Type of the Paper (Article)

Differential effects of red yeast rice, Berberis aristata and Morus alba extracts, active components of LopiGLIK®, on PCSK9 in HepG2 cells

Maria Giovanna Lupo[1], Chiara Macchi[2], Silvia Marchianò[2], Haixia Chen[3], Cesare Sirtori[4], Alberto Corsini[2,5], Massimiliano Ruscica[2], Nicola Ferri[1]

[1] Dipartimento di Scienze del Farmaco, Università degli Studi di Padova, Padua, ITALY
[2] Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, ITALY
[3] Tianjin Key Laboratory for Modern Drug Delivery & High-Efficiency, School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, China
[4] Dyslipidemia Center, A.S.S.T. Grande Ospedale Metropolitano Niguarda, Milan, Italy 5IRCCS Multimedica, Milan, Italy.

* Correspondence: Massimiliano Ruscica, Via Balzaretti 9, 20133, Milan, Italy; tel: 00390250318220

Email: massimiliano.ruscica@unimi.it

Abstract: Proprotein convertase subtilisin/kexin type 9 (PCSK9) is the key regulator of low-density lipoprotein cholesterol (LDL-C) plasma levels. We previously observed that treatment of dyslipidemic subjects with nutraceutical combination containing red yeast rice (monacolin K 3.3 mg), Berberis aristata cortex extract (Berberine 531.25 mg) and Morus alba leaves extract (1-deoxynojirimycin 4 mg) (LopiGLIK®) did not alter the plasma PCSK9 levels. Thus, the aim of the present study was to investigate the effect of these three components on PCSK9 expression in HepG2 cell line in relationship to their effects on LDL-C cellular uptake. HepG2 cell line were incubated with Berberis aristata cortex extract (BCE), red yeast rice (RYR) and Morus alba leaves extract (MLE) alone or in combination for 24h. RYR (50 μg/mL) increased PCSK9 protein expression (WB and ELISA assays), PCSK9 mRNA and its promoter activity. BCE (40 μg/mL) reduced PCSK9 expression, mRNA levels and promoter activity. MLE determined a concentration-dependent inhibition of PCSK9 at mRNA and protein levels, with a maximal reduction at 1 mg/mL; no significant changes in PCSK9 promoter activity were found. MLE also downregulates the expression of fatty acid synthase and HMG-CoA reductase mRNA levels. The combination of RYR, BCE and MLE reduced PCSK9 at mRNA, protein, and promoter activity. Finally, this combination induced the LDL receptor and LDL-C uptake by HepG2 cells. In conclusion, the positive effect of MLE on PCSK9 supports the rational of using this nutraceutical combination to control hyperlipidemic conditions.

Keywords: red yeast rice, berberis aristate, morus alba, PCSK9

1. Introduction

Innovative nutritional strategies to control dyslipidaemias have been developed and the ESC/EAS Guidelines for the Management of Dyslipidaemias encourages the consumption of the so-called nutraceuticals as alternatives or in addition to lipid-lowering drugs [1]. Several nutraceutical combinations have shown significant lipid lowering effects and potential positive impact on cardiovascular risk [2]. LopiGLIK® possesses a specific composition represented by red yeast rice

 doi:10.20944/preprints201810.0288.v1

(monacolin K 3.3 mg), Berberis aristata cortex extract (Berberine 531.25 mg) and Morus alba leaves extract (1-deoxynojirimycin 4mg). The clinical efficacy of this nutraceutical combination was recently evaluated in a randomized controlled trial that recruited subjects with mild hypercholesterolemia and not on statin therapy [3]. A 16 week treatment led to a 21.9% reduction of low-density-lipoprotein-cholesterol (LDL-C) with 72% subjects achieving LDL-C levels below 130 mg/dl [3]. The analysis of a subgroup of these subjects, demonstrated an improvement of the serum lipoprotein functional profile without any effect on proprotein convertase subtilisin/kexin type 9 (PCSK9) plasma levels [4].

PCSK9 plays a pivotal role on the regulation of LDL-C levels by acting on the degradation of the LDL receptor (LDLR), although many other physiological functions beyond lipid metabolism have also been reported [5-13]. The relevance of PCSK9 as new pharmacological target for controlling hypercholesterolemia and cardiovascular diseases (CVD) has been firmly demonstrated by the positive results of the FOURIER (Further Cardiovascular Outcomes Research with PCSK9 Inhibition in Subjects with Elevated Risk) trial, conducted by treating CVD patients with the monoclonal antibody anti PCSK9, evolocumab [14].

Many nutraceuticals have been shown to modulate PCSK9 expression, including berberine [15], curcumin [16], moracin C [17], and lignans [18]. In addition, statins induce PCSK9 in vitro [19,20], and in clinical trials [21-24]. The mechanism by which statin induce PCSK9 is dependent on the activation of the sterol responsive element binding protein (SREBP) transcriptional activity [25,26], and by the presence of the small G protein Rac1 [27]. On this regards, it should bear in mind that SREBP-2 preferentially transcribes for genes involved in the cholesterol biosynthetic pathway while SREBP-1a and SREBP-1c preferentially activate genes involved in the synthesis of fatty acids and triglycerides [28,29]. Differently from many of the SREBP-regulated genes, PCSK9 synthesis is controlled at the gene transcriptional level by both SREBPs [21,26] and the hepatocyte nuclear factor-1 (HNF-1) alpha [30,31].

Since monacolin K, the key component of RYR, is chemically indistinguishable from lovastatin, it is conceivable to hypothesize its inducible effect on PCSK9. On the contrary, berberine does not influence the SREBP pathway but rather increases the expression of the LDLR by stabilizing its mRNA [32]. Nevertheless, additional experiments suggest that berberine inhibits the PCSK9 promoter activity by reducing both the HNF1α and SREBP expression [15,30,31]. Indeed, at the promoter level, the inhibitory effect of berberine is partially abolished by each single mutation of SRE or HNF1 site of the PCSK9 promoter [30]. This effect counteracts the induction of PCSK9 by statins [30], and for this reason berberine may be consider useful in combination to statins [15]. The involvement of HNF1α transcription factor on the inhibitory effect of berberine on PCSK9 has also been confirmed in vivo and to be dependent from the proteasomal pathway [31]. Overall, the basic molecular mechanisms underlying the opposite effects of statins (positive) and berberine (negative) on the transcription of PCSK9 are defined. On the contrary, much less is known on Morus alba leaves extract, the third component of LopiGLIK®. The leaves of Morus alba have been used as a remedy since ancient times, and have many pharmacological effects, such as antidiabetic, hypolipidemic, antiatherogenic, antibacterial, anticancer, cardiovascular, antioxidant, and anti-inflammatory [33].

An additional property of the major component of Morus alba, i.e. 1-deoxynojirimycic (DNJ), is its inhibitory activity on α-glycosidase. This leads to a significant antiobesity effect of DNJ in models of experimental obesity [34]. Body weight reductions of around 20% are reported upon a one-month study. When glucose tolerance evaluations are carried out, these are characterized by improved glycemia and a markedly reduced insulinemic response. The α-glycosidase inhibitory activity appears to be superior to that of acarbose and potentially this treatment may reduce the development of diabetes in experimental animals, thus suggesting the application in clinical condition [35].

Experimental evidence also supports a positive effect of Morus alba on cholesterol and lipid metabolism. For instance, the 3% supplementation of dry Morus alba leaves powder in an atherogenic diet, determined hypolipidemic effects in LDLR null mice with a significant attenuation of atherosclerotic lesion development [36]. The lipid lowering and antiatherosclerotic effects could be related to the presence of quercetin 3-(6-Malonylglucoside), one of the most abundant flavonoid

 doi:10.20944/preprints201810.0288.v1

present in Morus alba leaves [36]. A hypolipidemic action was observed in hypercholesterolemic rats after supplementation of methanol fraction of Morus alba root bark extract [37]. The supplementation of Morus alba leaves in normal chow diet of apoE null mice also demonstrated an antiatherosclerotic effect, although no changes in lipid levels were observed [38]. Finally, Morus alba fruits water extracts showed a significant hypocholesterolemic effect in hamsters fed high fat (cholesterol diet [39]. Although the incubation of HepG2 cell line with Morus alba fruits water extracts was shown to affect the SREBP-transcriptional dependent genes, fatty acid synthase and 3-hydroxy-3-methyl-3-glutaryl coenzyme A (HMG-CoA) reductase, with a concomitant increase of LDLR and LDL uptake [39], the molecular mechanism underlying the hypolipidemic effect of Morus alba is still unknown.

Thus, the aim of the present study was to define the effect of the LopiGLIK® nutraceutical combination on genes involved in cholesterol homeostasis, including PCSK9, and thus the molecular mechanism underlying its hypocholesterolemic effect.

2. Materials and Methods

2.1 Reagents

Eagle's minimum essential medium (MEM) was purchased from Sigma, trypsin-EDTA, penicillin, streptomycin, sodium pyruvate, non-essential amino acid solution, fetal calf serum (FCS), plates and Petri dishes were purchased from EuroClone. RYR (220 mg contains 3.3 mg of monacolin K), Berberis aristata cortex extract (BCE; 625 mg contains 531.25 mg of berberine) and Morus alba leaves extract (MLE; 200 mg contains 4 mg of DNJ) powders were supplied by Akademy Pharma Srl (Milano, Italy). BCE and RYR were dissolved in DMSO while MLE was dissolved in H20, filtered and stored at -20°C. Actinomycin D was purchased from SIGMA, dissolved in DMSO and stored at -20°C.

2.2 Cell cultures

Human hepatic cancer cell line (HepG2) were cultured in MEM supplemented with 10% FCS, L-glutamine, sodium-pyruvate and non-essential amino acids, penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

2.3 Cell viability assay

Cell viability was determined by sulphorhodamine B (SRB) assay, as previously described [40]. Cells were seeded in a 96-well tray ($5*10^3$ cells/well) and after 24h treated with nutraceutical extracts at indicated concentrations. SRB assay was then performed after 48h incubation.

2.4 Retrotranscription and quantitative PCR (RT-qPCR)

RNA Preparation and Quantitative Real Time PCR-Total RNA was extracted with the iScriptTM RT-qPCR Sample Preparation Buffer (BIO-RAD) cDNA synthesis preparation reagents (Bio-Rad) according to manufacturer's instructions. Reverse transcription-polymerase first-strand cDNA synthesis was performed by using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) [41]. qPCR was then performed by using the PowerUpTM SYBRTM Green Master Mix (Thermo Scientific) and specific primers for selected genes. Primer sequences used for qPCR analysis are shown in table. The analyses were performed with the Mx3000P qPCR System (Agilent), with the following cycling conditions: 95°C, 2 min; 95°C, 15 sec and 60°C, 1 min for 40 cycles. Data were expressed as Ct values and used for the relative quantification of targets with the ΔΔCt calculation.

Table 1. Primer sequence utilized for the qPCR analysis.

Gene	Forward	Reverse
PCSK9	5′- CCTGCGCGTGCTCAACT-3′	5′- GCTGGCTTTTCCGAAACTC-3′

 doi:10.20944/preprints201810.0288.v1

HMG-CoA reductase	5′-CTTGTGTGTCCTTGGTATTAGAGCTT-3′	5′-GCTGAGCTGCCAAATTGGA-3′
LDLR	5′-TCTATGGAAGAACTGGCGGC-3′	5′-ACCATCTGTCTCGAGGGGTA-3′
FAS	5′-GCAAATTCGACCTTTCTCAGAAC-3′	5′-GGACCCCGTGGAATGTCA-3′
18S	5′-CGGCTACCACATCCACGGAA-3′	5′-CCTGAATTGTTATTTTTCGTCACTACC-3′

2.5 Western blot analysis

Cells were washed twice with PBS and lysed with a solution of 50mM Tris pH 7.5, 150mM NaCl, 0.5% Nonidet-P40, containing a protease and phosphatase inhibitor cocktails (SIGMA, Milan, Italy) for 30 min. on ice. Twenty μg of proteins and a molecular mass marker (Thermo Scientific) were separated on 4–12% SDS-PAGE (BIO-RAD) under denaturing and reducing conditions. Proteins were then transferred to a nitrocellulose membrane by using the Trans-Blot® Turbo™ Transfer System (BIO-RAD). The membranes were washed with Tris-buffered saline-Tween 20 (TBS-T), and nonspecific binding sites were blocked in TBS-T containing 5% nonfat dried milk for 60 min at room temperature. The blots were incubated overnight at 4 °C with a diluted solution (5% nonfat dried milk) of the following human primary antibodies: anti-PCSK9 (mouse monoclonal antibody, Abcam ab84041; dilution 1:1000), anti SREBP-2 (rabbit polyclonal antibody, Abcam ab30682; dilution 1:2000), anti HNF1α (rabbit polyclonal antibody, Abcam ab96777; dilution 1:1000), anti LDLR (mouse monoclonal antibody, Millipore clone 2H7.1; dilution 1:1000) and anti-α-tubulin (mouse monoclonal antibody, Sigma clone DM1A; dilution 1:2.000). Membranes were washed with TBS-T and then exposed for 90 min at room temperature to a diluted solution (5% nonfat dried milk) of the secondary antibodies (peroxidase-conjugate goat anti-rabbit, and anti-mouse, Jackson Immunoresearch). Immunoreactive bands were detected by exposing the membranes to ClarityTM Western ECL chemiluminescent substrates (Bio-Rad) for 5 min, and images were acquired with a VersaDoc 4000 Imaging System (Bio-Rad) [42]. Densitometric readings were evaluated using the ImageLabTM software as previously described.

2.6 ELISA assay

Conditioned media were cleared by centrifugation (13,000 rpm for 10 min at 4°C) and store at -20 °C. The amount of PCSK9 was then quantified by using the ELISA assays (R&D System) according to manufacturer's instructions and as previously described [9].

2.7 Luciferase reported promoted activities assay

HepG2 cells were seeded into 60mm Petri dished (6 x 105 cells/dish). The day after, the cells were transfected with pGL3-PCSK9-D4 and pGL3-PCSK9-D1 constructs [26] by using TurboFect reagent (Thermo Fisher), as previously described [43]. The plasmid pGL3-PCSK9-D4 contains the 5′ flanking region of the PCSK9 gene from -440 to -94 relative to the ATG start codon as, while the pGL3-PCSK9-D4 contains the region from -1711 to -94. Twenty-four hours post transfection, cells were seeded in a 48-well tray (8 x 104 cells/well) and treatments were performed after 24h. After 24h of treatment, luciferase activity was assessed by using NeoLite reagent (PerkinElmer), according to manufacturer's instructions.

 doi:10.20944/preprints201810.0288.v1

2.8 LDL-isolation and labelling

Total LDL (d >1.019 <1.063 g/mL) were isolated by ultracentrifugation at 4°C from the human plasma. To remove the excess of EDTA LDL samples were transferred to dialyzing tubes and dialyzed in physiologic solution (0.9% NaCl in deionized water) at 4°C, changing the solution three times (4h, 48h, 48h). Purified LDL were sterilized using a 0.22-µm filter and stored at 4°C. The protein content was evaluated by the BCA assay [44], using BSA as a standard. For the labeling, LDL were incubated with the fluorescent dye DiO (250 µg DiO/mg LDL protein) for 18h at 4°C. LDL-DiO were passed on a Sephadex G25 column (PD10) with 0.01% PBS-EDTA (pH 7.4), to remove unbound DiO [8,45].

2.9 Fluorescent LDL-uptake cell-based assay

HepG2 cells were were seeded into 96-well tray (25 x 103 cells/well in a complete medium) and after 24h treated in 0.4% FCS media. 24h after treatment, cells were incubated with 100 µg/mL of LDL-DiO (3,3'- dioctadecyloxacarbocyanine). After 3h of incubation at 37°C, cells were washed PBS and cells dissolved with 100µl of 2% SDS and the DiO fluorescence were measured by using the microplate fluorimeter (Victor MultiPlate Reader by PerkinElmer) at excitation and emission wavelength of 484nm and 501nm, respectively).

2.10 Statistical analysis

Statistical analysis was performed using the Prism statistical analysis package Version 5.01 (GraphPad Software, San Diego, CA). When possible, p values were determined by Student's t test. Otherwise, differences between treatment groups were evaluated by oneway ANOVA. A probability value of $p < 0.05$ was considered statistically significant. The data are representative of three independent experiments.

3. Results

3.1 Effect of RYR, BCE and MLE on PCSK9 expression

In a first series of experiments, we compared the effect of the three active components of LopiGLIK® on PCSK9 expression. HepG2 cells were incubated for 24h with non-cytotoxic concentrations of RYR (50µg/mL), MLE (1 mg/mL) or BCE (40µg/mL) (Figure 1A), and PCSK9 protein levels were determined by western blot analysis of total cell lysates. As expected, RYR significantly induced both proPCSK9 (74 kDa; 3.1 fold) and the active form (62 kDa; 2.1 fold), while BCE partially reduce their intracellular levels, compared to untreated control cells (-34.7% proPCSK9 and -64.3% PCSK9 vs control, respectively) (Figure 1B and C). The water-soluble extract of Morus alba leaves (MLE) acted similarly to BCE, such as determined a significant reduction of the intracellular levels of both proPCSK9 and active form of PCSK9 (-17.1% proPCSK9 and -59.3% PCSK9, respectively; Figure 1B and C). PCSK9 levels released in the conditioned media were significant raised upon RYR treatment (+9.8% ± 1.2% vs control), whilst were reduced by BCE (-64.8% ± 16.2% vs control) and MLE (-43.8% ± 23.6% vs control) treatments (Figure 1D).

The quantification of the mRNA levels, by real time qPCR, showed that RYR induced PCSK9 levels by 1.89 ± 0.11 fold, while MLE and BCE significantly suppressed its expression by 54.8% ± 0.7% and by 85.0% ± 0.5%, respectively (Figure 1E). To demonstrate the transcriptional inhibition of PCSK9 mRNA expression by MLE, HepG2 cells were transfected with a luciferase construct containing the 5'-flanking region of PCSK9 gene from -1711 to -94 (pGL3-PCSK9-D1), relative to ATG starting codon [30]. As expected, RYR significantly increased the luciferase activity (2.4±0.5 fold vs control) while BCE determined an opposite effect (-63.0 ± 11.8% vs control; Figure 1F). Importantly, MLE did not affect the PCSK9 promoter activity, thus indicating a different mechanism of action compared to BCE. Similar results were observed when a different plasmid was used, pGL3-PCSK9-D4 plasmid containing the Sp1, SRE and HNF1α sites [30]. Upon treatment with RYR and BCE, the luciferase activity was increased by 1.6 ± 0.08 fold and reduced by 63.6 ± 16.3% vs control, respectively (Figure

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 October 2018 doi:10.20944/preprints201810.0288.v1

1G). These results provide new evidence on the inhibitory effect of MLE on PCSK9 expression, without any interference with its promoter activity.

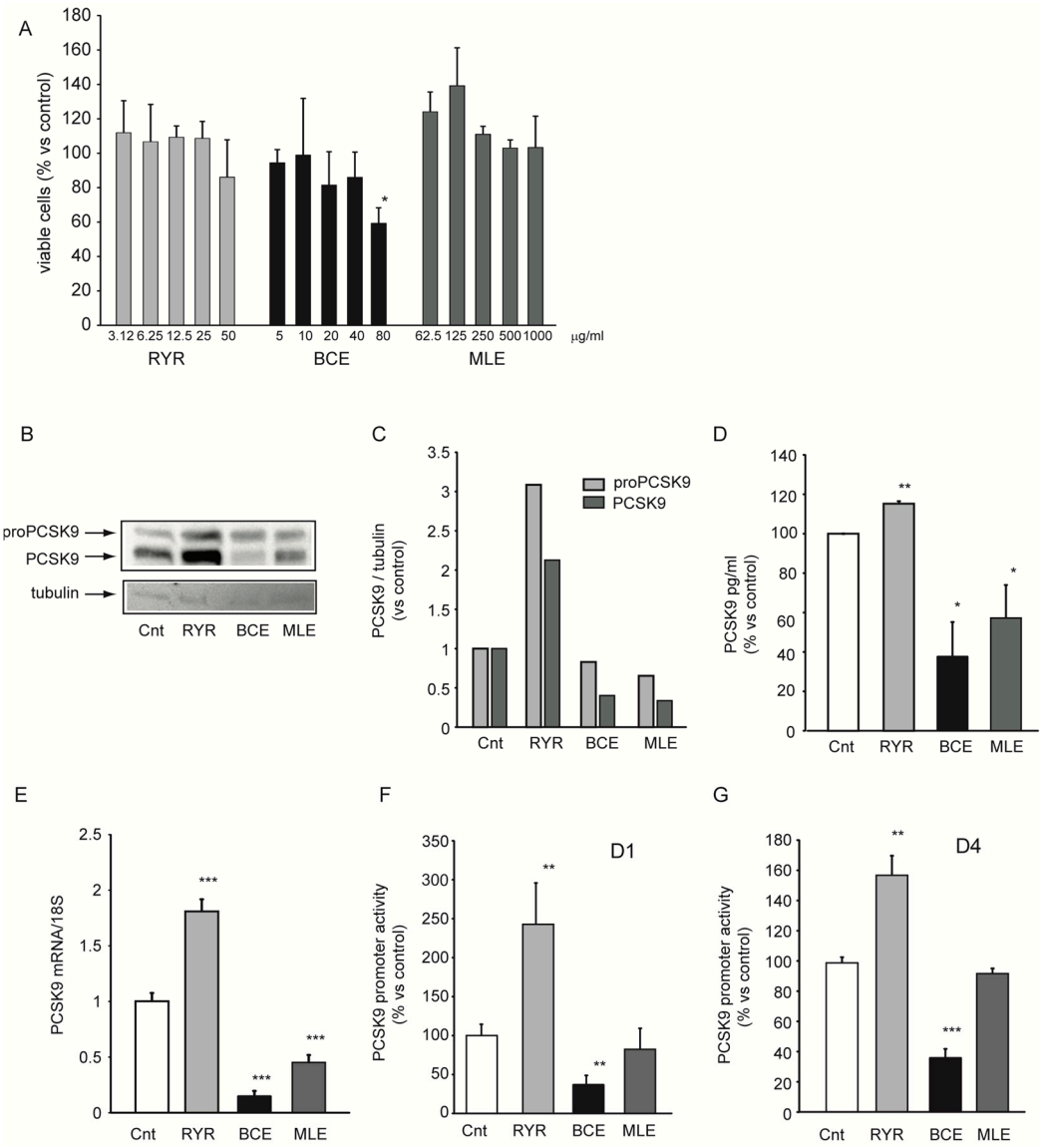

Figure 1. Effect of RYR, BCE and MLE on PCSK9 expression. A) HepG2 cells were incubated for 48h with indicated concentrations of RYR, MLE, and BCE. The cell viability was then determined by SRB assay. B-E) HepG2 cells were seeded in MEM/10% FCS and the day after incubated with MEM/10%FCS in the presence or absence of RYR (40 µg/mL), BCE (50 µg/mL) and MLE (1mg/mL). After 24h, total protein extract and RNA were prepared and conditioned media collected. B) PCSK9 protein expression was evaluated by western blot analysis from total protein extracts. Tubulin was used as loading control. C) Densitometric readings were evaluated using the ImageLabTM software. D) PCSK9 levels in the conditioned media were evaluated by ELISA assay. E) mRNA levels of PCSK9 were determined by quantitative real-time PCR. F and G) HepG2 cells were transfected with pGL3-PCSK9-D1 (G) or pGL3-PCSK9-D4 (H). The day after the transfection the cells were seeded in a 48well tray and after an additional 24h were incubated with RYR, BCE or MLE. After 24h, luciferase activities were determined by Neolite reagent. Differences between treatments were assessed by Student's t test, and one-way ANOVA (when necessary). *p<0.05; **p<0.01; ***p<0.001.

3.2 Effect of RYR, BCE, and MLE on mRNA levels of SREBP-regulated genes

As MLE reduces the expression of PCSK9 mRNA, we investigated its possible effect on additional genes regulated by the SREBP transcription factor, such as HMG-CoA reductase,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 October 2018 doi:10.20944/preprints201810.0288.v1

and FAS. This analysis revealed that MLE significantly reduces the expression of both FAS and HMG-CoA reductase at similar extent than BCE. On the contrary, RYR induced both transcripts in a significant manner (Figure 2A-B). In particular, the incubation of HepG2 cells with MLE significantly reduced HMG-CoA reductase expression (-33.7 ± 1.17% vs control), similarly to BCE (-54.5% ± 3.5% vs control); conversely, RYR led to opposite results: +1.77 ± 0.14-fold vs control. A similar effect was observed on FAS mRNA expression (-41.7 ± 3.9%, -33.9 ± 0.8% and 1.41 ± 0.07 fold vs control for MLE, BCE and RYR, respectively). At the protein levels, both RYR and BCE induced LDLR expression by 2.8 and 1.5 fold, respectively, while MLE showed a minor effect (1.2 fold) (Figure 2D). These data indicate that MLE elicit an inhibitory effect on additional genes, beyond PCSK9, that are involved in cholesterol metabolism and are transcriptionally regulated by SREBP pathway.

To further investigate the effect of MLE on gene expression, we determined, by Western blot analysis, the expression of SREBP2 and HNF1α from total cell lysates. RYR determined a significant increase of both transcription factors (1.9 and 1.4 fold for SREBP2 and HNF1α, respectively; Figure 2E and F), while BCE determined a significant downregulation (-30% for both SREBP2 and HNF1α). Interestingly, MLE did not determine any significant variation of SREBP and HNF1α (Figure 2E and F), suggesting that the inhibitory effects on PCSK9, HMG-CoA reductase and FAS by MLE is due to a different mechanism of action.

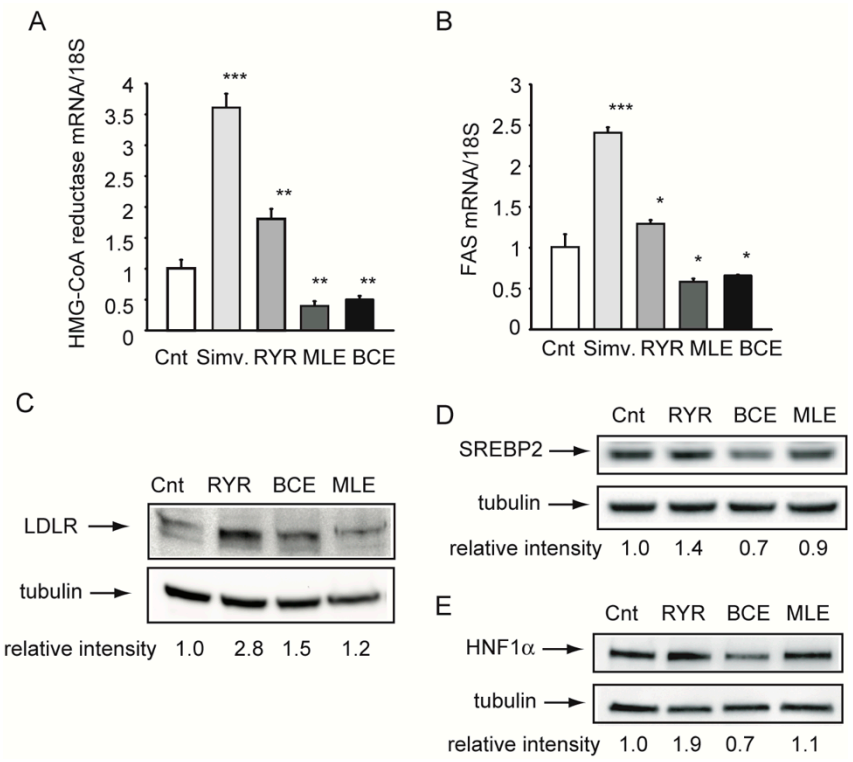

Figure 2. Effect of RYR, BCE and MLE on SREBP-regulated genes. A-E) HepG2 cells were seeded in MEM/10% FCS and the day after incubated with MEM/10% FCS in the presence or absence of simvastatin (40μM), RYR (40 μg/mL), BCE (50 μg/mL) and MLE (1mg/mL). After 24h total RNA and total protein extract were prepared. A and B) mRNA levels of HMG-CoA reductase and FAS mRNA were determined by quantitative real-time PCR. C-E) LDLR, SREBP2 and HNF1α protein expression were evaluated by western blot analysis from total protein extracts. Tubulin was used as loading control. Densitometric readings were evaluated using the ImageLabTM software and indicated below the representative western blot pictures. Differences between treatments were assessed by Student's t test, and one-way ANOVA (when necessary). *p<0.05; **p<0.01; ***p<0.001.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 October 2018 doi:10.20944/preprints201810.0288.v1

3.3 Concentration-dependent effect of MLE on PCSK9 and genes involved in cholesterol homeostasis

To better define the effect of MLE on genes involved in lipid metabolism, a series of experiments were conducted by incubating HepG2 cells with increasing concentration of MLE (0.25, 0.5, and 1.0 mg/mL). This analysis revealed a concentration dependent effect of MLE on PCSK9 mRNA and extracellular levels, determined by RT-qPCR and ELISA assay, respectively (Figure 3A and B). MLE significantly inhibited PCSK9 at any concentration utilized, while no effect was observed on the PCSK9 promoter activity, as assessed by luciferase assay (Figure 3C). The incubation with the transcriptional activity inhibitor, actinomycin D, showed that MLE did not alter the stability of PCSK9 mRNA (Figure 3D).

The inhibition on HMG-CoA reductase mRNA expression was observed at very similar range of concentration as those utilized for PCSK9 (Figure 3E), while the LDLR mRNA levels were not affected by MLE (Figure 3F) and only a minor, but significant inhibition was seen on FAS (-26.2 ± 15.9% vs control; Figure 3G).

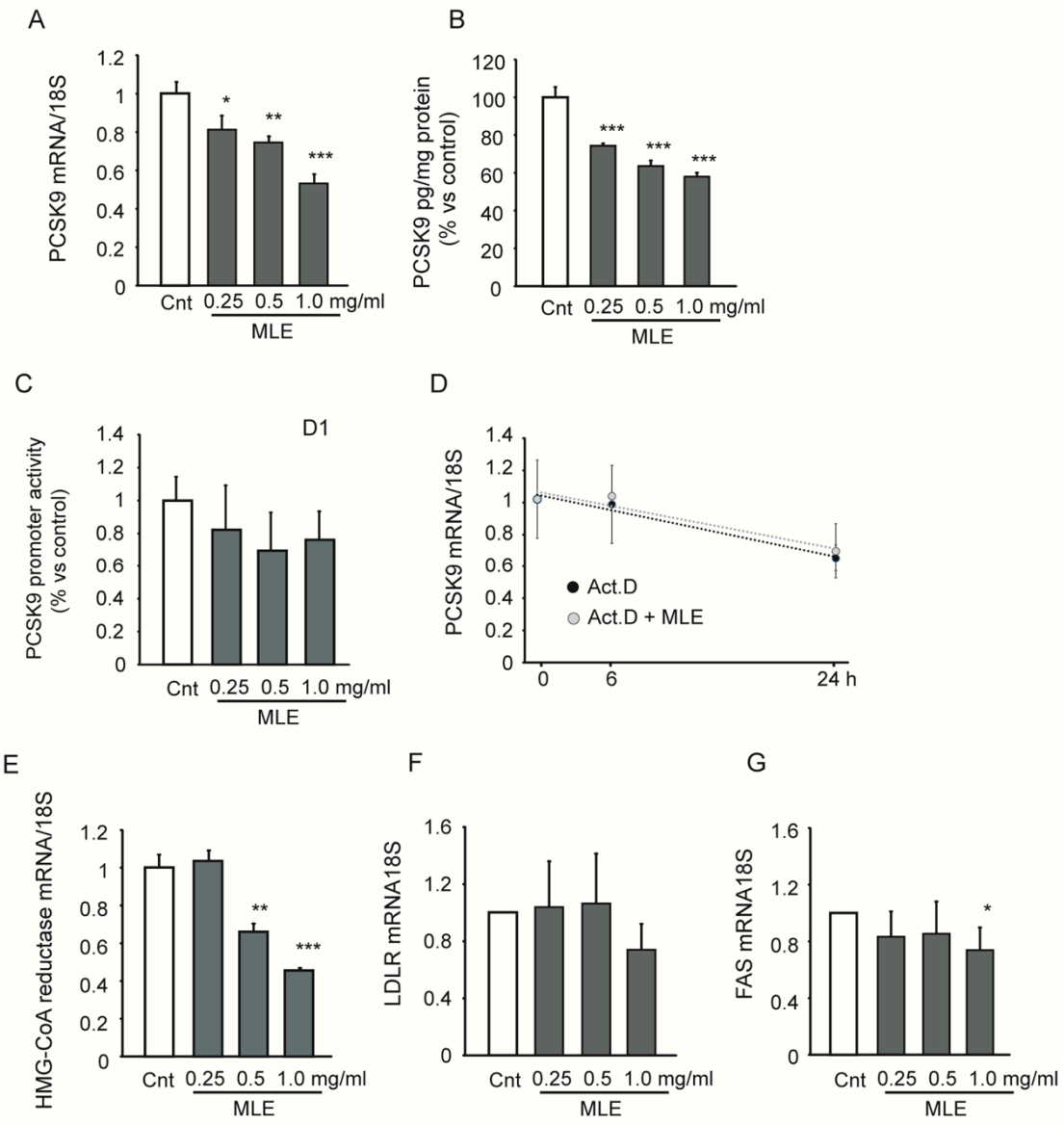

Figure 3. Concentration-dependent effect of MLE on PCSK9 and SREBP-related genes expression. A-B; E-G) HepG2 cells were seeded in MEM/10% FCS and the day after incubated with MEM/10% FCS in the presence or absence of increasing concentration of MLE (0.25, 0.5 and 1mg/mL). After 24h, total

 doi:10.20944/preprints201810.0288.v1

RNA was prepared and conditioned media collected. A) mRNA levels of PCSK9 were determined by quantitative real-time PCR. B) PCSK9 levels in the conditioned media were evaluated by ELISA assay. C) HepG2 cells were transfected with pGL3-PCSK9-D1. The day after the transfection the cells were seeded in a 48well tray and after an additional 24h were incubated with MLE. After 24h, luciferase activities were determined by Neolite reagent. D) HepG2 were incubated for the indicated times with MLE in the presence or absence of actinomycin D (5μg/mL) and PCSK9 mRNA levels were determined by qPCR. E-G) mRNA levels of HMG-CoA reductase, FAS, and LDLR mRNA were determined by quantitative real-time PCR. Differences between treatments were assessed by Student's t test, and one-way ANOVA (when necessary). *p<0.05; **p<0.01; ***p<0.001.

3.4 Effect of the combination of RYR, BCE and MLE on genes involved in cholesterol homeostasis.

Previous studies have shown that berberine counteract the effect of statins on PCSK9 expression [15,30]. For this reason, we performed a series of experiments in order to define the effect of the combination of RYR, MLE and BCE on cholesterol homeostasis in HepG2 cells.

The incubation with the three extracts resulted in a significant reduction of intracellular and secreted form of PCSK9, as determined by western blot analysis and ELISA assays, respectively (Figure 4A-C). In particular, LopiGLIK® components reduced by 96.6% and 93.3% the levels of proPCSK9 and PCSK9 respectively, and by 74.4 ± 14.9% the extracellular PCSK9 in the cultured media. A similar effect was also observed at transcriptional level, with lower mRNA levels (-77.3 ± 0.8% vs control) and PCSK9 promoter activities of pGL3-PCSK9-D1 and pGL3-PCSK9-D4 plasmids (-76.0 ± 9.6% and -67.4 ± 7.5% vs control respectively; Figure 4D-F). These data suggest that BCE and MLE actively counteract the induction of PCSK9 by RYR. We therefore investigated the effect of different combination of active component of LopiGLIK® on PCSK9 promoter activity. As shown in Figure 4G, MLE did not interfere with the induction PCSK9 promoter activity by RYR. However, the combination of three active components of LopiGLIK® reduced the PCSK9 promoter activity below the basal condition. These data suggest that the addition of BCE and MLE to a nutraceutical based on RYR could facilitate the LDL-C reduction by interfering with PCSK9 expression.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 October 2018 doi:10.20944/preprints201810.0288.v1

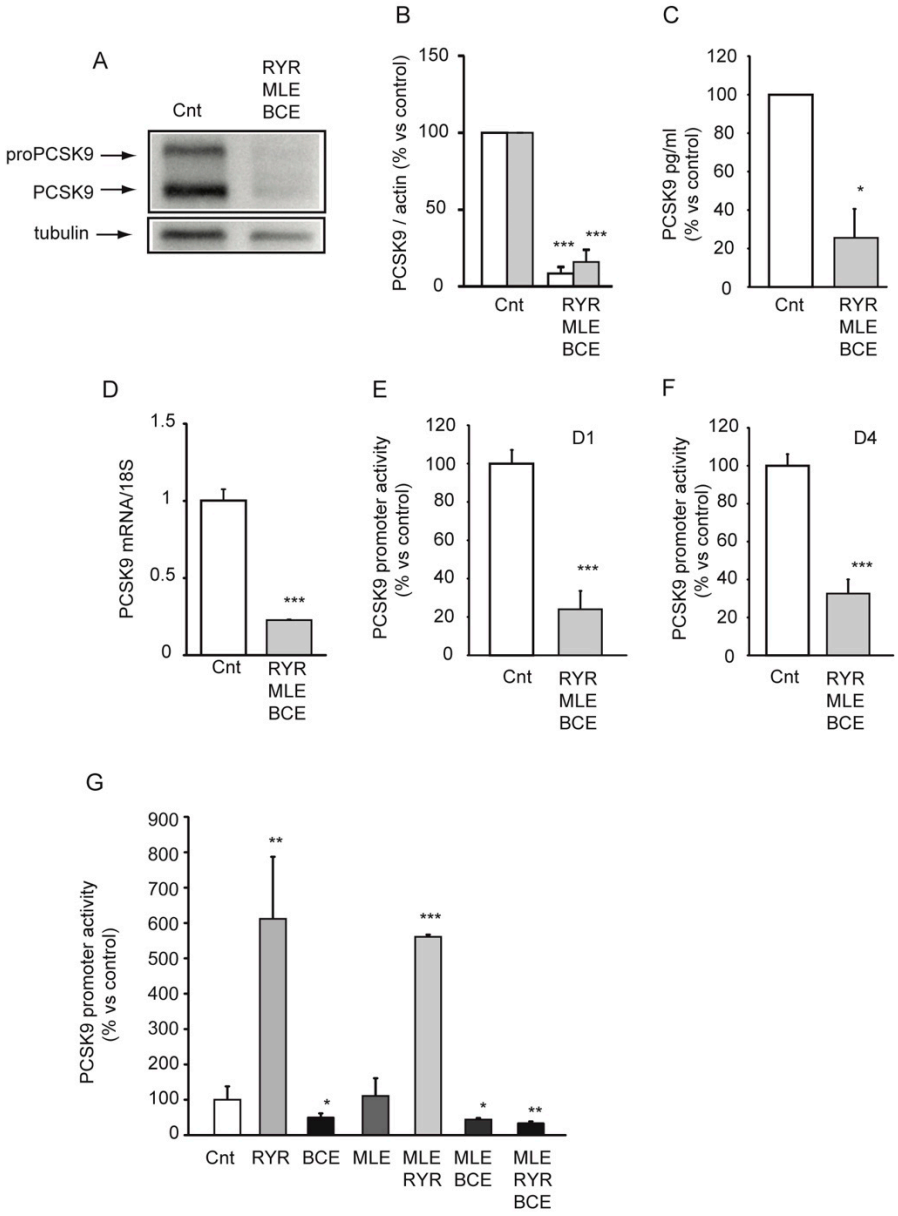

Figure 4. Effect of the combination of LopiGLIK® constituents on PCSK9 expression. A-D) HepG2 cells were seeded in MEM/10% FCS and the day after incubated with MEM/10% FCS in the presence or absence of three active components simultaneously (RYR, 40 µg/mL; BCE, 50 µg/mL and MLE, 1mg/mL). After 24h, total protein extract and RNA were prepared and conditioned media collected. A) PCSK9 protein expression was evaluated by western blot analysis from total protein extracts. Tubulin was used as loading control. B) Densitometric readings were evaluated using the ImageLabTM software. C) PCSK9 levels in the conditioned media were evaluated by ELISA assay. D) mRNA levels of PCSK9 were determined by quantitative real-time PCR. E and F) HepG2 cells were transfected with pGL3-PCSK9-D1 (E) or pGL3-PCSK9-D4 (F). The day after the transfection the cells were seeded in a 48well tray and after an additional 24h were incubated with RYR, BCE and MLE. After 24h, luciferase activities were determined by Neolite reagent. G) Under the same experimental conditions of panel F, the effect of different combinations of LopiGLIK® components on PCSK9 promoter activity were determined. Differences between treatments were assessed by Student's t test, and one-way ANOVA (when necessary). *p<0.05; **p<0.01; ***p<0.001.

In order to extend this evidence, we investigated, under the same experimental conditions, the effect of LopiGLIK® combination on additional SREBP-regulated genes. As shown in Figure 5, the combination of RYR, BCE and MLE reduced both the expression of HMG-CoA

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 October 2018 doi:10.20944/preprints201810.0288.v1

reductase (-41.1 ± 6.4% vs control) and FAS (-33.8 ± 3.7% vs control) mRNA. The western blot analysis of total cell lysates, revealed a significant induction of the LDLR after incubation of HepG2 cells with LopiGLIK® combination (1.3 fold; Figure 5Cand 5D).

Since the outcome for an efficient lipid lowering effect is the induction of the LDLR and the uptake of LDL-C, we determined the effect of RYR, BCE and MLE on this parameter by incubating HepG2 cells for 24h with different combination of these active components followed by 3h incubation of fluorescently labeled LDL-DiO. As expected, both RYR and BCE significantly improved the capacity of HepG2 cells to capture LDL (2.7 ± 0.9 and 2.1 ± 1.0 fold, respectively; Figure 5E). MLE also increased, in a concentration dependent manner, the uptake of LDL-C reaching a maximal induction at concentration of 1 mg/ml (2.4 ± 0.5 fold; figure 5E). Importantly, MLE partially improved the effect of both RYR and BCE on LDL-C uptake and the final combination of the three active components showed the maximal effect (4.3 ± 1.2 fold increase). These data support the use of these nutraceutical combination for an effective lipid-lowering action.

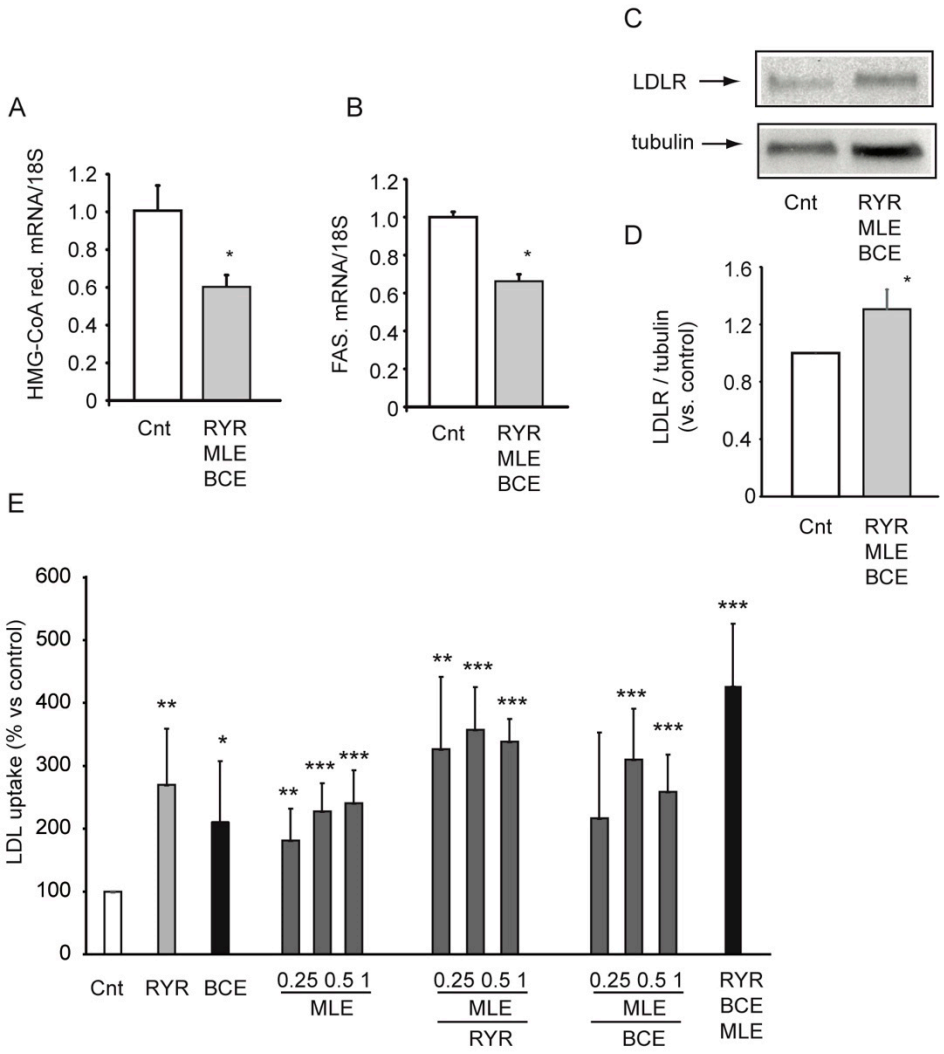

Figure 5. Effect of the combination of LopiGLIK® constituents on genes involved in cholesterol homeostasis and LDL-C uptake. A-D) HepG2 cells were seeded in MEM/10% FCS and the day after incubated with MEM/10%FCS in the presence or absence of three active components simultaneously (RYR, 40 µg/mL; BCE, 50 µg/mL and MLE ,1mg/mL). After 24h, total protein extract and RNA were prepared. A and B) mRNA levels of HMG-CoA reductase, and FAS mRNA were determined by quantitative real-time PCR. C) LDLR protein expression was evaluated by western blot analysis from total protein extracts. Tubulin was used as loading control. D) Densitometric readings were evaluated

 doi:10.20944/preprints201810.0288.v1

using the ImageLabTM software. E) HepG2 cells were seeded in MEM/10% FCS and the day after incubated with MEM/0.4% FCS in the presence or absence of different combination of LopiGLIK® active components. After 24h, HepG2 cells were incubated with 10μg/mL of LDL-DiO and fluorescence intensity determined by flowcytometry analysis after 3h incubation. Differences between treatments were assessed by Student's t test, and one-way ANOVA (when necessary). *p<0.05; **p<0.01; ***p<0.001.

4. Discussion

In the present study, we have investigated the effect of LopiGLIK® constituents, such as RYR, BCE and MLE, on PCSK9 expression and additional genes regulated by the SREBP transcription factors (LDLR, HMG-CoA reductase and FAS). The rational of the study was based on our previous observation on the lack of effect of LopiGLIK® on the plasma levels of PCSK9 treated for 16 weeks [4]. In particular, the active components of LopiGLIK® on cholesterol levels, such as RYR containing monacolin K and berberine have been previously shown to act in an opposite fashion on the transcription of PCSK9 [15,31]. Thus, the fact that RYR, present in LopiGLIK, did not induce PCSK9 plasma levels, as predicted by its ability to activate the SREBP pathway, could be due to berberine, a strong suppressor of PCSK9 expression [15,30]. Nonetheless, the contribution of MLE extract on the plasma levels of PCSK9 was not defined. Our results clearly demonstrated that the water-soluble extract of Morus alba leaves (MLE), significantly inhibited the expression of PCSK9, by reducing both the mRNA and the protein levels in HepG2 cells. However, differently from berberine [30], MLE did not affect the promoter activity of PCSK9, as determined by using both the pGL3-PCSK9-D1 and pGL3-PCSK9-D4 constructs, as well as the expression of both SREBP2 and HNF1α. However, it is important to point out that MLE negatively regulated the expression of both HMG-CoA reductase and FAS, two additional SREBP-reguated genes. We also exclude an effect of MLE on PCSK9 mRNA stability, as the degradation of PCSK9 was similar in control and MLE treated cells in the presence pf the transcriptional inhibitor actinomycin D. Thus, this data suggest that MLE may interfere with the SREBP pathway but at a post-transcriptional levels. Similar results have been observed with oncostatin M, a cytokine that effectively suppress the PCSK9 mRNA levels without affecting its promoter activity [46]. In addition, suppressor of cytokine signaling 3 (SOCS3) molecule has been shown to induce PCSK9 without activating the PCSK9 promoter activity [43]. Thus, additional experiments need to be performed to explore a possible effect of MLE on the mRNA stability of PCSK9 or on epigenetic regulation of PCSK9 expression.

Regarding the active components of MLE, it has been recently reported that moracin C, present in dried immature Morus alba fruits, reduced both the mRNA and protein of PCSK9 [17]. Moracin C was identified from a chloroform soluble extract of Morus alba fruits while in our study we have utilized water soluble extracts of Morus alba leaves. Interestingly, the water-soluble extracts of Morus alba fruits did not show any activity on PCSK9 expression [17]. Thus, although moracin C is present in Morus alba leaves [47], it is unlikely that was present in our water extract.

Regardless the mechanism of action and the active component/s of Morus alba responsible for the inhibitory expression of PCSK9, due to this effect, it was conceivable to hypothesize that MLE, together with BCE, may effectively counteract the induction of PCSK9 by RYR that contains monacolin K, ameliorating the final hypocholesterolemic action of LopiGLIK®. On this regards, we showed that the combination of MLE and BCE effectively counteracted the effect of RYR, by blocking the PCSK9 expression at protein, mRNA and promoter activity levels, together with a significant reduction of HMG-CoA reductase and FAS levels. The same combination induces the LDLR and LDL-DiO uptake in HepG2 cells, compared to control untreated cells.

Taken together, in the present study we demonstrated an effective combination of three active components of LopiGLIK® on genes regulating the cholesterol homeostasis, with a significant reduction of PCSK9, an increase of the LDLR and LDL-C uptake. In addition, we provided new

 doi:10.20944/preprints201810.0288.v1

evidence of the contribution of water soluble extracts of Morus alba leaves on the hypocholesterolemic effect of LopiGLIK® by reducing PCSK9 levels.

An important limitation of the present study is represented by the fact the molar ratio of the active components present in the extracts of RYR, BCE and MLE is potentially different from that observed in patients assuming LopiGLIK®. On this regard, it is relevant to point out that LopiGLIK® contains 3.3 mg di monacolin K (0.008 mmol) and 531.25 mg of berberine (1.5 mmol), thus with a molar ratio of 1 to 190. On the other hand, the bioavailability of monacolin K and berberine is approximately 20% [48,49] and 0.5% [50-52], respectively. Thus, the final molar ratio of these two active components that reached the circulation system is approximately 1 to 5 in favor of berberine. Under our experimental condition, we have utilized 50 μg/mL of RYR containing 15 μg/mL of monacolin K and 40 μg/mK BCE containing 34 μg/mL of berberine, thus with a molar ratio of 1 to 2.3 in favor of berberine, very similar that predicted to be present in human plasma. These in vitro concentrations are similar to those utilized in previous study [15,17,32,53], and have been shown to be not cytotoxic. Regarding the final concentration utilized for water-soluble extracts of Morus alba leaves, the interval of 0.25 ÷ 1 mg/mL, is lower than the previously tested 1 ÷ 6 mg/mL of mulberry water extracts on HepG2 cells [39]. In particular, we decided to utilize these concentrations since they show to be not cytotoxic in HepG2 cells. These concentrations appear to exceed those utilized for RYR and BCE and thus it is difficult to established if the observed effect on PCSK9 on HepG2 cells can be relevant for the clinical effects of LopiGLIK®. However, the reduction of PCSK9 was observed at concentration of 250 μg/mL, thus similar to the 40μg/mL of BCE or 50μg/mL of RYR. The relevant aspect is that the concentration and the bioavailability of the component/s with PCSK9 inhibitory activity present in MLE. On this regard, we tested the effect of DNJ that show no significant effect on PCSK9 expression in HepG2 cells.

5. Conclusions

The results of the present study indicate that the inhibitory effect of MLE on PCSK9 may contribute to the lipid-lowering action of LopiGLIK, supporting the rational of using this nutraceutical combination for controlling both a hyperlipidemic and hyperglycemic conditions [3,4].

Author Contributions: NF conceived the study and wrote the manuscript. MGL and CM conducted the experiments. SM and HC conducted some experiments related to figures 4 and 5. AC and CRS critically revised the manuscript. MR wrote the manuscript and critically revised it.

Acknowledgments: The authors acknowledge Akademy Pharma Srl (Milano, Italy) for supporting this study and Dr. Raffaella Marin, Dr. Daniela Vianello, and Prof. Alberto Zambon for providing the low-density lipoproteins (Department of Medicine; University of Padua). **Conflicts of Interest:** The authors declare no conflict of interest.

References

1. Catapano, A.L.; Graham, I.; De Backer, G.; Wiklund, O.; Chapman, M.J.; Drexel, H.; Hoes, A.W.; Jennings, C.S.; Landmesser, U.; Pedersen, T.R., et al. 2016 ESC/EAS Guidelines for the Management of Dyslipidaemias: The Task Force for the Management of Dyslipidaemias of the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS)Developed with the special contribution of the European Assocciation for Cardiovascular Prevention & Rehabilitation (EACPR). *Eur Heart J* 2016, 10.1093/eurheartj/ehw272, doi:10.1093/eurheartj/ehw272.

2. Li, J.J.; Lu, Z.L.; Kou, W.R.; Chen, Z.; Wu, Y.F.; Yu, X.H.; Zhao, Y.C.; Chinese Coronary Secondary Prevention Study, G. Beneficial impact of Xuezhikang on cardiovascular events and mortality in elderly hypertensive patients with previous myocardial infarction from the China Coronary Secondary Prevention Study (CCSPS). *Journal of clinical pharmacology* 2009, *49*, 947-956, doi:10.1177/0091270009337509.

3. Trimarco, V.; Battistoni, A.; Tocci, G.; Coluccia, R.; Manzi, M.V.; Izzo, R.; Volpe, M. Single blind, multicentre, randomized, controlled trial testing the effects of a novel nutraceutical compound on plasma lipid and cardiovascular risk factors: Results of the interim analysis. *Nutrition, metabolism, and cardiovascular diseases : NMCD* 2017, *27*, 850-857, doi:10.1016/j.numecd.2017.08.003.

4. Adorni, M.P.; Ferri, N.; Marchiano, S.; Trimarco, V.; Rozza, F.; Izzo, R.; Bernini, F.; Zimetti, F. Effect of a novel nutraceutical combination on serum lipoprotein functional profile and circulating PCSK9. *Therapeutics and clinical risk management* 2017, *13*, 1555-1562, doi:10.2147/TCRM.S144121.
5. Adorni, M.P.; Cipollari, E.; Favari, E.; Zanotti, I.; Zimetti, F.; Corsini, A.; Ricci, C.; Bernini, F.; Ferri, N. Inhibitory effect of PCSK9 on Abca1 protein expression and cholesterol efflux in macrophages. *Atherosclerosis* 2016, *256*, 1-6, doi:10.1016/j.atherosclerosis.2016.11.019.
6. Ferri, N.; Corsini, A.; Macchi, C.; Magni, P.; Ruscica, M. Proprotein convertase subtilisin kexin type 9 and high-density lipoprotein metabolism: experimental animal models and clinical evidence. *Translational research : the journal of laboratory and clinical medicine* 2016, *173*, 19-29, doi:10.1016/j.trsl.2015.10.004.
7. Ferri, N.; Marchiano, S.; Tibolla, G.; Baetta, R.; Dhyani, A.; Ruscica, M.; Uboldi, P.; Catapano, A.L.; Corsini, A. PCSK9 knock-out mice are protected from neointimal formation in response to perivascular carotid collar placement. *Atherosclerosis* 2016, *253*, 214-224, doi:10.1016/j.atherosclerosis.2016.07.910.
8. Ferri, N.; Tibolla, G.; Pirillo, A.; Cipollone, F.; Mezzetti, A.; Pacia, S.; Corsini, A.; Catapano, A.L. Proprotein convertase subtilisin kexin type 9 (PCSK9) secreted by cultured smooth muscle cells reduces macrophages LDLR levels. *Atherosclerosis* 2012, *220*, 381-386.
9. Ruscica, M.; Ferri, N.; Fogacci, F.; Rosticci, M.; Botta, M.; Marchiano, S.; Magni, P.; D'Addato, S.; Giovannini, M.; Borghi, C., et al. Circulating Levels of Proprotein Convertase Subtilisin/Kexin Type 9 and Arterial Stiffness in a Large Population Sample: Data From the Brisighella Heart Study. *Journal of the American Heart Association* 2017, *6*, doi:10.1161/JAHA.117.005764.
10. Zimetti, F.; Caffarra, P.; Ronda, N.; Favari, E.; Adorni, M.P.; Zanotti, I.; Bernini, F.; Barocco, F.; Spallazzi, M.; Galimberti, D., et al. Increased PCSK9 Cerebrospinal Fluid Concentrations in Alzheimer's Disease. *Journal of Alzheimer's disease : JAD* 2016, 10.3233/JAD-160411, doi:10.3233/JAD-160411.
11. Cariou, B.; Si-Tayeb, K.; Le May, C. Role of PCSK9 beyond liver involvement. *Curr Opin Lipidol* 2015, *26*, 155-161, doi:10.1097/MOL.0000000000000180.
12. Le May, C.; Kourimate, S.; Langhi, C.; Chetiveaux, M.; Jarry, A.; Comera, C.; Collet, X.; Kuipers, F.; Krempf, M.; Cariou, B., et al. Proprotein convertase subtilisin kexin type 9 null mice are protected from postprandial triglyceridemia. *Arterioscler Thromb Vasc Biol* 2009, *29*, 684-690.
13. Ricci, C.; Ruscica, M.; Camera, M.; Rossetti, L.; Macchi, C.; Colciago, A.; Zanotti, I.; Lupo, M.G.; Adorni, M.P.; Cicero, A.F.G., et al. PCSK9 induces a pro-inflammatory response in macrophages. *Scientific reports* 2018, *8*, 2267, doi:10.1038/s41598-018-20425-x.
14. Sabatine, M.S.; Giugliano, R.P.; Keech, A.C.; Honarpour, N.; Wiviott, S.D.; Murphy, S.A.; Kuder, J.F.; Wang, H.; Liu, T.; Wasserman, S.M., et al. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. *N Engl J Med* 2017, *376*, 1713-1722, doi:10.1056/NEJMoa1615664.
15. Cameron, J.; Ranheim, T.; Kulseth, M.A.; Leren, T.P.; Berge, K.E. Berberine decreases PCSK9 expression in HepG2 cells. *Atherosclerosis* 2008, *201*, 266-273, doi:10.1016/j.atherosclerosis.2008.02.004.
16. Tai, M.H.; Chen, P.K.; Chen, P.Y.; Wu, M.J.; Ho, C.T.; Yen, J.H. Curcumin enhances cell-surface LDLR level and promotes LDL uptake through downregulation of PCSK9 gene expression in HepG2 cells. *Molecular nutrition & food research* 2014, *58*, 2133-2145, doi:10.1002/mnfr.201400366.
17. Pel, P.; Chae, H.S.; Nhoek, P.; Kim, Y.M.; Chin, Y.W. Chemical Constituents with Proprotein Convertase Subtilisin/Kexin Type 9 mRNA Expression Inhibitory Activity from Dried Immature Morus alba Fruits. *Journal of agricultural and food chemistry* 2017, *65*, 5316-5321, doi:10.1021/acs.jafc.7b02088.
18. Pel, P.; Chae, H.S.; Nhoek, P.; Yeo, W.; Kim, Y.M.; Chin, Y.W. Lignans from the fruits of Schisandra chinensis (Turcz.) Baill inhibit proprotein convertase subtilisin/kexin type 9 expression. *Phytochemistry* 2017, *136*, 119-124, doi:10.1016/j.phytochem.2017.01.010.
19. Maxwell, K.N.; Soccio, R.E.; Duncan, E.M.; Sehayek, E.; Breslow, J.L. Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice. *J Lipid Res* 2003, *44*, 2109-2119, doi:10.1194/jlr.M300203-JLR200.
20. Dubuc, G.; Chamberland, A.; Wassef, H.; Davignon, J.; Seidah, N.G.; Bernier, L.; Prat, A. Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 2004, *24*, 1454-1459, doi:10.1161/01.ATV.0000134621.14315.43.
21. Careskey, H.E.; Davis, R.A.; Alborn, W.E.; Troutt, J.S.; Cao, G.; Konrad, R.J. Atorvastatin increases human serum levels of proprotein convertase subtilisin/kexin type 9. *J Lipid Res* 2008, *49*, 394-398, doi:10.1194/jlr.M700437-JLR200.
22. Berthold, H.K.; Seidah, N.G.; Benjannet, S.; Gouni-Berthold, I. Evidence from a randomized trial that simvastatin, but not ezetimibe, upregulates circulating PCSK9 levels. *PloS one* 2013, *8*, e60095, doi:10.1371/journal.pone.0060095.

23. Awan, Z.; Seidah, N.G.; MacFadyen, J.G.; Benjannet, S.; Chasman, D.I.; Ridker, P.M.; Genest, J. Rosuvastatin, proprotein convertase subtilisin/kexin type 9 concentrations, and LDL cholesterol response: the JUPITER trial. *Clin Chem* 2012, *58*, 183-189, doi:10.1373/clinchem.2011.172932.
24. Guo, Y.L.; Liu, J.; Xu, R.X.; Zhu, C.G.; Wu, N.Q.; Jiang, L.X.; Li, J.J. Short-term impact of low-dose atorvastatin on serum proprotein convertase subtilisin/kexin type 9. *Clinical drug investigation* 2013, *33*, 877-883, doi:10.1007/s40261-013-0129-2.
25. Costet, P.; Cariou, B.; Lambert, G.; Lalanne, F.; Lardeux, B.; Jarnoux, A.L.; Grefhorst, A.; Staels, B.; Krempf, M. Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c. *J Biol Chem* 2006, *281*, 6211-6218.
26. Jeong, H.J.; Lee, H.S.; Kim, K.S.; Kim, Y.K.; Yoon, D.; Park, S.W. Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2. *J Lipid Res* 2008, *49*, 399-409, doi:10.1194/jlr.M700443-JLR200.
27. Ferri, N.; Marchiano, S.; Lupo, M.G.; Trenti, A.; Biondo, G.; Castaldello, P.; Corsini, A. Geranylgeraniol prevents the simvastatin-induced PCSK9 expression: Role of the small G protein Rac1. *Pharmacological research* 2017, *122*, 96-104, doi:10.1016/j.phrs.2017.05.021.
28. Goldstein, J.L.; DeBose-Boyd, R.A.; Brown, M.S. Protein sensors for membrane sterols. *Cell* 2006, *124*, 35-46, doi:10.1016/j.cell.2005.12.022.
29. Horton, J.D.; Goldstein, J.L.; Brown, M.S. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 2002, *109*, 1125-1131, doi:10.1172/JCI15593.
30. Li, H.; Dong, B.; Park, S.W.; Lee, H.S.; Chen, W.; Liu, J. Hepatocyte nuclear factor 1alpha plays a critical role in PCSK9 gene transcription and regulation by the natural hypocholesterolemic compound berberine. *J Biol Chem* 2009, *284*, 28885-28895, doi:10.1074/jbc.M109.052407.
31. Dong, B.; Li, H.; Singh, A.B.; Cao, A.; Liu, J. Inhibition of PCSK9 transcription by berberine involves down-regulation of hepatic HNF1alpha protein expression through the ubiquitin-proteasome degradation pathway. *J Biol Chem* 2015, *290*, 4047-4058, doi:10.1074/jbc.M114.597229.
32. Kong, W.; Wei, J.; Abidi, P.; Lin, M.; Inaba, S.; Li, C.; Wang, Y.; Wang, Z.; Si, S.; Pan, H., et al. Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. *Nature medicine* 2004, *10*, 1344-1351, doi:10.1038/nm1135.
33. Chan, E.W.; Lye, P.Y.; Wong, S.K. Phytochemistry, pharmacology, and clinical trials of Morus alba. *Chinese journal of natural medicines* 2016, *14*, 17-30, doi:10.3724/SP.J.1009.2016.00017.
34. Kong, W.H.; Oh, S.H.; Ahn, Y.R.; Kim, K.W.; Kim, J.H.; Seo, S.W. Antiobesity effects and improvement of insulin sensitivity by 1-deoxynojirimycin in animal models. *Journal of agricultural and food chemistry* 2008, *56*, 2613-2619, doi:10.1021/jf073223i.
35. Takasu, S.; Parida, I.S.; Onose, S.; Ito, J.; Ikeda, R.; Yamagishi, K.; Higuchi, O.; Tanaka, F.; Kimura, T.; Miyazawa, T., et al. Evaluation of the anti-hyperglycemic effect and safety of microorganism 1-deoxynojirimycin. *PloS one* 2018, *13*, e0199057, doi:10.1371/journal.pone.0199057.
36. Enkhmaa, B.; Shiwaku, K.; Katsube, T.; Kitajima, K.; Anuurad, E.; Yamasaki, M.; Yamane, Y. Mulberry (Morus alba L.) leaves and their major flavonol quercetin 3-(6-malonylglucoside) attenuate atherosclerotic lesion development in LDL receptor-deficient mice. *The Journal of nutrition* 2005, *135*, 729-734.
37. El-Beshbishy, H.A.; Singab, A.N.; Sinkkonen, J.; Pihlaja, K. Hypolipidemic and antioxidant effects of Morus alba L. (Egyptian mulberry) root bark fractions supplementation in cholesterol-fed rats. *Life sciences* 2006, *78*, 2724-2733, doi:10.1016/j.lfs.2005.10.010.
38. Harauma, A.; Murayama, T.; Ikeyama, K.; Sano, H.; Arai, H.; Takano, R.; Kita, T.; Hara, S.; Kamei, K.; Yokode, M. Mulberry leaf powder prevents atherosclerosis in apolipoprotein E-deficient mice. *Biochem Biophys Res Commun* 2007, *358*, 751-756, doi:10.1016/j.bbrc.2007.04.170.
39. Liu, L.K.; Chou, F.P.; Chen, Y.C.; Chyau, C.C.; Ho, H.H.; Wang, C.J. Effects of mulberry (Morus alba L.) extracts on lipid homeostasis in vitro and in vivo. *Journal of agricultural and food chemistry* 2009, *57*, 7605-7611, doi:10.1021/jf9014697.
40. Keepers, Y.P.; Pizao, P.E.; Peters, G.J.; van Ark-Otte, J.; Winograd, B.; Pinedo, H.M. Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for in vitro chemosensitivity testing. *Eur J Cancer* 1991, *27*, 897-900.
41. Rimoldi, I.; Facchetti, G.; Lucchini, G.; Castiglioni, E.; Marchiano, S.; Ferri, N. In vitro anticancer activity evaluation of new cationic platinum(II) complexes based on imidazole moiety. *Bioorganic & medicinal chemistry* 2017, *25*, 1907-1913, doi:10.1016/j.bmc.2017.02.010.
42. Raines, E.W.; Garton, K.J.; Ferri, N. Beyond the endothelium: NF-kappaB regulation of smooth muscle function. *Circ Res* 2004, *94*, 706-708, doi:10.1161/01.RES.0000125646.08156.4D.

578 43. Ruscica, M.; Ricci, C.; Macchi, C.; Magni, P.; Cristofani, R.; Liu, J.; Corsini, A.; Ferri, N. Suppressor of
579 Cytokine Signaling-3 (SOCS-3) Induces Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9) Expression in
580 Hepatic HepG2 Cell Line. *J Biol Chem* 2016, *291*, 3508-3519, doi:10.1074/jbc.M115.664706.
581 44. Morton, R.E.; Evans, T.A. Modification of the bicinchoninic acid protein assay to eliminate lipid interference
582 in determining lipoprotein protein content. *Analytical biochemistry* 1992, *204*, 332-334.
583 45. Pirillo, A.; Reduzzi, A.; Ferri, N.; Kuhn, H.; Corsini, A.; Catapano, A.L. Upregulation of lectin-like oxidized
584 low-density lipoprotein receptor-1 (LOX-1) by 15-lipoxygenase-modified LDL in endothelial cells. *Atherosclerosis*
585 2011, *214*, 331-337, doi:10.1016/j.atherosclerosis.2010.11.006.
586 46. Cao, A.; Wu, M.; Li, H.; Liu, J. Janus kinase activation by cytokine oncostatin M decreases PCSK9 expression
587 in liver cells. *J Lipid Res* 2011, *52*, 518-530, doi:10.1194/jlr.M010603.
588 47. Yang, Z.Z.; Wang, Y.C.; Wang, Y.; Zhang, Y. Bioassay-guided screening and isolation of α-glucosidase and
589 tyrosinase inhibitors from leaves of Morus alba. *Food Chemistry* 2012, *131*.
590 48. Chen, C.H.; Yang, J.C.; Uang, Y.S.; Lin, C.J. Improved dissolution rate and oral bioavailability of lovastatin
591 in red yeast rice products. *International journal of pharmaceutics* 2013, *444*, 18-24, doi:10.1016/j.ijpharm.2013.01.028.
592 49. Bellosta, S.; Paoletti, R.; Corsini, A. Safety of statins: focus on clinical pharmacokinetics and drug
593 interactions. *Circulation* 2004, *109*, III50-57, doi:10.1161/01.CIR.0000131519.15067.1f.
594 50. Maeng, H.J.; Yoo, H.J.; Kim, I.W.; Song, I.S.; Chung, S.J.; Shim, C.K. P-glycoprotein-mediated transport of
595 berberine across Caco-2 cell monolayers. *Journal of pharmaceutical sciences* 2002, *91*, 2614-2621,
596 doi:10.1002/jps.10268.
597 51. Pan, G.Y.; Wang, G.J.; Liu, X.D.; Fawcett, J.P.; Xie, Y.Y. The involvement of P-glycoprotein in berberine
598 absorption. *Pharmacology & toxicology* 2002, *91*, 193-197.
599 52. Wang, X.; Wang, R.; Xing, D.; Su, H.; Ma, C.; Ding, Y.; Du, L. Kinetic difference of berberine between
600 hippocampus and plasma in rat after intravenous administration of Coptidis rhizoma extract. *Life sciences* 2005,
601 *77*, 3058-3067, doi:10.1016/j.lfs.2005.02.033.
602 53. Huang, C.H.; Shiu, S.M.; Wu, M.T.; Chen, W.L.; Wang, S.G.; Lee, H.M. Monacolin K affects lipid metabolism
603 through SIRT1/AMPK pathway in HepG2 cells. *Archives of pharmacal research* 2013, *36*, 1541-1551,
604 doi:10.1007/s12272-013-0150-2.