Nutrition, Metabolism & Cardiovascular Diseases (2019) **29**, 1245–1253

Available online at www.sciencedirect.com

Nutrition, Metabolism & Cardiovascular Diseases

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

Differential effects of red yeast rice, *Berberis aristata* and *Morus alba* extracts on PCSK9 and LDL uptake

Maria G. Lupo [a], Chiara Macchi [b], Silvia Marchianò [b], Riccardo Cristofani [b], Maria F. Greco [b], Stefano Dall'Acqua [a], Haixia Chen [c], Cesare R. Sirtori [d], Alberto Corsini [b,e], Massimiliano Ruscica [b,*], Nicola Ferri [a]

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

Received 30 April 2019; received in revised form 26 May 2019; accepted 8 June 2019
Handling Editor: M Averna
Available online 14 June 2019

KEYWORDS
Berberis aristate;
Monacolin K;
Morus alba;
PCSK9 and red yeast rice

Abstract *Background and aims:* The novel 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) is effective in the management of elevated plasma low-density lipoprotein cholesterol (LDL-C) levels. The aim of the present study was to investigate the effects of the three components on proprotein convertase subtilisin/kexin type 9 (PCSK9), a key regulator of LDL receptor (LDLR) expression, in hepatocyte cell lines and to compare their effects on LDL cellular uptake.

Methods and results: HepG2 and Huh7 cells were incubated with *B. aristata* cortex extract (BCE), red yeast rice (RYR) and *M. alba* leaves extract (MLE) alone or in combination for 24 h. RYR (50 μg/mL) increased PCSK9 protein expression (Western blot analysis and ELISA), PCSK9 mRNA (qPCR) and its promoter activity (luciferase reporter assay). BCE (40 μg/mL) reduced instead PCSK9 expression, mRNA levels and promoter activity. MLE determined a concentration-dependent reduction of PCSK9 at the mRNA and protein levels, with a maximal reduction at 1 mg/mL, without significant changes of PCSK9 promoter activity. MLE also downregulated the expression of 3-hydroxy-3-methyl-3-glutaryl coenzyme A reductase and fatty acid synthase mRNA levels. The combination of RYR, BCE and MLE reduced the PCSK9 mRNA and protein levels, as well as the promoter activity. Finally, the single components and their combination induced LDL receptor and LDL uptake by the hepatocytes.

Conclusion: The positive effect of MLE on PCSK9 supports the rationale of using the nutraceutical combination of RYR, BCE and MLE to control hyperlipidemic conditions.

* Corresponding author. Università degli Studi di Milano, Via Balzaretti 9, 20133, Milano, Italy.
E-mail address: massimiliano.ruscica@unimi.it (M. Ruscica).

https://doi.org/10.1016/j.numecd.2019.06.001
0939-4753/

Introduction

In recent years, nutritional approaches to control dyslipidemias have been developed [1,2] and some Guidelines for the management of hyperlipidemias encourage the use of nutraceuticals as alternatives [3] or in addition to lipid-lowering drugs [4]. Several nutraceutical combinations have shown significant lipid lowering effects and potential positive impact on cardiovascular (CV) risk [5] including the one 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). The clinical efficacy of this combination was evaluated in subjects with mild hypercholesterolemia and not on statin therapy [6].

PCSK9 plays a pivotal role on low-density lipoprotein cholesterol (LDL-C) levels by regulating the degradation of the LDL receptor (LDLR), although other physiological functions beyond lipid metabolism have been reported [7–12]. In addition, PCSK9 is regulated by different factors implicated in cardiovascular diseases (CVD), *e.g.* including a condition of insulin resistance [13–15]. The relevance of PCSK9 as a pharmacological target to control hypercholesterolemia and CVD has been demonstrated by the positive results of the main cardiovascular outcome trials with evolocumab [16] and alirocumab [17].

Statin therapy raises PCSK9 [18] by activating the sterol responsive element binding protein (SREBP) transcriptional activity [19,20] or inhibiting the small G protein Rac1 [21]. Besides statins, many nutraceuticals modulate PCSK9 expression [22], including berberine [23], curcumin [24], moracin C [25], and lignans [26].

Since monacolin K, the key component of RYR, is chemically indistinguishable from lovastatin, it is conceivable to hypothesize an inducible effect on PCSK9. Conversely, berberine does not influence SREBP pathway but rather increases expression of LDLR by stabilizing its mRNA [27]. Additional evidence suggests that berberine inhibits the PCSK9 promoter activity by reducing HNF1α and SREBP expression [23,28,29]. This effect counteracts the induction of PCSK9 by statins [29], and for this reason berberine may be considered useful in combination to statins [23].

The major component of *M. alba*, i.e. 1-deoxynojirimycin (DNJ), shows an inhibitory activity on α-glycosidase leading to a significant hypoglycemic and antiobesity effect [30,31]. Experimental evidence also indicates that MLE reduces cholesterol levels and attenuate the progression of atherosclerotic lesions [32–34], although the basic molecular mechanism has not been explored. The dual role of *M. alba* extracts on lipid and glucose levels support the rational of studying its effect on PCSK9.

Thus, the aim of the present study was to define the effects of the active components of a novel nutraceutical combination on genes involved in cholesterol homeostasis, including PCSK9, and thus the molecular mechanisms underlying its hypocholesterolemic effect.

Methods

For detailed descriptions of the Materials and methods, please see Supplemental material.

Results

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 the novel nutraceutical combination on PCSK9 expression. HepG2 cells were incubated for 24 h with non-cytotoxic concentrations of RYR (50 μg/mL), MLE (1 mg/mL) or BCE (40 μg/mL) (Supplemental Fig. 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), whereas BCE partially reduced their intracellular levels, compared to untreated control cells (−34.7% proPCSK9 and −64.3% PCSK9 *vs* control, respectively) (Fig. 1A and B). Simvastatin was used as the positive control. The water-soluble extract of MLE acted similarly to BCE, leading to a significant reduction of the intracellular levels of both proPCSK9 and active form of PCSK9 (−17.1% proPCSK9 and −59.3% PCSK9, respectively; Fig. 1A and B). Western blot analysis from the Huh7 hepatic cell line showed similar results with a significant reduction of PCSK9 expression by BCE and MLE and increased levels in response to RYR (Supplemental Fig. 2A and B). PCSK9 levels released in the conditioned media were significant raised upon RYR treatment (+9.8% ± 1.2% *vs* control), whilst they were reduced by BCE (−64.8% ± 16.2% *vs* control) and MLE (−43.8% ± 23.6% *vs* control) treatments (Fig. 1C).

