Investigations on the hypocholesterolaemic activity of LILPKHSDAD and LTFPGSAED, two peptides from lupin β-conglutin: focus on LDLR and PCSK9 pathways

Chiara Zanoni, Gilda Aiello, Anna Arnoldi,* Carmen Lammi

Department of Pharmaceutical Sciences, University of Milan, 20133 Milan, Italy

*Corresponding author: Anna Arnoldi, Department of Pharmaceutical Sciences, University of Milan, via Mangiagalli 25, 20133 Milan, Italy. Tel +390250319372, anna.arnoldi@unimi.it

ABSTRACT.

P5 (LILPKHSDAD) and **P7** (LTFPGSAED) are two peptides from lupin protein that are absorbed in Caco-2 cells. Recent *in silico* docking studies had suggested that they could potentially function as inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoAR) activity. This paper confirms that both peptides inhibit *in vitro* the HMGCoAR functionality and it reports the characterisation of the molecular mechanism through which they modulate the cholesterol metabolism in HepG2 cells. Through the inhibition of HMGCoAR activity, **P5** and **P7** are able to improve the LDLR protein levels leading to a better ability of HepG2 cells to uptake extracellular LDL molecules with a final hypocholesterolaemic effects. The modulation of PCSK9 intracellular processing was also evaluated: only **P5** was able to decrease the PCSK9 and HNF1-alpha protein levels leading to a decrease of mature PCSK9 secretion by HepG2 cells.

Keywords: bioactive peptides, functional foods, LDL receptor, PCSK9, plant protein.

Abbreviations: **AMPK**, adenosine monophosphate-activated protein kinase; **DMEM**, Dulbecco's modified Eagle's medium; **FBS**, foetal bovine serum; **HMGCoAR**, 3-hydroxy-3-methylglutaryl CoA reductase; **HNF1-alpha**, hepatocyte nuclear factor 1-alpha; **LDL**, low density lipoprotein; **LDLR**, LDL receptor; **PCSK9**, proprotein convertase subtilisin/kexin type 9; **SREBP2**, sterol regulatory element binding protein 2.

1. Introduction

Lupin protein and peptides provide useful health benefits (Arnoldi, Boschin, Zanoni, & Lammi, 2015; Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008), particularly in the area of cholesterol reduction (Bähr, Fechner, Kiehntopf, & Jahreis, 2015; Sirtori, Triolo, Bosisio, Bondioli, Calabresi, De Vergori, et al., 2012), hypertension control (Arnoldi, Boschin, Zanoni, & Lammi, 2015), and hyperglycaemia prevention (Duranti et al., 2008).

Convinced that these activities should be ascribed to specific peptides initially encrypted in the protein sequences and released by digestion, in the last few years, we have dedicated much effort to investigate the biological activity and the bioavailability of lupin peptides, with particular focus on cholesterol reduction. Working on human hepatic HepG2 cells, it was possible to demonstrate that tryptic and peptic hydrolysates from a total lupin protein extract are able to interfere with the HMGCoAR activity, up-regulating the LDL receptor (LDLR) and sterol regulatory element binding protein 2 (SREBP2) proteins via the activation of phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), and glycogen synthase kinase-3β (GSK3β) pathways and increasing the LDL-uptake of HepG2 cells (Lammi, Zanoni, Scigliuolo, D'Amato, & Arnoldi, 2014). As indicated by chip-HPLC-ESI-MS/MS analysis, the composition of both hydrolysates was very complex, with a prevalence of peptides deriving from β-conglutin, a major storage protein belonging to the vicilin-like family (Duranti et al., 2008).

In order to sort out potentially bioavailable peptides among those detected, a model of the intestine based on differentiated human intestinal Caco-2 cells was used (Ferruzza, Rossi, Scarino, & Sambuy, 2012; Sambuy, De Angelis, Ranaldi, Scarino, Stammati, & Zucco, 2005). The hydrolysates were added in the apical chamber of a two-chambers system and after 4 h the basolateral solutions were collected and analysed by HPLC-Chip-MS/MS. This permitted the identification of eleven tryptic and eight peptic peptides. Interestingly, a specific assay showed that the basolateral samples still kept up the capacity to inhibit the activity of HMGCoAR (Lammi, Aiello, Vistoli, Zanoni, Arnoldi, Sambuy, et al., 2016).

A further experiment, conducted in a co-culture system in which Caco2 cells and HepG2 cells were combined, showed that the basolateral solutions from the peptic and tryptic hydrolysate treatments are still bioactive on HepG2 cells, with an intensity that was even improved by the crosstalk between the two cell systems in co-culture (Lammi, Zanoni, Ferruzza, Ranaldi, Sambuy, & Arnoldi, 2016). All these evidences suggest that the basolateral samples should contain very active peptides. In order to identify the most active ones, it was decided to perform *in silico* docking simulations with the catalytic site of HMGCoAR: two peptides LILPKHSDAD (**P5**) and LTFPGSAED (**P7**), derived from β-conglutin, appeared to be potentially the major amino acid sequences responsible of the observed

activity, because they bind very efficiently to the catalytic site of this enzyme showing the best normalised Plp95, APBS and MLP score values in the docking simulations (Lammi, et al., 2016). Based on these considerations, this work had two main objectives: 1) the experimental confirmation that indeed **P5** and **P7** are able to inhibit the HMGCoAR activity as suggested by the preceding *in silico* simulations; 2) the detailed investigation of the mechanism of action through which these peptides exert their hypocholesterolaemic effects in HepG2 cells at molecular and functional levels.

2. Materials and methods

2.1 Materials

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, foetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin/streptomycin, chemiluminescent reagent, and 96-well plates were purchased from Euroclone (Milan, Italy). Bovine serum albumin (BSA), RIPA buffer, and the antibody against β-actin were bought from Sigma-Aldrich (St. Louis, MO, USA). The antibody against HMGCoAR was bought from Abcam (Cambridge, UK). The antibody against phospho-HMGCoAR (Ser872) was purchased from Bioss Antibodies (Woburn, MA, USA). The antibody against proprotein convertase subtilisin/kexin type 9 (PCSK9) and hepatocyte nuclear factor 1-alpha (HNF1-alpha) were bought from GeneTex (Irvine, CA, USA). Phenylmethanesulfonyl fluoride (PMSF), Na-orthovanadate inhibitors, and the antibodies against rabbit Ig-horseradish peroxidase (HRP), mouse Ig-HRP, and SREBP-2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The antibodies against the LDLR and the phospho-AMPK (Thr172) were bought from Pierce (Rockford, IL, USA). The inhibitor cocktail Complete Midi from Roche (Basel, Swiss). Mini protean TGX pre-cast gel 7.5% and Mini nitrocellulose Transfer Packs were purchased from BioRad (Hercules, CA, USA). The Human Proprotein Convertase 9 Immunoassay (Quantikine ELISA) was bought from R&D System (Minneapolis, MN, USA). Synthetic peptides **P5** and **P7** were synthesized by the company PRIMM (Milan, Italy) at >95% purity.

2.2 Cell culture conditions

HepG2 cell line was bought from ATCC (HB-8065, ATCC from LGC Standards, Milan, Italy) and was cultured in DMEM high glucose with stable L-glutamine, supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (complete growth medium) with incubation at 37 °C under 5% CO_2 atmosphere. HepG2 cells were used for no more than 20 passages after thawing, because the increase in number of passages may change the cell characteristics and impair assay results.

99

2.3 HMGCoAR activity assay

The assay buffer, NADPH, substrate solution, and HMGCoAR were provided in the HMGCoAR Assay Kit (Sigma). The experiments were carried out following the manufacturer's instructions at 37 °C. In particular, each reaction (200 µL) was prepared adding the reagents in the following order: 1 X assay buffer, a concentration range of **P5** and **P7** from 10^{-5} to 10^{-3} M or vehicle (C), the NADPH (4 µL), the substrate solution (12 µL), and finally the HMGCoAR (catalytic domain) (2 µL). Subsequently, the samples were mixed and the absorbance at 340 nm read by a microplate reader Synergy H1 from Biotek at time 0 and 10 min. The HMGCoAR-dependent oxidation of NADPH and the inhibition properties of lupin peptides were measured by absorbance reduction, which is directly proportional to enzyme activity.

2.4 Western blot analysis

A total of 1.5×10^5 HepG2 cells/well (24-well plate) were treated with 50 and 100 µM of **P5** and 10 and 50 µM of **P7** for 24 h. After each treatment, cells were scraped in 40 µL ice-cold lysis buffer [RIPA buffer + inhibitor cocktail + 1:100 PMSF + 1:100 Na-orthovanadate] and transferred in an ice-cold microcentrifuge tube. After centrifugation at 16,060 g for 15 min at 4 °C, the supernatant was recovered and transferred into a new ice-cold tube. Total proteins were quantified by Bradford method and 50 µg of total proteins loaded on a pre-cast 7.5% Sodium Dodecyl Sulphate - Polyacrylamide (SDS-PAGE) gel at 130 V for 45 min. Subsequently, the gel was pre-equilibrated with 0.04% SDS in H_2O for 15 min at room temperature (RT) and transferred to a nitrocellulose membrane (Mini nitrocellulose Transfer Packs,) using a Trans-Blot Turbo at 1.3 A, 25 V for 7 min. Target proteins, on milk blocked membrane, were detected by primary antibodies as follows: rabbit anti-SREBP2, rabbit anti-LDLR, anti-HMGCoAR, anti-pospho AMPK (Thr172), anti-pospho HMGCoAR (Ser872), anti-HNF1 alpha, anti-PCSK9, and anti-β-actin. Secondary antibodies conjugated with HRP and a chemiluminescent reagent were used to visualize target proteins and their signal was quantified using the Image Lab Software (Biorad). The internal control β-actin was used to normalise loading variations.

2.5 Assay for evaluation of fluorescent LDL uptake by HepG2 cells

A total of 3×10^4 HepG2 cells/well were seeded in 96-well plates and kept in complete growth medium for 2 d before treatment. On the third day, cells were treated with 10 and 50 µM of **P5** and **P7**, respectively, or vehicle (H_2O) for 24 h. At the end of the treatment period, the culture medium was replaced with 75 µL/well LDL-DyLight™ 550 working solution. The cells were additionally

incubated for 2 h at 37 °C and then the culture medium was aspirated and replaced with PBS (100 µL/well). The degree of LDL uptake was measured using the Synergy H1 fluorescent plate reader from Biotek (excitation and emission wavelengths 540 and 570 nm, respectively).

2.6 Quantification of PCSK9 excreted by HepG2 cells through ELISA

The supernatants collected from HepG2 cells were centrifuged at 600 g for 10 min at 4 °C. They were recovered and diluted to the ratio 1:10 with DMEM in a new ice-cold tube. PCSK9 was quantified by ELISA (R&D System, Minneapolis, MN, USA). Briefly, the experiments were carried out at 37 °C, following the manufacturer's instructions. Before starting the assay, human PCSK9 standards (20.0, 10.0, 5.0, 2.5, 1.25, and 0.625 ng/mL) were prepared from the stock PCSK9 standard solution (40 ng/mL) with serial dilutions (for building the standard curve) and meanwhile 100 µL of the Assay Diluent RD1-9 (provided into the kit) were added to each wells. Afterward, standards and samples (50 µL) were pipetted into the wells and the ELISA plate was allowed to incubate for 2 h at RT. Subsequently, wells were washed 4 times with Wash Buffer, and 200 µL of human PCSK9-Conjugate (HRP-labelled anti-PCSK9) was added to each wells for a 2 h incubation at RT. Following aspiration, well was washed 4 times with Wash Buffer provided by the kit. After the last wash, 200 µL of Substrate Solution were added to the wells and allowed to incubate for 30 min at RT. The reaction was stopped with 50 µL of Stop Solution (2 N sulfuric acid) and the absorbance at 450 nm was measured using Synergy H1 (Biotek, Bad Friedrichshall, Germany). The lower limit of detection was 0.096 ng/mL.

2.7 Statistically Analysis

Statistical analyses were carried out by One-way ANOVA (Graphpad Prism 6) followed by Dunnett's test. Values were expressed as means ± sem; P-values < 0.05 were considered to be significant.

3. Results

3.1. Inhibition of the catalytic activity of HMGCoAR

In order to confirm the *in silico* prediction according to which **P5** and **P7** might be potential inhibitors of the catalytic activity of HMGCoAR (Lammi, et al., 2016), an *in vitro* assay was performed using the purified catalytic domain of this enzyme. Peptide concentrations ranging from 10^{-5} to 10^{-3} M were tested. Each peptide inhibited HMGCoAR in a dose-dependent manner (Figure 1). The IC_{50} value of **P5** was equal to 147.2 ± 1.34 µM and that of **P7** was equal to 68.4 ± 1.53 µM. Based on these results, **P7** appeared to be a more potent inhibitor of the HMGCoAR catalytic activity.
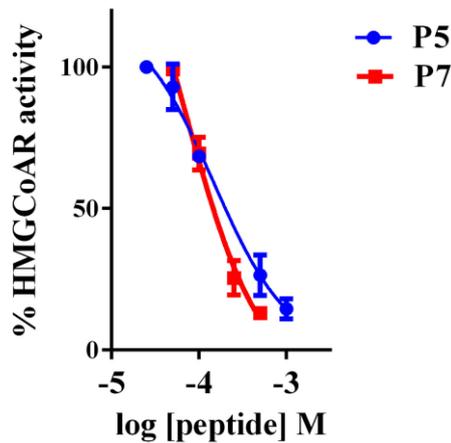
Figure 1. Concentration-response curves of the inhibitory effect of P5 and P7 on HMGCoAR activity.
The HMGCoAR, physiologically, catalyzes the four-electron reduction of HMGCoA to coenzyme A (CoA)
and mevalonate (HMGCoA + 2NADPH + 2H+ > mevalonate + 2NADP+ + CoA-SH). In this assay, the
decrease in absorbance at 340 nm, which represents the oxidation of NADPH by the catalytic subunit of
HMGCoAR in the presence of the substrate HMGCoA, was measured spectrophotometrically for each
peptide dose tested. IC50 value for P5 is equal to 147.2 ± 1.34 μM, for P7 is equal to 68.4 ± 1.53 μM. Each
point represents the average ± SEM of three experiments in duplicate.

3.2 Activation of the LDLR pathway

HepG2 cells were treated with **P5** (50 and 100 μM) and **P7** (10 and 50 μM) and each sample was
investigated with immunoblotting experiments. Figure 2A,C shows that the treatment with **P5**
induced an up-regulation of the protein level of N-terminal fragment of the SREBP2 (mature form
with a molecular weight of 68 kDa) by $52.3 \pm 18\%$ ($p < 0.05$) and $79.6 \pm 29.5\%$ ($p < 0.001$) at 50 μM
and 100 μM, respectively, *versus* the control. As a consequence, up-regulations of the LDLR and
HMGCoAR protein levels were also observed. In particular, after treatment with **P5** at 50 μM and
100 μM (Figure 2A,C), the LDLR protein level was increased by $53.5 \pm 9.4\%$ ($p < 0.05$) and $94.8 \pm
46.3\%$,($p < 0.0001$) respectively, *versus* the control, whereas the HMGCoAR protein level was
augmented by $51.2 \pm 29.8\%$ ($p < 0.05$) and $57.5 \pm 15.2\%$ ($p < 0.05$), respectively, *versus* the control.
Figure 2 B,D shows, instead, the variations induced by treatments with 10 μM and 50 μM **P7** *versus*
the control. The SREBP2 protein level was increased by $52.8 \pm 1.69\%$ ($p < 0.05$) and $62.46 \pm 28.18\%$
($p < 0.001$) respectively; the LDLR protein level was augmented by $38.4 \pm 7.9\%$ ($p < 0.001$) and $102.7
\pm 16.8\%$ ($p < 0.0001$), respectively; whereas the HMGCoAR protein level was enhanced by $98.3 \pm
52.6\%$ ($p < 0.001$) and $67.8 \pm 33.7\%$ ($p < 0.05$), respectively, versus the control.
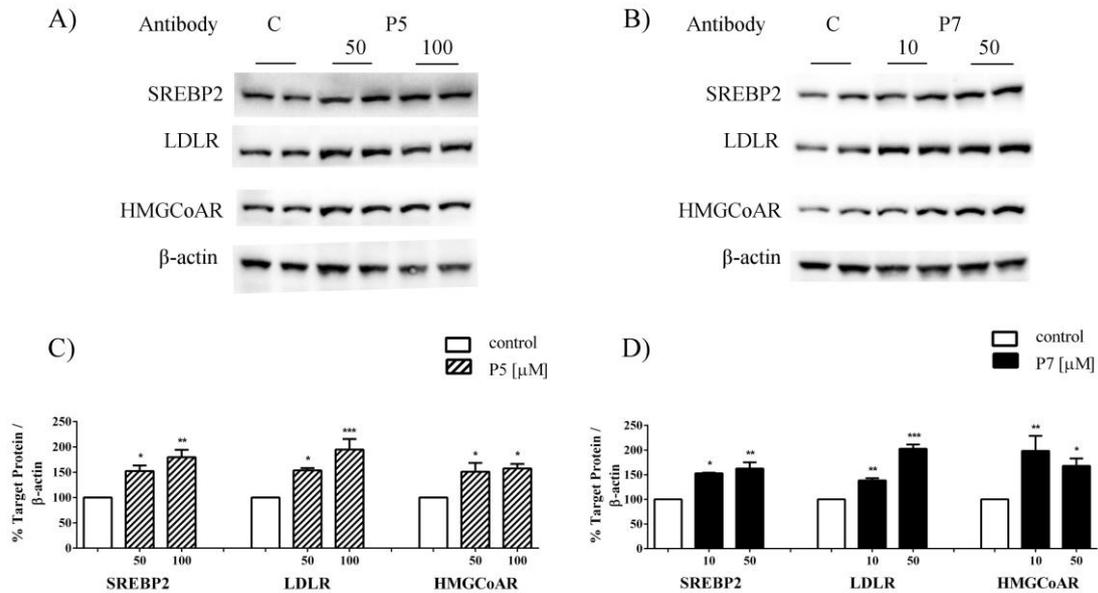
Figure 2. Effects of P5 and P7 on SREBP2, LDLR, and HMGCoAR protein levels. HepG2 cells (1.5 × 10⁵) were treated with **P5** (50 and 100 μM) (A-C) and **P7** (10 and 50 μM) (B-D) for 24 h. SREBP2, LDLR, HMGCoAR, and β-actin immunoblotting signals were detected using specific anti-SREBP2, anti-LDLR, anti-HMGCoAR, and anti-β-actin primary antibodies, respectively. Each protein signal was quantified by ImageLab software (Biorad) and normalised with β-actin signals. Bars represent averages ± SEM of six independent experiments (two duplicates per sample). (∗) *p <0.05,* (∗∗) *p < 0.001,* and (∗∗∗) *p < 0.0001 versus* control (C).

3.3 The inactivation of HMGCoAR depends on activation of the AMPK pathway

Suitable immunoblotting experiments were performed in order to evaluate the effect of the treatment with the same peptides on AMPK activation and HMGCoAR inactivation (AMPK substrate). The lysates from treated and untreated HepG2 cells were therefore analysed using specific antibodies for AMPK phosphorylated at threonine 172 (Figure 3A) and for HMGCoAR phosphorylated at serine 872 (AMPK phosphorylation site) (Figure 3B). Figure 3C shows that treatment with **P5** significantly increased AMPK phosphorylation by 36.5 ± 8.3% (50 μM, *p <0.001*) and by 60.5 ± 2.8% (100 μM, *p <0.001*), whereas the treatment with **P7** significantly increased the AMPK phosphorylation by 106.6 ± 6.42% (10 μM, *p <0.05*) and 196.4 ± 69.3% (50 μM, *p <0.0001*) *versus* the control. As a consequence of the AMPK activation, the phosphorylation levels of HMGCoAR were also increased (Figure 3D): after treatment with **P5** by 157.0 ± 21.1% (50 μM, *p <0.0001*) and 169.9 ± 35.0% (100 μM, *p<0.0001)*, whereas after treatment with **P7** by 75.3 ± 2.9% (10 μM, *p <0.05*) and 90.4 ± 19.2% (50 μM, *p <0.001) versus* the control.

214
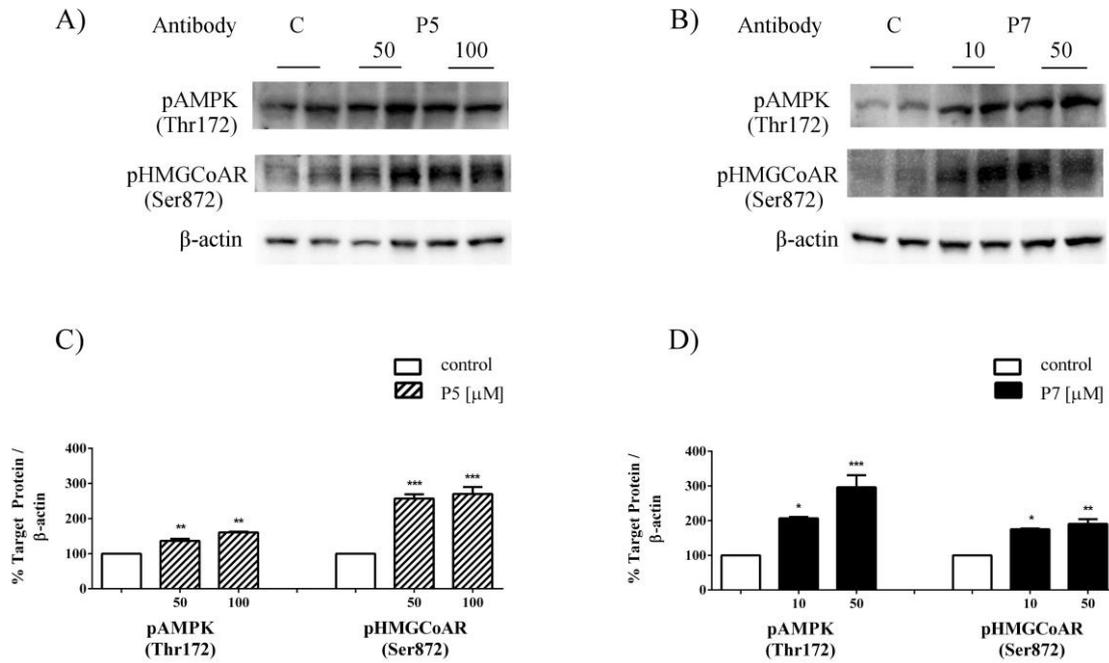
Figure 3. Effect of P5 and P7 on AMPK activation and HMGCoAR inactivation. HepG2 cells ($1.5 \times$ 10^5) were treated with **P5** (50 and 100 µM) (A-C) and **P7** (10 and 50 µM) (B-D) for 24 h. The phosphorylation levels of AMPK (Thr172) and HMGCoAR (Ser872) and β-actin immunoblotting signals were detected using specific anti-phospho AMPK (Thr172), anti-phospho HMGCoAR (Ser872), and anti-β-actin primary antibodies, respectively. Each protein signal was quantified by ImageLab software (Biorad) and normalised with β-actin signals. Bars represent averages ± SEM of six independent experiments (two duplicates per sample). (∗) *p <0.05,* (∗∗) *p < 0.001,* and (∗∗∗) *p < 0.0001 versus* control (C).

Figure 4 clearly shows that the treatments with **P5** and **P7** led to an elevation of the p-HMGCoAR/HMGCoAR protein ratio indicating a diminished enzyme activity. In particular, the ratio phospho (p)-HMGCoAR/HMGCoAR protein increased by $86.2 \pm 16.2\%$ (50 µM, *p<0.0001*) and $129.1 \pm 13.4\%$ (100 µM, *p <0.0001*) *versus* the control after treatment with **P5**, and by $68.2 \pm 39.1\%$ (10 µM, *p< 0.001*) and $104.2 \pm 57.8\%$ (50 µM, *p< 0.0001*) after treatment with **P7**.
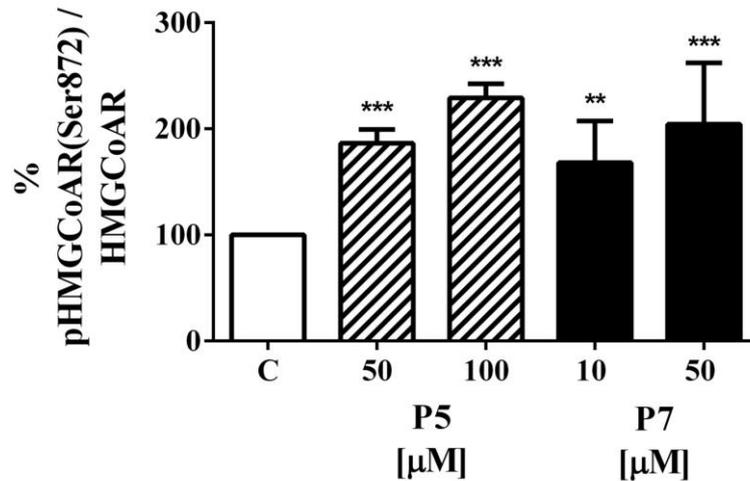
Figure 4. Ratios between p-HMGCoAR (Ser872) and HMGCOAR protein levels. Bars represent the ratios between p-HMGCoAR (Ser872) and HMGCoAR, each normalised with β-actin signals, after **P5** (50 and 100 μM) and **P7** (10 and 50 μM) treatments. Bars represent averages ± SEM of six independent experiments (two duplicates per sample). ($\ast\ast$) *p < 0.001,* and ($\ast\ast\ast$) *p < 0.0001 versus* control (C).

3.4 *Modulation of the LDL-uptake in HepG2 cells*

The change of the functional capability of HepG2 cells to uptake extracellular LDL after the treatments with the peptides was investigated by performing fluorescent-LDL uptake experiments. As shown in Figure 5, each peptide increased the LDL-uptake in a statistically significant way *versus* the control. In fact, after treatments with 10 μM and 50 μM **P7**, the LDL-uptake was increased by 72.4 ± 21.6% (*p < 0.05*) and 235.9 ± 29.2% (*p < 0.001*), respectively (Figure 5B), whereas the treatments with **P5** at a concentration of 50 μM led to an increase of the LDL-uptake by 99.5 ± 48.51% (*p < 0.001*) (Figure 5A). At the concentration of 10 μM, **P5** led to a 66 ± 21.4% (*p < 0.001*) increase as reported in another publication (Lammi, Zanoni, Aiello, Arnoldi, & Grazioso, 2016).
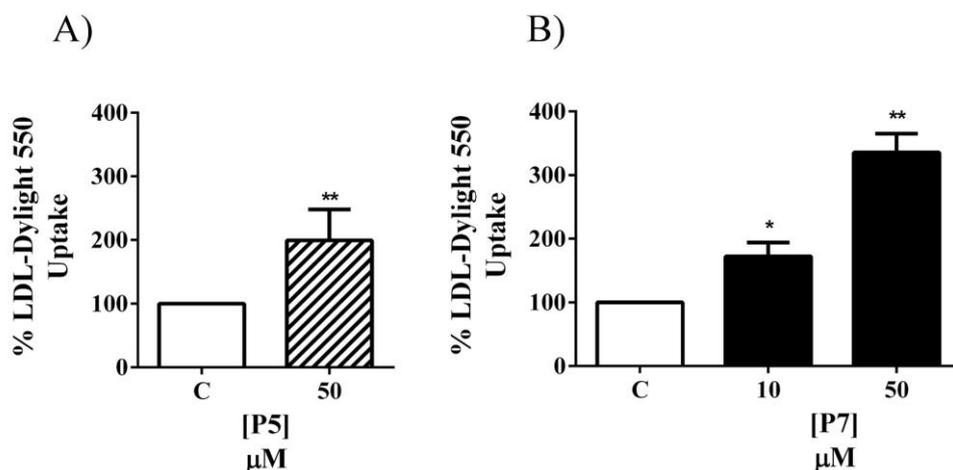
Figure 5. Fluorescent LDL-uptake assay after treatments of HepG2 with P5 and P7. Cells (3×10^4) were treated with **P5** (50 μM) (A) and **P7** (10 and 50 μM) (B) for 24 h. LDL-Dylight 549 (10 μg/mL) was incubated for an additional 2 h. Excess LDL-Dylight 549 was removed and cells were washed two times with PBS. Specific fluorescent LDL-uptake signal was analysed by Synergy H1 (Biotek). Data points represent averages ± SEM of three independent experiments in triplicate. (∗) *p <0.05* and (∗∗) *p < 0.001 versus* control (C).

3.5 Modulation of the PCSK9 and HNF1-alpha protein levels

Further experiments were conducted with the objective of evaluating the effects of the treatments of HepG2 cells with **P5** (50 and 100 μM) and **P7** (10 and 50 μM) on the modulation of PCSK9. Mature PCSK9 (molecular weight ~62 kDa, PCSK9-M) was detected by immunoblotting using a specific antibody. Whereas the treatment with **P7** did not produce any statistically significant change (Figure 6D), the treatment with **P5** (Figure 6A,B) induced a 40.8 ± 12.6% (*p<0.001*) increase in PCSK9-M protein level at a 50 μM concentration and a 63.3 ± 10.2% (*p<0.001*) augmentation at 100 μM *versus* the untreated sample (Figure 6C). In parallel, the same peptides had different effects on HNF1-alpha protein levels (Figure 6A,B), since **P5** decreased HNF-1 alpha level by 51.7 ± 18.4% (*p<0.001*) at 50 μM and by 59.8 ± 14.3% (*p<0.001*) at 100 μM, *versus* the untreated sample (Figure 6C), whereas **P7** was essentially inactive (Fig 6D).
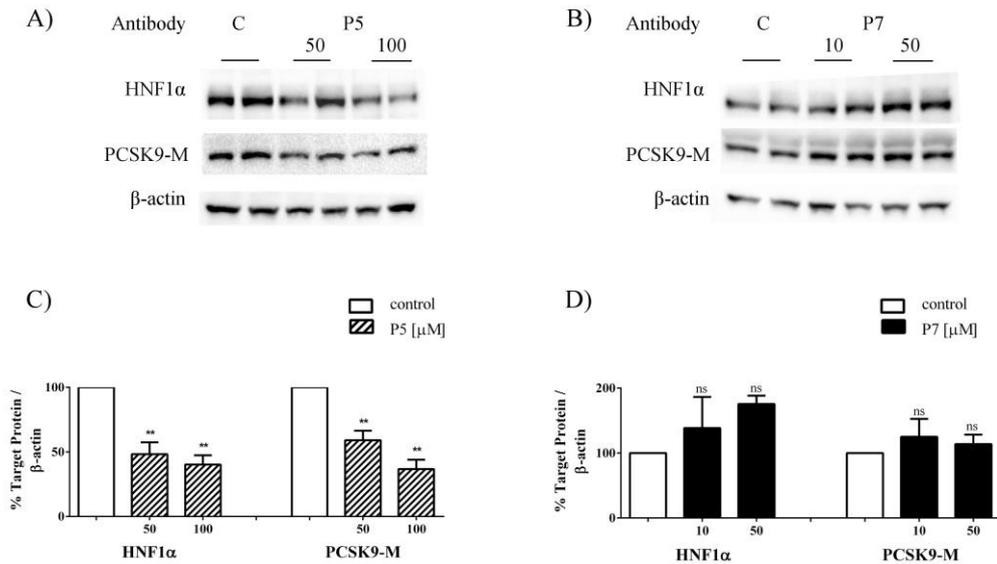
Figure 6. Effects of P5 and P7 on PCSK9 and HNF1-alpha protein levels. HepG2 cells (1.5×10^5) were treated **P5** (50 μM) (A-C) and **P7** (10 and 50 μM) (B-D) for 24 h. PCSK9, HNF1-alpha and β-actin immunoblotting signals were detected using specific anti-PCSK9, anti-HNF1-alpha and anti-β-actin primary antibodies, respectively. PCSK9-M represents the cleaved mature form of PCSK9. PCSK9-M and HNF1-alpha signals were quantified by ImageLab software (Biorad) and normalised with β-actin signals. Bars represent averages ± SEM of six independent experiments (two duplicates per sample). (∗∗) *p < 0.001 versus* control (C).

3.6. P5 reduces the secretion of PCSK9-M by HepG2 cells

It was then decided to evaluate whether **P5** was also able to modulate the secretion of PCSK9-M by HepG2 cells. For this scope, the same cells were treated with **P5** (50 and 100 μM) for 24 h. On the second day, the cell culture medium was collected and secreted PCSK9-M measured using a specific ELISA kit. The data of Table 1 clearly indicate that **P5** induced a small but significant reduction of the secretion of PCSK9-M *versus* the untreated samples: at 50 μM concentration secreted PCSK9 was lessened by 19.7% (63.7 ± 4.0 ng/mL, *p <0.05*) and at 100 μM concentration by 27.3% (57.7 ± 13. ng/mL, *p < .001*) *versus* control (79.4 ± 1.0 ng/mL). **P7** was not investigated here, since it had been shown to be inactive during modulation of PCSK9 and HNF1-alpha protein levels.

Table 1 – Effect of **P5** and **P7** peptides on secreted PCSK9 media levels in HepG2 cells

Parameter	C	P 5	
		50 μM	100 μM

Secreted PCSK9 levels (ng/mL)	79.4± 1.0	63.7 ± 4.0 *	57.7 ± 13.4 **

<center>* <i>P<0.05 vs</i> untreated sample (C); ** <i>P<0.001 vs</i> untreated sample (C)</center>

4. Discussion

4.1 Modulation of cholesterol homeostasis

The first objective of the work was successfully achieved, since indeed the experimental assay confirmed that **P5** and **P7** can function as HMGCoAR inhibitors, because both gave statistically significant concentration-response curves, with calculated IC_{50} values equal to 147.2 μM for **P5** and 68.4 μM for **P7** (Figure 1). As explained in the introduction, these peptides had been identified in the basolateral solution of the transport experiments in Caco2 cells by HPLC-chip-ESI-MS/MS and selected through *in silico* docking simulation experiments as the best candidate for HMGCoAR inhibition. In fact, their specific structures favour efficient bindings to the catalytic site of this enzyme (Lammi, et al., 2016). The peptide-HMGCoAR complexes appear to be stabilised particularly by the following interactions: A) The negatively charged residues located at C-terminal tail are engaged in ionic contacts with Arg568, Arg571, and Lys722; B) The positively charged N-terminus elicits ion-pairs with Glu559 and Asp767, reinforced by H-bonds with Thr557 and Thr558; C) The hydrophobic residues located at N-terminus are involved in hydrophobic interactions with apolar residues of HMGCoAR. Additionally, **P5** contains a central positively charged residue (Lys5), stabilising a salt bridge with Asp690. Their activities were postulated to be similar, with a slight preference for **P7**, since their docking scores, i.e. normalized Plp95, APBS and MPL scores, fell in a very small range (Lammi, et al., 2016)**.**

As indicated in the introduction, in view of better understanding their mode of action, the second objective was a detailed investigation of their effects on the LDLR pathway in HepG2 cells. To this scope, **P7** was tested at lower concentrations than **P5**, being a more efficient inhibitor of HMGCoAR activity.

Our findings clearly suggest that both peptides are able to enhance the protein level of the transcriptional N-terminal active part of the SREBP2 protein with a statistical significance (Figure 2). In agreement with the increase of active SREBP2 protein, enhancements of the LDLR and HMGCoAR protein levels were also detected. to These molecular results are in line with functional results according to which both **P5** and **P7** are able to increase the HepG2 ability to uptake LDL from the extracellular environment with a final hypocholesterolemic effect. This last result is in agreement

with those published in a recently work in which the treatment with **P5** at a 10 µM concentration led to an increase of the LDL-uptake by 66±21.4% (Lammi, Zanoni, Aiello, Arnoldi, & Grazioso, 2016). Finally, the results suggest that the activity of **P7** on cholesterol modulation in HepG2 cells is slightly better than that of **P5**.

SREBP2 is responsible for the LDLR and HMGCoAR transcription and SREBP2 maturation is regulated by the intracellular cholesterol homeostasis (Lammi et al., 2014). By inhibiting the HMGCoAR activity, **P5** and **P7** modulate the intracellular cholesterol biosynthesis and homeostasis, leading to an increase of the LDLR and HMGCoAR protein levels through the activation of mature SREBP2.

In particular, HMGCoAR is a highly regulated enzyme (Goldstein & Brown, 1990): it can be long-term regulated by the control of its synthesis and its degradation or short-term regulated through phosphorylation or dephosphorylation (Pallottini, Martini, Pascolini, Cavallini, Gori, Bergamini, et al., 2005). The HMGCoAR transcription and translation increased when the concentrations of the products of the mevalonate pathway are low. Conversely, when the sterol concentrations are high, the intracellular HMGCoAR concentration decreases (Istvan, Palnitkar, Buchanan, & Deisenhofer, 2000). A third level of regulation is achieved through phosphorylation of Ser872 by AMPK, which decreases the enzyme activity (Ching, Davies, & Hardie, 1996). HMGCoAR is present physiologically in the cell in an active non-phosphorylated form (30%) and an inactive phosphorylated one (70%) (Pallottini et al., 2005).

AMPK is expressed in numerous tissues, particularly in the liver, brain and skeletal muscle. Literature reports that some natural compounds, such as policosanols, are able to increase the phosphorylation of AMPK with a direct inhibition of HMGCoAR (Oliaro-Bosso, Calcio Gaudino, Mantegna, Giraudo, Meda, Viola, et al., 2009). Furthermore, it is also known that statins are able to activate AMPK (Sun, Lee, Zhu, Gu, Wang, Zhu, et al., 2006), with the consequence of a synergistic inhibition of HMGCoAR activity.

Our results provide some evidence according to which **P5** and **P7** are able to increase the phosphorylation level of AMPK at the Thr172 residue of the catalytic α subunit, indicating AMPK activation, which in turn produces an inhibition of HMGCoAR activity (Figure 3). In fact, the AMPK activation mediated by **P5** and **P7** led to a significant increase of the phosphorylation levels of the HMGCoAR at Ser872 residue, which is the phosphorylation site of AMPK. For this reason, these two peptides are able not only to act as competitive inhibitors of the HMGCoAR, but also to inhibit HMGCoAR activity by enhancing AMPK activation (Figure 3). In line with these findings, there is an agreement between the molecular data and *in vitro* results on the **P5** and **P7** ability to interfere with HMGCoAR activity, since the molecular data shows that they led to an elevation of the p-

HMGCoAR/HMGCoAR protein ratio with the consequence of a diminished enzyme activity, as reported in literature (Levy, Ben Djoudi Ouadda, Spahis, Sane, Garofalo, Grenier, et al., 2013). Thus, although **P5** and **P7** determine an increment of HMGCoAR protein levels through SREBP2 activation (Figure 2), the findings clearly suggest that they are also able to negatively regulate the HMGCoAR activity by a direct inhibition of the enzyme catalytic domain (Figure 1) and increase of its phosphorylation through activation of the AMPK pathway (Figure 3), which leads to an increase of p-HMGCoAR/HMGCoAR protein ratio (Figure 4). Other plant peptides share the same mechanism of HMGCoAR inhibition, in particular IAVPGEVA, IAVPTGVA, and LPYP, deriving from soy protein (Lammi, Zanoni, & Arnoldi, 2015).

Such detailed studies on the hypocholesterolemic activity of food peptides are very scarce in literature. The first investigated peptide was LRVPAGTTFYVVNPDNDENLRMIA, corresponding to position 301-324 of α' subunit of β-conglycinin (UNIProtKB P11827), a major storage protein of soybean seed belonging to the vicilin-like family. A paper (Lovati, Manzoni, Gianazza, Arnoldi, Kurowska, Carroll, et al., 2000) has demonstrated that it increases the ^{125}I-LDL uptake in human HepG2 cells by 41% and its degradation by 10% *versus* the vehicle at the concentration of 100 μM. Interestingly, absorption experiments conducted in human enterocytes (Amigo-Benavent, Clemente, Caira, Stiuso, Ferranti, & del Castillo, 2014) have shown that YVVNPDNDEN, corresponding to the central sequence of LRVPAGTTF**YVVNPDNDEN**LRMIA, is potentially an absorbed peptide. This prompted a following study (Lammi, Zanoni, Arnoldi, & Vistoli, 2015) showing that it inhibits *in vitro* the activity of HMGCoAR with a dose response behaviour and an IC_{50} value equal to 150 μM. Comparing this activity with those of lupin peptides, **P5** is roughly equivalent to YVVNPDNDEN, whereas **P7** appears to be a more efficient inhibitor (Figure 1). *In silico* simulations have shown that also in this case the peptide-HMGCoAR complex is stabilised by the same kind of interactions reported for **P5** and **P7** (Lammi, et al., 2016). Another paper has investigated the effects of YVVNPDNDEN on cholesterol metabolism in HepG2 cells at the concentration of 350 μM (Lammi, Zanoni, Arnoldi, & Vistoli, 2015). YVVNPDNDEN up-regulated the mature SREBP2 protein level by $134.0 \pm 10.5\%$, increased the LDLR protein level by $152.0 \pm 20.0\%$, and enhanced the HMGCoAR protein level by $171 \pm 29.9\%$ *versus* control. Considering the higher concentrations of these experiments, it seems possible to affirm that **P5** and especially **P7** are much more effective than YVVNPDNDEN in regulating cholesterol metabolism.

The direct comparison of the functional effects of these peptides on the capability of HepG2 of up-taking extracellular LDL is facilitated by the fact that they were tested all at the same concentration, i.e. 50 μM: again **P7** was the most active, since **P7** increased the LDL uptake by $235.0 \pm 29.2\%$, whereas **P5** produced only $99.5 \pm 48.51\%$ increase (Figure 5), and YVVNPDNDEN by $64.0 \pm 29.9\%$

383 (Lammi, Zanoni, Arnoldi, & Vistoli, 2015). Instead, at the concentration of 10 μM **P7** and **P5** are

384 almost equivalent.

385 In light with all these data, the conclusions of this experimentation may be summarised in this way:

386 A) both **P5** and **P7** are able to positively modulate the cholesterol metabolism through the inhibition

387 of HMGCoAR activity; B) **P7** is slightly more active than **P5**; C) these lupin peptides are more active

388 than all known soy peptides with which they apparently share the same mechanism of action.

389

390

391 *4.2 Modulation of PCSK9*

392 Proprotein convertase subtilisin/kexin type 9 (PCSK9) has been recently identified as a new target

393 for hypercholesterolemia treatment (Seidah & Prat, 2007). It is an extracellular protein that is

394 expressed primarily in liver, kidney, and intestine (Seidah & Prat, 2012) and plays an important role

395 in regulating hepatic LDLR degradation (Gencer, Lambert, & Mach, 2015; D. W. Zhang, Lagace,

396 Garuti, Zhao, McDonald, Horton, et al., 2007). Notably, since PCSK9 and LDLR are co-regulated by

397 SREBP-2 (Dubuc, Chamberland, Wassef, Davignon, Seidah, Bernier, et al., 2004), increased PCSK9

398 expression in response to statin-induced cellular cholesterol depletion may limit the efficacy of statin

399 treatment (Careskey, Davis, Alborn, Troutt, Cao, & Konrad, 2008; Welder, Zineh, Pacanowski,

400 Troutt, Cao, & Konrad, 2010). The development of therapies that inhibit PCSK9 function holds

401 promise for improved management of hypercholesterolemia and cardiovascular disease risk.

402 Particularly, some evidence supports the direct binding of secreted PCSK9 to LDLR, resulting in

403 receptor degradation (Cameron, Holla, Ranheim, Kulseth, Berge, & Leren, 2006; Lagace, Curtis,

404 Garuti, McNutt, Park, Prather, et al., 2006). The PCSK9 binding site in the LDLR is located at the

405 first epidermal growth factor-like repeat (EGF-A) of the extracellular domain (Zhang, et al., 2007)

406 and this protein-protein interaction (PPI) is necessary for LDLR degradation.

407 The idea of investigating PCSK9 modulation in this case was stimulated by recent literature. A

408 clinical study has demonstrated that consuming dietary bars containing 30 g lupin protein per day

409 changed the plasma levels of PCSK9 by -8.5% ($p = 0.0454$) *versus* the control group (casein bar) in

410 mild hypercholesterolemic subjects (Lammi, Zanoni, Calabresi, & Arnoldi, 2016). In addition, peptic

411 and tryptic hydrolysates from lupin protein positively influenced intracellular PCSK9 processing in

412 hepatocytes (Lammi, Zanoni, Calabresi, & Arnoldi, 2016) and the basolateral samples from the

413 absorption experiments on Caco-2 cells were able to interfere with the PCSK9/LDLR PPI (Lammi,

414 Zanoni, Aiello, Arnoldi, & Grazioso, 2016). This activity may be primarily attributable to **P5**, which

415 was demonstrated to be a potent inhibitor of the interaction of PCSK9 with the LDLR, with a very

416 low IC_{50} value equal to 1.6 μM. In particular, we have demonstrated that **P5** is able to impair the

417   interaction of PCSK9 with the LDLR in a dose-dependent manner using a PCSK9-LDLR *in vitro*

418   binding assay (Lammi, Zanoni, Aiello, Arnoldi, & Grazioso, 2016). It is useful to observe that Pep2-

419   8 (Ac-TVFTSWEEYLDWV-amide), the best inhibitor of this PPI singled out in a recent paper (

420   Zhang, Eigenbrot, Zhou, Shia, Li, Quan, et al., 2014), has an IC_{50} value equal of 0.81 ± 0.08 μM, i.e.

421   only slightly better than that of **P5**. Interestingly, Pep2-8 contains 13 amino acid residues and its C-

422   terminal truncated analogues lose activity, whereas **P5** contains only 10 residues. **P5** is thus one of

423   the shortest peptides ever described in literature endowed with this specific activity.

424   This stimulated us to investigate whether **P5** as well as **P7** are able to modulate PCSK9 intracellular

425   processing. Indeed, **P5** induced a reduction of mature PCSK9 protein level and a consequent decrease

426   of mature PCSK9 secretion. In order to elucidate the mechanism through which this peptide positively

427   affect the LDLR pathway by reducing the PCSK9 protein level and secretion, a crucial aspect is the

428   regulation of PCSK9 transcription. Several transcription factors, such as SREBPs and HNF-1, have

429   been identified as transcriptional activators of PCSK9 gene expression (Horton, Shah, Warrington,

430   Anderson, Park, Brown, et al., 2003; Li, Dong, Park, Lee, Chen, & Liu, 2009). PCSK9 and LDLR

431   both contain functional sterol regulatory elements (SREs) in their promoters that respond to change

432   in intracellular cholesterol levels through the activation of the SREBP pathway (Dubuc, et al., 2004;

433   Maxwell, Soccio, Duncan, Sehayek, & Breslow, 2003). However, since the HNF1-alpha binding site

434   is unique to the PCSK9 promoter and is not present in the LDLR promoter, modulation of PCSK9

435   transcription through HNF1-alpha sequence does not affect LDLR gene expression. Thus, the HNF1-

436   alpha binding site represents a divergent point to disconnect the co-regulation of PCSK9 from LDLR

437   and other SREBP target genes (Dong, Li, Singh, Cao, & Liu, 2015). Interestingly, we have shown

438   here that only **P5** decreased hepatic PCSK9 production (Figure 6 A,C) and extra cellular secretion

439   (Table 1), without increasing LDLR protein levels and other SREBP-2 target genes (such as

440   HMGCoAR), and down-regulates HNF1-alpha protein content in HepG2 cells (Figure 6 A,C).

441   The behaviour of **P7** instead is quite different, since it increased the intracellular LDLR protein levels

442   through activation of SREBP2 leading to an increased ability of HepG2 cells to uptake extracellular

443   LDL, without affecting PCSK9 and HNF1-alpha protein levels (Figure 6 B-D).

444   In conclusion, we have identified in lupin protein hydrolysates **P5** and **P7,** two peptides that share the

445   capacity to up-regulate the LDLR-SREBP2 pathway, leading to an improved HepG2 capability to

446   uptake LDL, but greatly diverge in the modulation of intracellular PCSK9 processing and

447   extracellular excretion where only **P5** is active. The molecular mechanisms of these peptides may in

448   part explain the hypocholesterolemic activity observed in clinical trials et al., 2015; Lammi, Zanoni,

449   Calabresi, & Arnoldi, 2016; Sirtori, et al., 2012) and in animal studies (Bettzieche, Brandsch,

450   Schmidt, Weisse, Eder, & Stangl, 2008; Marchesi, Parolini, Diani, Rigamonti, Cornelli, Arnoldi, et

451 al., 2008; Parolini, Rigamonti, Marchesi, Busnelli, Cinquanta, Manzini, et al., 2012; Sirtori, Lovati,

452 Manzoni, Castiglioni, Duranti, Magni, et al., 2004).

453

AUTHORS CONTRIBUTIONS

455 Experiment ideation and design: CL. Experiments & data analysis: biological experiments CL & CZ;

456 peptide identification and analysis GA. Figure preparation: CZ. Grant retrieval: AA. Manuscript

457 writing: CL & AA.

458

466

467

REFERENCES

Amigo-Benavent, M., Clemente, A., Caira, S., Stiuso, P., Ferranti, P., & del Castillo, M. D. (2014). Use of phytochemomics to evaluate the bioavailability and bioactivity of antioxidant peptides of soybean β-conglycinin. *Electrophoresis, 35*(11), 1582-1589.

Arnoldi, A., Boschin, G., Zanoni, C., & