molecular and physiological effects. This is confirmed by evidence that the use of BPF in patients is accompanied by an enhanced elevation of urinary mevalonic acid excretion [5].

3.3. PDE inhibition and lipolysis

Other potential targets of bergamot flavonoids could be cyclic nucleotide phosphodiesterases (PDEs). PDEs catalyze hydrolysis of cAMP (cyclic adenosine monophosphate) or cGMP, regulate their intracellular concentrations and, consequently, the physiological effects of these second messengers. PDEs belong to a complex and diverse superfamily of at least structurally related, highly regulated, and functionally distinct gene families (PDE1–PDE11) [60]. PDE3B, and certain PDE4 isoforms are characterized by their high affinity for cAMP and play critical roles in regulating energy metabolism and lipolysis [4]. The rate of triglyceride (TG) hydrolysis (lipolysis) is positively regulated by increase in intracellular cAMP. In fact, cAMP activates, a cAMP dependent protein kinase, PKA which stimulates hormone-sensitive lipase (HSL), one of the key molecules controlling lipolysis, by promoting its phosphorylation. Certain flavonoids can induce lipolysis in adipose tissue, likely through inhibition of PDEs and antagonism of cAMP degradation. This could explain weight control and anti-obesity effects of dietary supplements rich in citrus flavonoids [61]. cAMP signaling pathways also modulate gluconeogenesis, glycogenolysis and insulin secretion [62].

The structure–activity relationships of flavonoids with regard to their inhibitory effects on PDE isozymes are subject of an intensive research. Computational chemistry studies indicate that certain flavonoids can sterically fit in the cyclic nucleotide binding pocket of PDE and mediate competitive inhibition of the enzyme activity. The positions of hydroxyl groups on polyphenol ring C of flavones and flavanones are important for differential PDE inhibition. For example, the C-4′ hydroxyl group of flavones is very important for PDE3 inhibition; hydroxylation at the C-5 position is important for inhibition of PDE1, PDE2, PDE4, and PDE5; and C-7 hydroxylation is important for inhibition of PDE1, PDE3, and PDE4 [63,64]. The most potent phosphodiesterase (PDE) inhibitors are aglycones that have a C2.3 double bond, a keto group at C4 and hydroxyls at C3′ and/or C4′. However, when the C-ring is opened the requirement for the C2.3 double bond is eliminated.

Accordingly, flavonoids such as apigenin, genistein, daidzein, and quercet in fit very well in the catalytic site of x-ray crystallographic models of human PDE3B, PDE4B, and PDE4D. Similarly, computational chemistry considerations suggest that neoeriocitrin, rich in bergamot juice and albedo, can be also a potent inhibitor of PDE3.

There are two different PDE3 isoforms A and B. They exhibit cell-specific differences in expression. PDE3A is highly expressed primarily in the cardiovascular system, for example in platelets,

smooth muscle cells and cardiac myocytes [65]. PDE3B is expressed in cells of importance for energy metabolism including hepatocytes, brown and white adipocytes, pancreatic β cells [66,67] and it is also found in the cardiovascular system [65]. One characteristic of PDE3B isoform is that the pharmacological attenuation of PDE3B activity increases catecholamine-induced and cAMP mediated catabolism, decreases fat accumulation and glucose storage [68]. PDE3B is activated in response to insulin, IGF-1, and leptin [69] *via* PI3K-dependent signals [68]. PDE3B is also involved in regulation of insulin-induced glucose uptake, glucose transporter–4 (GLUT-4) translocation, and lipogenesis [70,71]. Inhibition of PDE3B in adipocytes favors lipolysis and prevents lipogenic action of insulin, while in hepatocytes, it promotes cAMP-induced lipolysis together with glycogenolysis [4]. Molecular events leading to induction of lipolysis in the liver, according to Peluso et al. [4], are depicted in Fig. 5.

Interestingly, lack of functional of PDE3B in the liver leads to a severe dysregulation of glucose, triglyceride and cholesterol metabolism, suggesting that the modulatory role of PDE3B is crucial for lipid and glucose homeostasis [68,72]. Hepatocytes from PDE3B knock out mice display increased glucose, triglyceride and cholesterol levels, which was associated with increased expression of gluconeogenic and lipogenic genes/enzymes including, HMG-CoA reductase, phosphoenolpyruvate carboxykinase, peroxisome proliferator-activated receptor γ (PPARg) and sterol regulatory element-binding protein 1c (SREBP 1c). Dysregulation of PDE3B can have a role in the development of fatty liver, which is very common in metabolic syndrome and type 2 diabetes patients [72]. It is very likely that bergamot flavonoids mediate their beneficial effects on lipid and glucose homeostasis by PDE3B modulation, but formally this hypothesis needs to be verified by experimental results.

3.4. QR2 and other targets of flavonoids

One of the highest affinity targets of flavonoids that has been identified up-to-date is quinone oxidoreductase 2 (QR2), also known as NRH:quinone oxidoreductase 2 (NQO2). The physical interaction between resveratrol and QR2 has been characterized by crystallography [21]. In addition, resveratrol, quercetin and other flavonoids, including those found in bergamot juice are potent inhibitors of QR2 with IC50 values between 50 nM and 2 μM [22]. QR2 is highly flexible flavoenzyme in terms of substrate and co-substrate and may catalyze many oxido-reduction reactions, involving xenobiotic and biogenic substrates [73]. The typical reaction catalyzed by this enzyme is a two-electron reduction of quinones and oxidation of *N*-alkyl and *N*-ribosyl nicotinamide (NADH) derivatives, such as NRH [74]. Biological functions of QR2 are largely unkown, but it has been suggested that QR2 is involved in detoxification processes of endogenous toxic quinone derivatives of dopamine and estrogen [75] and it has been proposed to act as tumor suppressor [76] and as a regulator of autophagy and oxidative stress [77]. Interestingly, this enzyme plays totally opposite functions with respect to several exogenous substrates

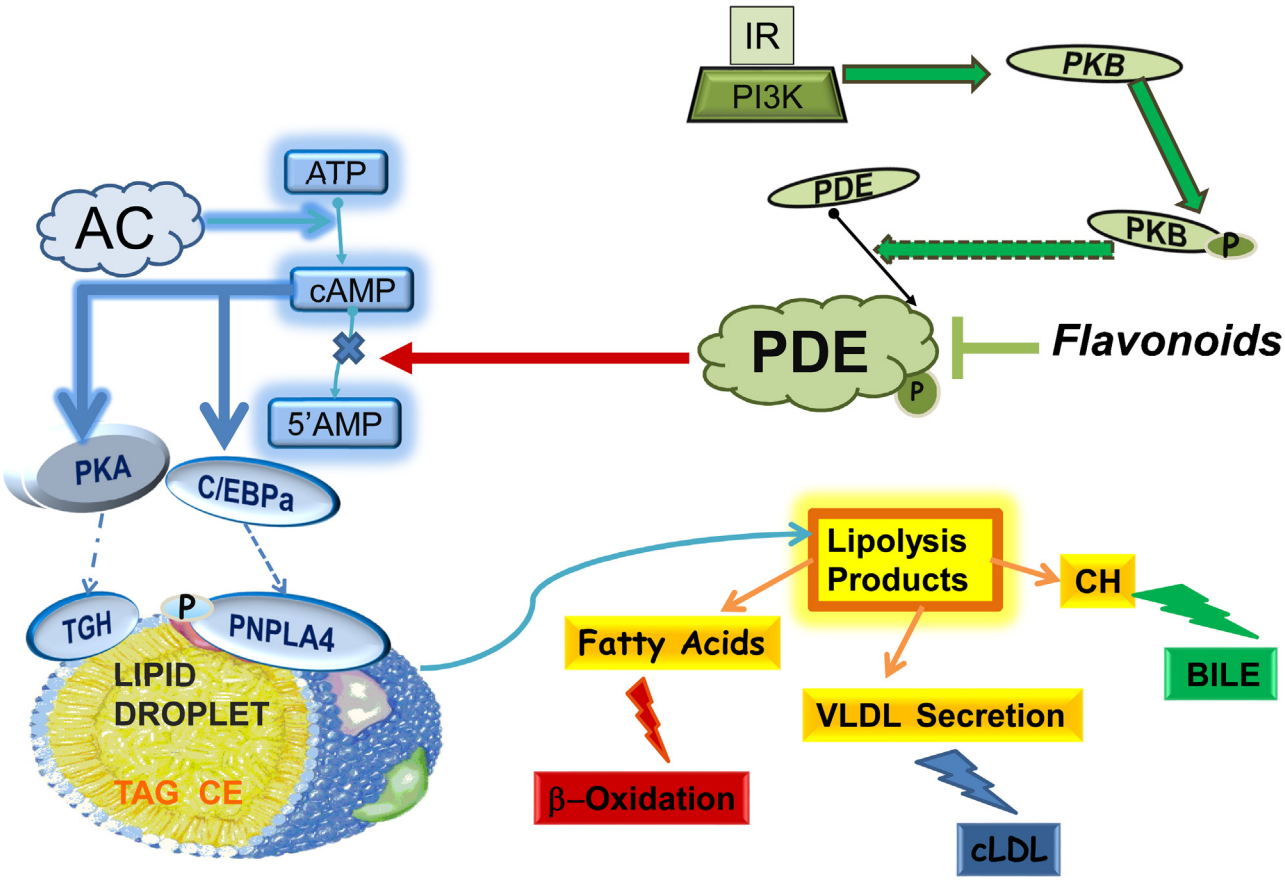

Fig. 5. Regulation of lipolysis by phosphodiesterasen (PDE) inhibition in the liver. PDE3B or related PDE isoforms are expressed in hepatocytes and are involved in the regulation of lipolysis (see text). *Citrus* flavonoids or resveratrol inhibit PDE causing an increase in cAMP levels that activates PKA (Protein Kinase A) and C/EBPα (CCAAT/Enhancer Binding Protein α). PKA stimulates triacylglycerol hydrolase (TGH) leading to lipid hydrolysis, while C/EBPα is a transcription factor regulating a series of genes involved in lipid catabolism. Activation of both pathways leads to lipid droplet breakdown and liberation of free fatty acids (FA) and cholesterol (CH) in the liver. FA can be catabolized in the beta oxidation process to produce energy, while majority of CH is used for the bile synthesis or bound to VLDL and released to the bloodstream.

(mainly quinone or nitrogen compounds) and many reaction products of QR2 appear to be toxic. In fact, QR2 mediates toxicity of menadione, a toxic variant of vitamin K. QR2 is crucially involved in the activation of mitomycin C, an anticancer antibiotic used for treatment of bladder cancer and other tumors, and for the bioactivation of the synthetic anticancer agent CB1954 [78]. Bioactivation of these drugs occurs *via* either 2- or 4- electron reductions. QR2 also mediates toxicity of certain herbicides like paraquat. However, in this case paraquat is not a direct substrate of QR2- mediated oxidoreduction, but QR2 is crucially involved in the generation of oxidative stress in the presence of the herbicide [77,79]. Thus, QR2 is capable of metabolically activating various quinones and other compounds, which generates oxidative stress and can ultimately lead to cytotoxicity and cell death [73,79]. In addition, according to our observations, QR2 is likely involved in the regulation of ROS levels and metabolic oxidative stress in the absence of toxic exogenous substrates [77,79]. Therefore inhibition of QR2 by resveratrol or citrus flavonoids might protect cells from harmful metabolically-activated compounds and resulting oxidative stress, which is a common denominator of several metabolic dysfunctions, including metabolic syndrome, diabetes, atherosclerosis and many others. Up to date, it is not clear if QR2 can be directly involved in lipid and glucose metabolism, but our preliminary data suggest that the inhibition of QR2 by specific inhibitors (NMDPEF) and flavonoids (resveratrol, BPF) reduces glucose utilization and glycolysis by favoring mitochondrial oxidative phosphorylation. This potently reduces lactic acid generation and resulting acidification of cell environment (Janda, unpublished observations).

These findings may suggest that, beside regulation of drug metabolism, QR2 is crucially involved in basic energy and glucose metabolism and the modulation of QR2 activity by flavonoids likely contributes to beneficial anti-diabetic and hypolipemic effects of these compounds. Future experimental data in cellular and animal models of metabolic syndrome, should test this attractive hypothesis.

4. Which works better: single active polyphenol or a mixture of natural compounds?

The review of literature on hypolipemic and hypoglycemic effects of natural polyphenols in animal models of diabetes or metabolic syndrome or in few human trials suggest a discrepancy between rather poor or no benefits from a single flavonoid-based therapy and good results with fitocomplex mixtures. The study addressing the efficacy of individual citrus flavonoids, naringin, hesperidin on totChol, cLDL, cHDL and blood triglycerides (TC) reported lack of significant effects in patients with moderate hypercholesterolemia [59]. This placebo-controlled human study recruited a total of 204 healthy men and women with moderately elevated serum levels. During the 4-wk intervention, the participants consumed 4 capsules/d providing either placebo

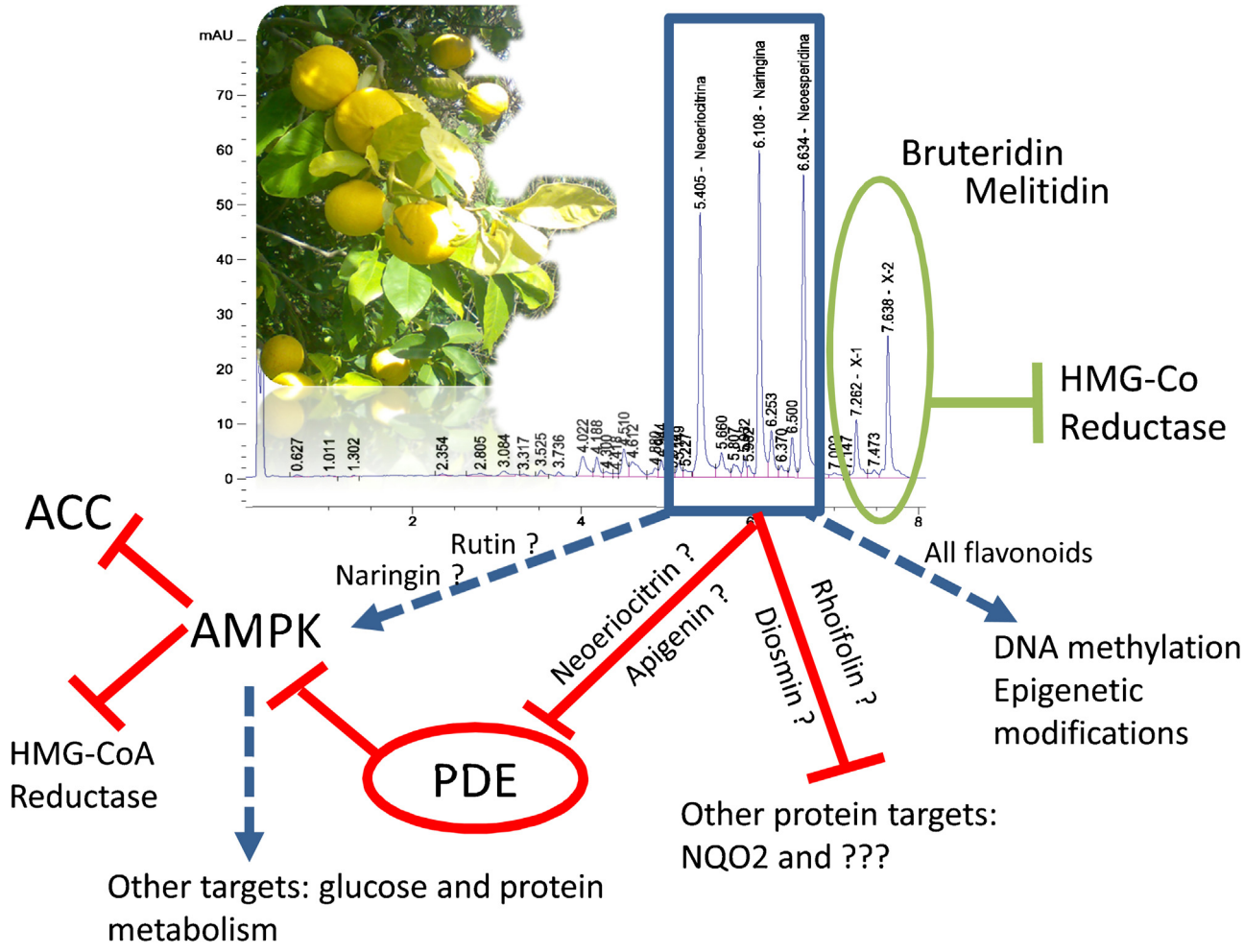

Fig. 6. Possible molecular targets of bergamot flavonoids.
Summary of possible molecular mechanisms which are likely triggered by bergamot polyphenols and are discussed in this review.

(cellulose) or a daily dose of 800 mg hesperidin or 500 mg naringin. The citrus flavonoids did not affect blood parameters with endpoint cLDL concentrations of 4.00 ± 0.04, 3.99 ± 0.04, and 3.99 ± 0.04 mmol/L for control, hesperidin, and naringin groups, respectively. Naringin and hesperidin also did not affect serum HDL-cholesterol and triglyceride concentrations. In contrast, the results obtained with the mixture of bergamot juice polyphenols *i.e.* BPF, in clinical trial by Mollace et al. and further confirmed in studies of R. Walker are much better than "statistically significant". These result are even more remarkable, if we consider that the low dose of BPF, such as 500 mg/day/patient, containing as little 200 mg total bergamot polyphenols, was able to induce a significant improvement in blood tChol, cLDL, cHDL and tryglicerides, in 3 independent groups of patients suffering from hypercholesterolemia, mixed hyperlipidemia or metabolic syndrome. The results of both clinical trials may suggest that neohesperidin and naringin, highly abundant in BPF, are not the active compounds in humans, in contrast to mice (see Section 3.1 and 3.2) and other less abundant bergamot polyphenols exert the hypolipemic effects. However, the enhanced activity of compounds in a natural *fitocomplex* can be another possible explanation. In fact, there are many other studies in literature showing good results obtained with natural polyphenol mixtures [48], but rather poor results with a single active compound, for example: the effect of naringenin *vs* citrus peel extract in animal models of dyslipidemia [53,58] or rather poor results obtained with resveratrol in clinical trials addressing anti-diabetic effects and very good antilipemic effects obtained with grape juice extract [80,81]. We propose that good performance of mixtures like BPF in human studies can be explained by pleiotropic and synergistic effects of constituents in the mixture of natural polyphenols, acting on different pathways and molecular targets, that ultimately lead to a clear reduction in cardiovascular risk factors.

As far as we know, this simple concept has never been properly addressed by direct comparison of pharmacological effects of a mixture *vs* a single compound and thus remains as a plausible hypothesis to be tested in the future.

5. Conclusion

Citrus and bergamot polyphenols, in particular, may exert numerous effects on cellular and organism homeostasis, including the regulation of fat, cholesterol and carbohydrate metabolism. Given the fact, that pharmacological effects of nutraceutical preparations based on bergamot flavonoids appear to be comparable to low-dose synthetic drugs, while side-effects are negligible, they represent a valid alternative for conventional drugs. In fact, bergamot BPF has a potential to become a "one pill" natural therapy against metabolic syndrome and dyslipidemias. However, up to date there is limited scientific evidence on how bergamot polyphenols can act at the molecular level. As summarized in Fig. 6, bergamot polyphenols can exert their beneficial effects through AMPK activation or HMG-Co reductase, PDE and QR2 inhibition or by other unknown signaling or metabolic regulatory proteins and downstream pathways.

In this context, the strongest evidence is for AMPK activation by certain flavonoids, including naringin and rutin, but it is not clear what is the molecular mechanism of this activation, since flavonoids do not bind directly to AMPK. However, with the exception of naringin, and few reports on rutin effects, other individual polyphenols present in bergamot have not been studied thoroughly. This is mainly because the majority of flavonoids present in bergamot are relatively rare. Structure-activity relationship studies performed with other polyphenolic compounds offer many predictions to explain the mechanism of action of bergamot flavonoids, but as long as they are not tested empirically, they remain as scientific hypotheses. In addition, as many reports suggest, the pharmacological and molecular effects of a "fitocomplex" or a mixture of active compounds as they occur in natural plants, may be quite different then the sum of individual activities, due to compensatory or synergistic effects of the mixture.

Thus, it appears clear that bergamot polyphenols and BPF itself should be tested in established model systems *in vitro* and *in vivo* to gain insights into the mechanisms of their action. Therefore, beside the need for further testing of BPF in multicentered double-blind clinical trials, a translational study addressing possible mechanisms of action of BPF is required.

Conflict of interest

The authors declare no conflict of interest.

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