The quantification of the mRNA levels by real time qPCR showed that RYR induced PCSK9 levels by 2.98 ± 0.73-fold, while MLE and BCE significantly suppressed its expression by 69.2% ± 11.0% and by 93.7% ± 3.9%, respectively (Fig. 1D). 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 the PCSK9 gene from −1711 to −94 (pGL3-PCSK9-D1), relative to the ATG starting codon [29]. 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; Fig. 1E). 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, *i.e.* the pGL3-PCSK9-D4 plasmid containing Sp1, SRE and HNF1α sites [29]. Upon combined 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 (Fig. 1F). These results provide new evidence on the inhibitory effect of MLE on PCSK9 expression, without any interference with its promoter activity.

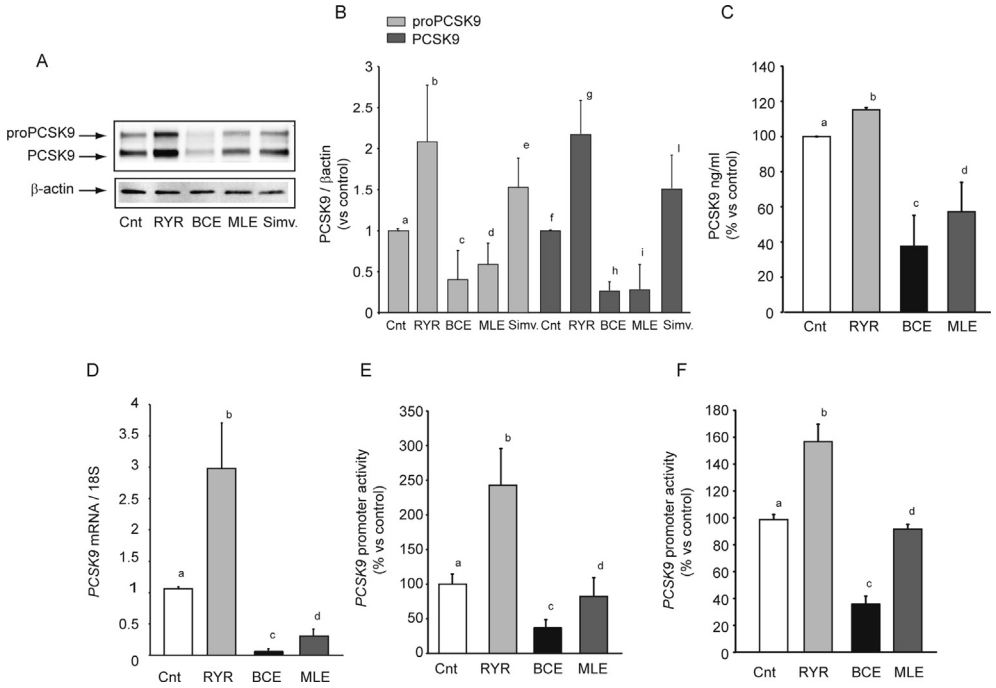

Figure 1 Effect of RYR, BCE and MLE on PCSK9 expression. A–D) HepG2 cells incubated with MEM/10%FCS in the presence or absence of RYR (40 μg/mL), BCE (50 μg/mL), MLE (1 mg/mL), and simvastatin (40 μM). After 24 h, total protein extract and mRNA were prepared, and conditioned media collected. A) PCSK9 protein expression was evaluated by Western blot analysis. β-actin was used as loading control. B) Densitometric readings were evaluated using the ImageLab™ software. C) PCSK9 levels in the conditioned media were evaluated by ELISA. D) mRNA levels of *PCSK9* were determined by quantitative real-time PCR. F and G) HepG2 cells were transfected with pGL3-PCSK9-D1 (E) or pGL3-PCSK9-D4 (F) and the effect of RYR, BCE or MLE on PCSK9 promoter activity was evaluated by the luciferase assay. Data are given as the mean ± S.D. of at least three independent experiments. Differences between treatments were assessed by one-way ANOVA. B) a *vs* b $^{**}p < 0.01$; a *vs* e $^{*}p < 0.05$; b *vs* c $^{***}p < 0.001$; b *vs* d $^{***}p < 0.001$; f *vs* g $^{**}p < 0.01$; f *vs* h $^{**}p < 0.01$; f *vs* i $^{**}p < 0.01$; f *vs* l $^{**}p < 0.01$; g *vs* h $^{***}p < 0.001$; g *vs* i $^{***}p < 0.001$; C) a *vs* b $^{*}p < 0.05$; a *vs* c $^{***}p < 0.001$; a *vs* d $^{**}p < 0.01$; b *vs* c $^{***}p < 0.001$; b *vs* d $^{***}p < 0.001$; D) a *vs* b $^{***}p < 0.001$; a *vs* c$^{**}p < 0.01$; a *vs* d $^{*}p < 0.05$; b *vs* c $^{***}p < 0.001$; b *vs* d $^{***}p < 0.001$; E) a *vs* b $^{***}p < 0.001$; a *vs* c $^{***}p < 0.001$; b *vs* c $^{***}p < 0.001$; b *vs* d $^{***}p < 0.001$; c *vs* d $^{***}p < 0.001$; F) a *vs* b $^{***}p < 0.001$; a *vs* c $^{**}p < 0.01$; b *vs* c $^{***}p < 0.001$; b *vs* d $^{***}p < 0.001$; c *vs* d $^{***}p < 0.001$; Cnt: control; RYR: red yeast rice; BCE: *Berberis aristata* cortex extract; MLE: *Morus alba* leaves extract; Simv.: simvastatin.

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 HMGCR, and FAS. This analysis revealed that MLE significantly reduces the expression of both FAS and HMGCR at a similar extent as BCE. On the contrary, RYR induced both transcripts in a significant manner (Fig. 2A and B). In particular, the incubation of HepG2 cells with MLE significantly reduced HMGCR mRNA expression (−51.0 ± 7.2% *vs* control), similarly to BCE (−41.1% ± 5.1% *vs* control); conversely, RYR led to opposite results: +1.58 ± 0.11-fold *vs* control. A similar effect was observed on FAS mRNA expression (−37.2 ± 0.9%, −30.0 ± 0.7% and 1.25 ± 0.04-fold *vs* control for MLE, BCE and RYR, respectively). At the protein levels, both RYR, BCE and MLE induced LDLR expression in HepG2 cells by 1.6, 1.7 and 1.8-fold, respectively (Fig. 2C). These results were also confirmed in the Huh7 cell line (Fig. S2C). Overall, these findings indicate that MLE elicits an inhibitory effect on additional genes, beyond PCSK9, that are both involved in cholesterol metabolism and transcriptionally regulated by the SREBP pathway.

To further investigate the effects of MLE on gene expression, we determined the protein expression of SREBP2 and HNF1α from total cell lysates. RYR determined a significant increase of both transcription factors (1.6- and 1.8-fold for SREBP2 and HNF1α, respectively), whereas BCE determined a significant downregulation (−70% and 20% for SREBP2 and HNF1α, respectively). Interestingly, MLE determined a minor reduction of SREBP2 expression (−40%) and no effect on HNF1α (Fig. 2D and E), suggesting that the inhibitory effects on PCSK9, HMGCR reductase and FAS by MLE are due to a different mechanism of action.

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

To better define the effects of MLE on genes involved in lipid metabolism, a series of experiments were conducted with increasing concentrations of MLE (0.25, 0.5, and 1.0 mg/mL). This analysis revealed a negative effect of MLE on PCSK9 mRNA on both HepG2 and Huh7 cell lines, at any concentration utilized (Fig. 3A and S2D). In addition, by ELISA, we observed a significant reduction of extracellular concentrations of PCSK9 in HepG2 cell line (−42% at 1 mg/

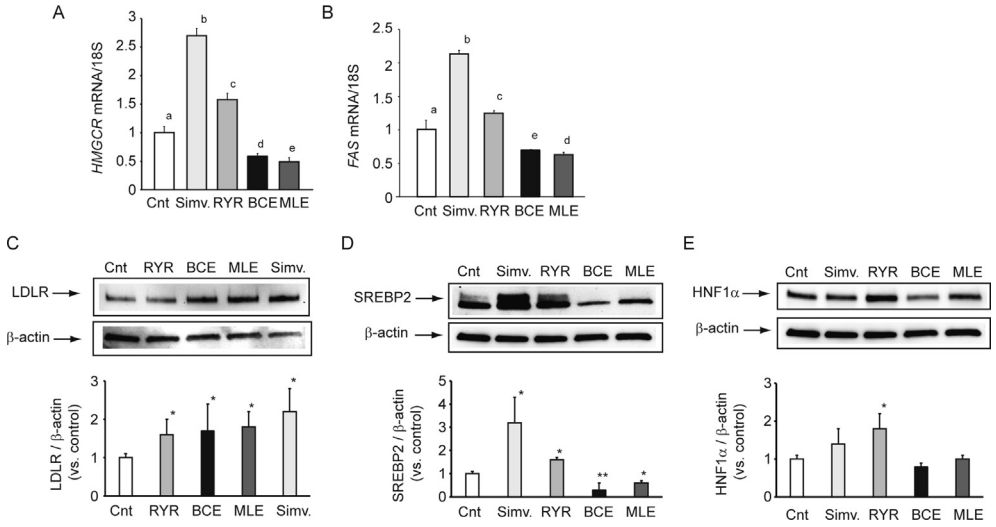

Figure 2 Effect of RYR, BCE and MLE on SREBP-regulated genes. A–E) HepG2 were incubated with MEM/10% FCS in the presence or absence of simvastatin (40 μM), RYR (40 μg/mL), BCE (50 μg/mL), MLE (1 mg/mL). After 24 h total RNA and total protein extract were prepared. A and B) mRNA levels of *HMGCR* and *FAS* mRNA were determined by qPCR. C–E) LDLR, SREBP2 and HNF1α protein expression were evaluated by Western blot analysis from total protein extracts. β-actin was used as loading control. Densitometric readings were evaluated using the ImageLab™ software. Data are given as the mean ± S.D. of at least three independent experiments. Differences between treatments were assessed by Student's t test, and one-way ANOVA (when needed). A) a *vs* b ***$p < 0.001$; a *vs* c ***$p < 0.001$; a *vs* d ***$p < 0.01$; a *vs* e **$p < 0.001$; c *vs* d ***$p < 0.001$; c *vs* e ***$p < 0.001$; B) a *vs* b ***$p < 0.001$; a *vs* c **$p < 0.01$; a *vs* d ***$p < 0.001$; a *vs* e ***$p < 0.001$; c *vs* d ***$p < 0.001$; c *vs* e ***$p < 0.001$. Cnt: control; RYR: red yeast rice; BCE: *Berberis aristata* cortex extract; MLE: *Morus alba* leaves extract; Simv.: simvastatin.

mL; Fig. 3B). MLE significantly inhibited PCSK9 at any concentration utilized, whereas no effect was observed on the PCSK9 promoter activity, as assessed by the luciferase assay (Fig. 3C). The incubation with the transcriptional activity inhibitor, actinomycin D, showed that MLE did not alter the stability of PCSK9 mRNA (Fig. 3D).

The inhibition of HMGCR mRNA expression was observed in a very similar range of concentrations as those utilized for PCSK9 (Fig. 3E), whereas only 1 mg/mL of MLE reduced, although only marginally, the expression of LDLR (−26.0 ± 18.0% *vs* control; Fig. 3F) and FAS (−23.2 ± 14.9% *vs* control; Fig. 3G).

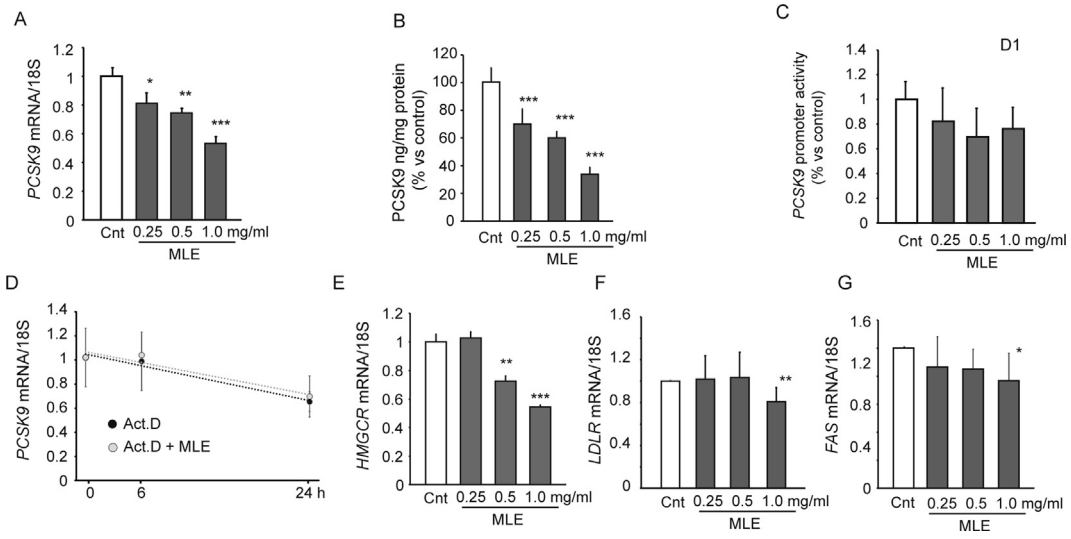

Figure 3 Concentration-dependent effect of MLE on PCSK9 and SREBP-related genes expression. HepG2 cells were incubated with MEM/10% FCS in the presence or absence of increasing concentrations of MLE (0.25, 0.5 and 1 mg/mL). After 24 h total RNA was prepared, and conditioned media collected. A) mRNA levels of *PCSK9* were determined by quantitative real-time PCR. B) PCSK9 levels in the conditioned media were evaluated by ELISA and values corrected for total proteins from cell monolayers. C) HepG2 cells were transfected with pGL3-PCSK9-D1 and the effect of RYR, BCE or MLE on PCSK9 promoter activity was evaluated by the luciferase assay. D) HepG2 were incubated for the indicated times with MLE in the presence or absence of actinomycin D (5 μg/mL) and *PCSK9* mRNA levels were determined by qPCR. E–G) mRNA levels of *HMGCR*, *FAS*, and *LDLR* mRNA were determined by quantitative real-time PCR. Data are given as the mean ± S.D. of at least three independent experiments. Differences between treatments were assessed by Student's t test, and one-way ANOVA (when necessary). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Cnt: control; MLE: *Morus alba* leaves extract.

The analytical determination of the major active components in MLE, showed the presence of 0.299 ± 0.002 mg/mL of DNJ, 18.0 ± 0.5 μg/mL of quercetin malonyl glucoside and 3-, 4-, 5-caffeoyl quinic acid (30.2, 26.2 and 39.7 μg/mL, respectively, Table 1).

Thus, the calculated final concentrations of DNJ and quercetin in 1 mg/mL of MLE added to cultured media were 18 μmol/L and 0.32 μmol/L, respectively. We then tested increasing concentrations of DNJ and quercetin on PCSK9 expression in HepG2 cells. As shown in Fig. 4, both DNJ and quercetin did not affect PCSK9 mRNA levels at concentrations similar to those present in MLE. However, DNJ slightly raised mRNA PCSK9 levels at 200 μmol/L concentration whereas quercetin reduced the expression at concentrations above 6.3 μmol/L.

Effects of the combination of RYR, BCE and MLE on genes involved in cholesterol homeostasis

Previous studies have shown that berberine counteracts the effects of statins on PCSK9 expression [23,29]. 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 of HepG2 cells with RYR (40 μg/mL), BCE (50 μg/mL) and MLE (1 mg/mL) did not shown any significant cytotoxic effect, as assessed by the SRB assay (Fig. S1B).

The incubation with the three extracts resulted in a significant reduction of intracellular and secreted forms of PCSK9, as determined by Western blot analysis and ELISA, respectively (Fig. 5A and C). In particular, the three 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. The effect of these components on intracellular PCSK9 levels were also confirmed in Huh7 cells (Figs. S2E and F). A similar effect was also observed at the 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; Fig. 5D–F). Under the same experimental conditions, treatments did not show any significant changes of β-Galactosidase activity after transfection with the pCMV-β vector, suggesting a specific effect on the *PCSK9* promoter and the lack of interference with protein expression (Supplemental Fig. 3). These data suggest that BCE and MLE actively counteract the induction of *PCSK9* by RYR. We therefore investigated the effect of different active components of the novel nutraceutical combination on *PCSK9* promoter activity. As shown in Fig. 5G, MLE did not interfere with the induction of the *PCSK9* promoter activity by RYR. However, the combination of three active components reduced the *PCSK9* promoter activity below basal conditions. 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.

In order to extend this evidence, we investigated, under the same experimental conditions, the effect of active components of the novel nutraceutical combination on additional SREBP-regulated genes. As shown in Fig. 6, the combination of RYR, BCE and MLE reduced both the expression of *HMGCR* (−32.5 ± 5.5% *vs* control) and *FAS* (−30.0 ± 3.4% *vs* control) mRNA. The Western blot analysis showed a significant induction of the LDLR after incubation of HepG2 and Huh7 cells with RYR, BCE and MLE combination (1.54 ± 0.38 and 4.3 ± 1.9-fold, respectively; Fig. 6C and D, S2G and H). As expected, simvastatin induced the LDLR (Fig. 6C and S2C). Since lipid lowering effect has to rely on the induction of LDLR and uptake of LDL-C, we determined the effects of RYR, BCE and MLE on this parameter by incubating HepG2 cells for 24 h with different combination of these active components followed by 3 h 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; Fig. 6E). 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; Fig. 6E). Importantly, MLE partially improved the effects of both RYR and BCE on LDL 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 this nutraceutical combination for an effective lipid-lowering action.

Discussion

In the present study, we investigated the effects of active constituents of a nutraceutical combination, *i.e.* RYR, BCE and MLE, on PCSK9 expression and additional genes regulated by the SREBP transcription factors (*LDLR*, *HMGCR* and *FAS*). In particular, the active components of this nutraceutical combination on cholesterol levels, such as RYR containing monacolin K and berberine, reportedly reduce

Table 1 Concentrations of active components of MLE.

Component	Concentration in MLE stock extract	MW (molecular weight)	Molar quantity in 1 mg/mL of MLE in cultured media
DNJ	0.299 ± 0.002 mg/mL	163.17 g/mol	18 μmol/L
Quercetin malonyl glucoside	18.0 ± 0.5 μg/mL	550.42 g/mol	0.32 μmol/L
3-Caffeoyl quinic acid	30.2 ± 0.5 μg/mL	354.31 g/mol	0.85 μmol/L
4-Caffeoyl quinic acid	26.2 ± 0.5 μg/mL	354.31 g/mol	0.74 μmol/L
5-Caffeoyl quinic acid	39.7 ± 0.5 μg/mL	354.31 g/mol	1.12 μmol/L

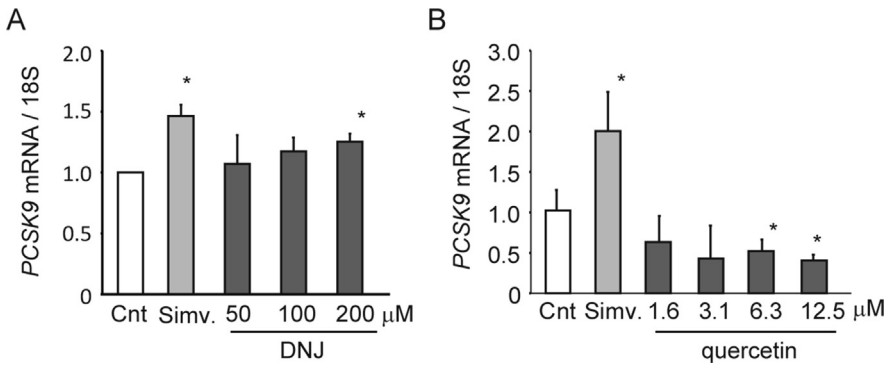

Figure 4 Effect of DNJ and quercetin on PCSK9 mRNA expression in HepG2 cells. HepG2 cells were seeded in MEM/10% FCS and the day after incubated with MEM/10%FCS in the presence or absence of simvastatin (40 μM) or indicated concentrations of DNJ (A) and quercetin (B). After 24 h, total RNA was prepared and mRNA levels of PCSK9 were determined by quantitative real-time PCR. Differences between treatments were assessed by Student's t test. *p < 0.05 *vs* control. Simv.: simvastatin.

the transcription of *PCSK9* [23,28], whereas the activity of MLE on this pivotal regulator of cholesterol homeostasis is still unclear.

Our results demonstrate that the water-soluble extract of MLE significantly inhibited the expression of PCSK9, by reducing both mRNA and protein levels in hepatic cells. However, differently from berberine [29], MLE does not significantly affect the promoter activity of *PCSK9*, as determined by using 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

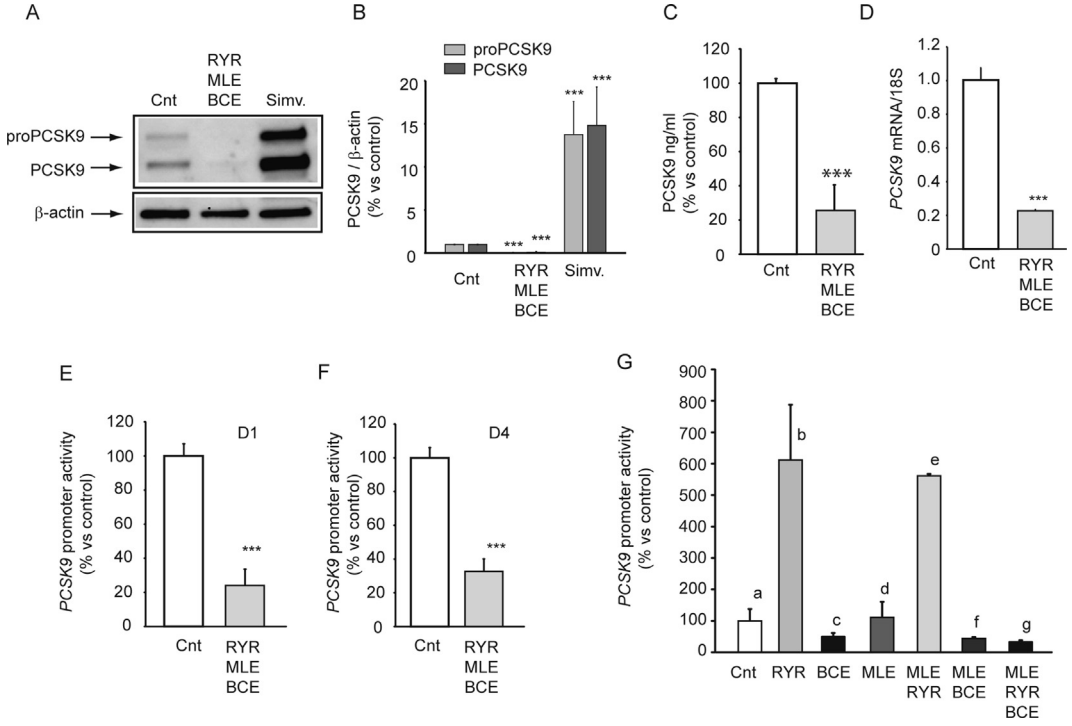

Figure 5 Effect of the active components of a novel nutraceutical combination on PCSK9 expression. A–D) HepG2 were incubated with MEM/10% FCS in the presence or absence of three active components simultaneously (RYR, 40 μg/mL; BCE, 50 μg/mL and MLE, 1 mg/mL) or simvastatin (40 μM). After 24 h, total protein extract and RNA were prepared, and conditioned media collected. A) PCSK9 protein expression was evaluated by Western blot analysis from total protein extracts. β-actin was used as loading control. B) Densitometric readings were evaluated using the ImageLab™ software. C) PCSK9 levels in the conditioned media were evaluated by ELISA and values corrected for total proteins from cell monolayers. 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, cells were seeded in a 48-well tray and, after an additional 24 h, they were incubated with RYR, BCE and MLE. After 24 h, luciferase activities were determined by NeoLite reagent. G) Under the same experimental conditions of panel F, the effect of different combinations of active components on *PCSK9* promoter activity was determined. Data are given as the mean ± S.D. of at least three independent experiments. Differences between treatments were assessed by Student's t test (panels B–F), and one-way ANOVA (panel G). G) a *vs* b ***p < 0.001; a *vs* e ***p < 0.001; a *vs* f *p < 0.05; a *vs* g *p < 0.05; b *vs* c ***p < 0001; b *vs* d ***p < 0.01; b *vs* f ***p < 0.001; b *vs* g ***p < 0.001; c *vs* e ***p < 0.001; d *vs* e ***p < 0.001; e *vs* f ***p < 0.001; e *vs* g***p < 0.001. Cnt: control; RYR: red yeast rice; BCE: *Berberis aristata* cortex extract; MLE: *Morus alba* leaves extract; Simv.: simvastatin.

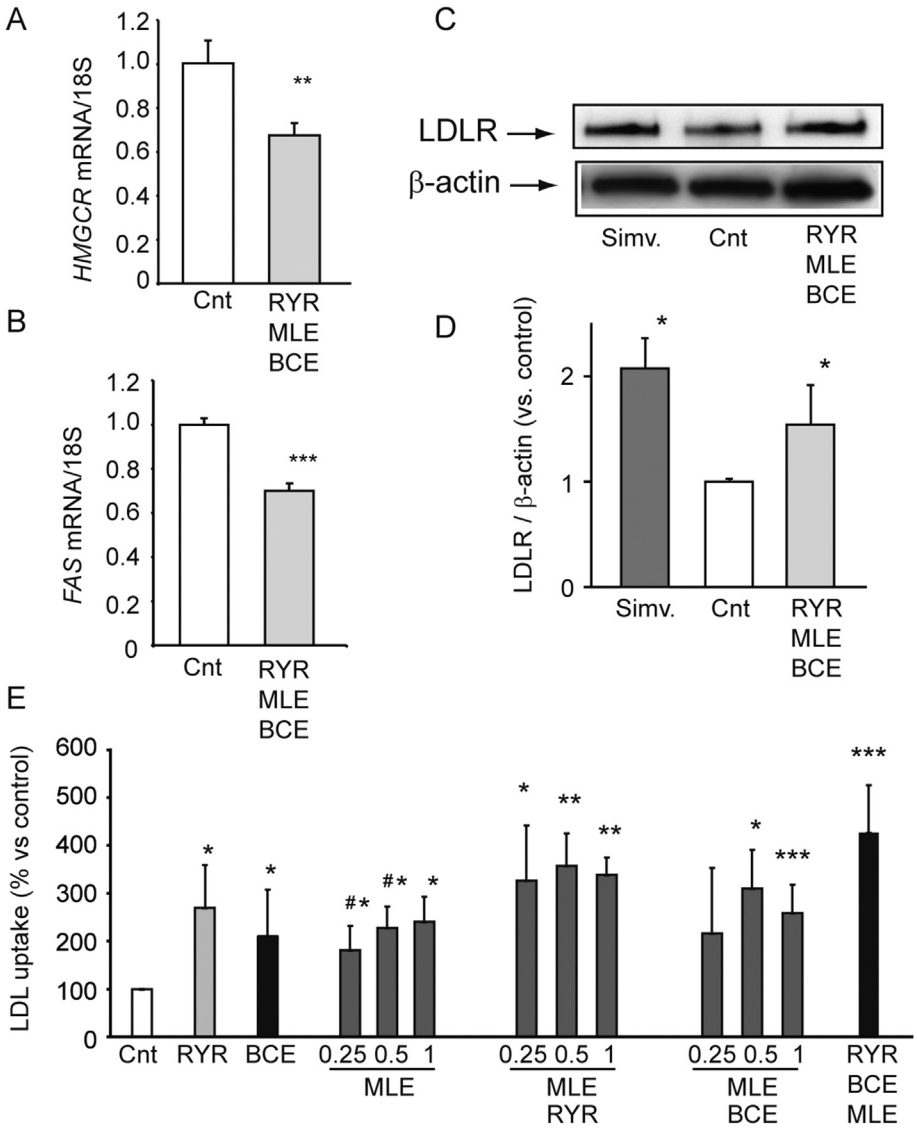

Figure 6 Effect of the active components of a novel nutraceutical combination on genes involved in cholesterol homeostasis and LDL uptake. HepG2 (A–D) cells were seeded in MEM/10% FCS and the day after incubated with MEM/10%FCS in the presence or absence of the three active components simultaneously (RYR, 40 μg/mL; BCE, 50 μg/mL and MLE,1 mg/mL). After 24 h, total protein extract and RNA were prepared. A and B) mRNA levels of *HMGCR*, and *FAS* mRNA were determined by quantitative real-time PCR. C) LDLR protein expression was evaluated by Western blot analysis from total protein extracts. β-actin was used as loading control. D) Densitometric readings were evaluated using the ImageLab™ 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 active components. After 24 h, HepG2 cells were incubated with 10 μg/mL of LDL-DiO and fluorescence intensity determined by flow cytometry analysis after 3 h incubation. Data are given as the mean ± S.D. of at least three independent experiments. Differences between treatments were assessed by Student's t test, and one-way ANOVA (panel E). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs* control; #$p < 0.05$ *vs* RYR/BCE/MLE. Cnt: control; RYR: red yeast rice; BCE: *Berberis aristata* cortex extract; MLE: *Morus alba* leaves extract; Simv.: simvastatin.

HMGCR and *FAS*, two additional SREBP-regulated genes. We also excluded an effect of MLE on *PCSK9* mRNA stability, since the degradation of PCSK9 was similar in control and MLE treated cells in the presence of the transcriptional inhibitor actinomycin D. These data suggest that MLE may interfere with the SREBP pathway but at the post-transcriptional levels. Similar results have been observed with oncostatin M, a cytokine that effectively suppresses the *PCSK9* mRNA levels without affecting promoter activity [35]. In addition, the suppressor of cytokine signaling 3 (SOCS3) molecule has been shown to induce *PCSK9* without activating the PCSK9 promoter activity [36]. 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.

The relevant aspect that should be taken into account is the concentration of the component/s with PCSK9 inhibitory activity present in water soluble MLE. The analytical determination of the major active components of MLE showed that DNJ is the most abundant, being quercetin malonyl glucoside and different isomers of caffeoyl quinic acid in minor amounts. The effects of DNJ that elicit a

significant increase of PCSK9 expression in HepG2 cells occur at 200 μmol/L; on the contrary, we observed a significant reduction of PCSK9 mRNA after incubation with quercetin, although this effect was statistically significant at concentrations 20-fold higher than those detected in MLE. At similar concentrations, quercetin-3-glucoside reduces the secretion of PCSK9 in Huh7 [37].

In a separate evaluation of the active components of MLE, it has been recently reported that moracin C, present in dried immature *M. alba* fruits, reduces both the mRNA and protein of PCSK9 [25]. Moracin C was identified from a chloroform soluble extract of *M. alba* fruits, whereas in our study we utilized water soluble extracts of *M. alba* leaves. Interestingly, the water-soluble extracts of *M. alba* fruits did not show any activity on PCSK9 expression [25]. Thus, although moracin C is present in *M. alba* leaves [38], it is unlikely to be present in our water extract. Overall, these results demonstrated a significant effect of MLE on PCSK9, although the active component/s still need to be identified.

The final concentrations of water-soluble extracts of *M. alba* leaves, *i.e.* 0.25 ÷ 1 mg/mL, are lower than the previously tested 1 ÷ 6 mg/mL of mulberry water extracts on HepG2 cells [34] and 0.5 ÷ 2 mg/mL of mulberry leaf extract in cultured aortic vascular smooth muscle cells [39]. We chose these concentrations since they were not cytotoxic in HepG2 cells. These concentrations appear to exceed those utilized for RYR and BCE and thus it is difficult to establish if the observed effect on PCSK9 on HepG2 cells can be relevant for the clinical effects of this novel combination. However, the reduction of PCSK9 was observed at the concentration of 250 μg/mL, thus similar to the 40 μg/mL of BCE or 50 μg/mL of RYR.

Regardless of the mechanism of action and active components of *M. alba* responsible for the inhibited expression of PCSK9, it is conceivable that MLE, together with BCE, may effectively counteract the induction of PCSK9 by monacolin K in RYR, ameliorating the final hypocholesterolemic action of the nutraceutical combination. We further demonstrated that the combination of MLE and BCE effectively counteracts the effect of RYR, by blocking the PCSK9 expression at protein, mRNA and promoter activity levels, together with a significant reduction of *HMGCR* and *FAS* levels. The same combination induces the LDLR and LDL-DiO uptake in HepG2 cells, compared to control untreated cells.

In the present study, we thus demonstrated that the combination of three active components can markedly affect genes that regulate the cholesterol homeostasis, with a significant reduction of PCSK9 and increased LDLR and LDL uptake. In addition, we provided new evidence of the contribution of water-soluble extracts of *M. alba* leaves on the hypocholesterolemic effect of this nutraceutical by reducing PCSK9 levels.

It remains obviously unclear by what mechanism these active components can induce LDLR and LDL uptake; it may be dependent upon a variation of the intracellular pool of cholesterol, consequent either by a variation of VLDL secretion by the cells or to the inhibition of cholesterol synthesis. Further analyses will need to be performed to address these points. The results of the present study indicate that the inhibitory effect of MLE on PCSK9 may contribute to the lipid-lowering action of this nutraceutical, supporting the rationale for the use of this combination in the management of hyperlipidemias [4,5].

Author contributions

NF conceived the study and wrote the manuscript. MGL and CM conducted the experiments. SM and HC conducted some experiments related to Figs. 4 and 5. RC performed the new experiments on β-Galactosidase activity. MFG conducted the experiments on quercetin. SDA determined the concentrations of active components of *M. alba* leaves extract. AC and CRS critically revised the manuscript. MR wrote the manuscript and critically revised it.

Acknowledgments

NF acknowledges Akademy Pharma Srl (Milan, 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).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2019.06.001.

Conflicts of interest

The authors declare no conflict of interest.

References

[1] Cicero AFG, Fogacci F, Colletti A. Food and plant bioactives for reducing cardiometabolic disease risk: an evidence based approach. Food Funct 2017;8:2076–88. https://doi.org/10.1039/c7fo00178aI.
[2] Poli A, Barbagallo CM, Cicero AFG, Corsini A, Manzato E, Trimarco B, et al. Nutraceuticals and functional foods for the control of plasma cholesterol levels. An intersociety position paper. Pharmacol Res 2018;134:51–60. https://doi.org/10.1016/j.phrs.2018.05.015I.
[3] Catapano AL, Graham I, De Backer G, Wiklund O, Chapman MJ, Drexel H, 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. https://doi.org/10.1093/eurheartj/ehw272I.
[4] Cicero AFG, Colletti A, Bajraktari G, Descamps O, Djuric DM, Ezhov M, et al. Lipid-lowering nutraceuticals in clinical practice: position paper from an international lipid expert panel. Nutr Rev 2017;75:731–67. https://doi.org/10.1093/nutrit/nux047I.
[5] Li JJ, Lu ZL, Kou WR, Chen Z, Wu YF, Yu XH, et al. 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). J Clin Pharmacol 2009;49:947–56. https://doi.org/10.1177/0091270009337509I.
[6] Trimarco V, Battistoni A, Tocci G, Coluccia R, Manzi MV, Izzo R, et al. 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. Nutr Metab Cardiovasc Dis 2017;27:850–7. https://doi.org/10.1016/j.numecd.2017.08.003I.
[7] Adorni MP, Cipollari E, Favari E, Zanotti I, Zimetti F, Corsini A, et al. Inhibitory effect of PCSK9 on Abca1 protein expression and cholesterol efflux in macrophages. Atherosclerosis 2016;256:1–6. https://doi.org/10.1016/j.atherosclerosis.2016.11.019I.
[8] Ferri N, Marchiano S, Tibolla G, Baetta R, Dhyani A, Ruscica M, et al. PCSK9 knock-out mice are protected from neointimal formation in response to perivascular carotid collar placement. Atherosclerosis 2016;253:214–24. https://doi.org/10.1016/j.atherosclerosis.2016.07.910I.
[9] Ruscica M, Ferri N, Fogacci F, Rosticci M, Botta M, Marchiano S, 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. J Am Heart Assoc 2017;6. https://doi.org/10.1161/JAHA.117.005764I.
[10] Ricci C, Ruscica M, Camera M, Rossetti L, Macchi C, Colciago A, et al. PCSK9 induces a pro-inflammatory response in macrophages. Sci Rep 2018;8:2267. https://doi.org/10.1038/s41598-018-20425-xI.
[11] Macchi C, Ferri N, Favero C, Cantone L, Vigna L, Pesatori AC, et al. Long-term exposure to air pollution raises circulating levels of proprotein convertase subtilisin/kexin type 9 in obese individuals. Eur J Prev Cardiol 2019;26:578–88. https://doi.org/10.1177/2047487318815320I.
[12] Cicero AFG, Toth PP, Fogacci F, Virdis A, Borghi C. Improvement in arterial stiffness after short-term treatment with PCSK9 inhibitors. Nutrition, metabolism, and cardiovascular diseases. Nutr Metab Cardiovasc Dis 2019;29:527–9. https://doi.org/10.1016/j.numecd.2019.01.010I.
[13] Ferri N, Ruscica M. Proprotein convertase subtilisin/kexin type 9 (PCSK9) and metabolic syndrome: insights on insulin resistance, inflammation, and atherogenic dyslipidemia. Endocrine 2016;54:588–601. https://doi.org/10.1007/s12020-016-0939-0I.
[14] Cariou B, Langhi C, Le Bras M, Bortolotti M, Le KA, Theytaz F, et al. Plasma PCSK9 concentrations during an oral fat load and after short term high-fat, high-fat high-protein and high-fructose diets. Nutr Metab 2013;10:4. https://doi.org/10.1186/1743-7075-10-4I.
[15] Guoa W, Gong Y, Li J, Qin P, Lu J, Li X, et al. Association of serum proprotein convertase subtilisin/kexin type 9 with early atherosclerosis in newly diagnosed type 2 diabetes mellitus. Nutr Metab Cardiovasc Dis 2019;29(8):815–21. https://doi.org/10.1016/j.numecd.2019.04.006.
[16] Sabatine MS, Giugliano RP, Keech AC, Honarpour N, Wiviott SD, Murphy SA, et al. Evolocumab and clinical outcomes in patients with cardiovascular disease. N Engl J Med 2017;376:1713–22. https://doi.org/10.1056/NEJMoa1615664I.
[17] Schwartz GG, Steg PG, Szarek M, Bhatt DL, Bittner VA, Diaz R, et al. Alirocumab and cardiovascular outcomes after acute coronary syndrome. N Engl J Med 2018;379:2097–107. https://doi.org/10.1056/NEJMoa1801174I.
[18] Macchi C, Banach M, Corsini A, Sirtori CR, Ferri N, Ruscica M. Changes in circulating pro-protein convertase subtilisin/kexin type 9 levels - experimental and clinical approaches with lipid-lowering agents. Eur J Prev Cardiol 2019 Jun;26(9):930–49. https://doi.org/10.1177/2047487319831500.
[19] Costet P, Cariou B, Lambert G, Lalanne F, Lardeux B, Jarnoux AL, et al. Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c. J Biol Chem 2006;281:6211–8.
[20] Jeong HJ, Lee HS, Kim KS, Kim YK, Yoon D, Park SW. 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. https://doi.org/10.1194/jlr.M700443-JLR200I.
[21] Ferri N, Marchiano S, Lupo MG, Trenti A, Biondo G, Castaldello P, et al. Geranylgeraniol prevents the simvastatin-induced PCSK9 expression: role of the small G protein Rac1. Pharmacol Res 2017;122:96–104. https://doi.org/10.1016/j.phrs.2017.05.021I.
[22] Momtazi AA, Banach M, Pirro M, Katsiki N, Sahebkar A. Regulation of PCSK9 by nutraceuticals. Pharmacol Res 2017;120:157–69. https://doi.org/10.1016/j.phrs.2017.03.023I.
[23] Cameron J, Ranheim T, Kulseth MA, Leren TP, Berge KE. Berberine decreases PCSK9 expression in HepG2 cells. Atherosclerosis 2008;201:266–73. https://doi.org/10.1016/j.atherosclerosis.2008.02.004I.
[24] Tai MH, Chen PK, Chen PY, Wu MJ, Ho CT, Yen JH. Curcumin enhances cell-surface LDLR level and promotes LDL uptake through downregulation of PCSK9 gene expression in HepG2 cells. Mol Nutr Food Res 2014;58:2133–45. https://doi.org/10.1002/mnfr.201400366I.
[25] Pel P, Chae HS, Nhoek P, Kim YM, Chin YW. Chemical constituents with proprotein convertase subtilisin/kexin type 9 mRNA expression inhibitory activity from dried immature *Morus alba* fruits. J Agric Food Chem 2017;65:5316–21. https://doi.org/10.1021/acs.jafc.7b02088I.
[26] Pel P, Chae HS, Nhoek P, Yeo W, Kim YM, Chin YW. Lignans from the fruits of *Schisandra chinensis* (Turcz.) Baill inhibit proprotein convertase subtilisin/kexin type 9 expression. Phytochemistry 2017;136:119–24. https://doi.org/10.1016/j.phytochem.2017.01.010I.
[27] Kong W, Wei J, Abidi P, Lin M, Inaba S, Li C, et al. Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. Nat Med 2004;10:1344–51. https://doi.org/10.1038/nm1135I.
[28] Dong B, Li H, Singh AB, 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;290:4047–58. https://doi.org/10.1074/jbc.M114.597229I.
[29] Li H, Dong B, Park SW, Lee HS, Chen W, Liu J. Hepatocyte nuclear factor 1 alpha plays a critical role in PCSK9 gene transcription and regulation by the natural hypocholesterolemic compound berberine. J Biol Chem 2009;284:28885–95. https://doi.org/10.1074/jbc.M109.052407I.
[30] Kong WH, Oh SH, Ahn YR, Kim KW, Kim JH, Seo SW. Antiobesity effects and improvement of insulin sensitivity by 1-deoxynojirimycin in animal models. J Agric Food Chem 2008;56:2613–9. https://doi.org/10.1021/jf073223iI.
[31] Takasu S, Parida IS, Onose S, Ito J, Ikeda R, Yamagishi K, et al. Evaluation of the anti-hyperglycemic effect and safety of microorganism 1-deoxynojirimycin. PLoS One 2018;13:e0199057. https://doi.org/10.1371/journal.pone.0199057I.
[32] Enkhmaa B, Shiwaku K, Katsube T, Kitajima K, Anuurad E, Yamasaki M, et al. Mulberry (*Morus alba* L.) leaves and their major flavonol quercetin 3-(6-malonylglucoside) attenuate atherosclerotic lesion development in LDL receptor-deficient mice. J Nutr 2005;135:729–34. https://doi.org/10.1093/jn/135.4.729.
[33] El-Beshbishy HA, Singab AN, Sinkkonen J, Pihlaja K. Hypolipidemic and antioxidant effects of *Morus alba* L. (*Egyptian mulberry*) root bark fractions supplementation in cholesterol-fed rats. Life Sci 2006;78:2724–33. https://doi.org/10.1016/j.lfs.2005.10.010I.
[34] Liu LK, Chou FP, Chen YC, Chyau CC, Ho HH, Wang CJ. Effects of mulberry (*Morus alba* L.) extracts on lipid homeostasis in vitro and in vivo. J Agric Food Chem 2009;57:7605–11. https://doi.org/10.1021/jf9014697I.
[35] Cao A, Wu M, Li H, Liu J. Janus kinase activation by cytokine oncostatin M decreases PCSK9 expression in liver cells. J Lipid Res 2011;52:518–30. https://doi.org/10.1194/jlr.M010603I.
[36] Ruscica M, Ricci C, Macchi C, Magni P, Cristofani R, Liu J, et al. Suppressor of cytokine signaling-3 (SOCS-3) induces proprotein convertase subtilisin kexin type 9 (PCSK9) expression in hepatic HepG2 cell line. J Biol Chem 2016;291:3508–19. https://doi.org/10.1074/jbc.M115.664706I.
[37] Mbikay M, Sirois F, Simoes S, Mayne J, Chretien M. Quercetin-3-glucoside increases low-density lipoprotein receptor (LDLR) expression, attenuates proprotein convertase subtilisin/kexin 9 (PCSK9) secretion, and stimulates LDL uptake by Huh7 human hepatocytes in culture. FEBS Open Bio 2014;4:755–62. https://doi.org/10.1016/j.fob.2014.08.003I.
[38] Yang ZZ, Wang YC, Wang Y, Zhang Y. Bioassay-guided screening and isolation of α-glucosidase and tyrosinase inhibitors from leaves of *Morus alba*. Food Chem 2012;131. https://doi.org/10.1016/j.foodchem.2011.09.040.
[39] Chan KC, Yang MY, Lin MC, Lee YJ, Chang WC, Wang CJ. Mulberry leaf extract inhibits the development of atherosclerosis in cholesterol-fed rabbits and in cultured aortic vascular smooth muscle cells. J Agric Food Chem 2013;61:2780–8. https://doi.org/10.1021/jf305328dI.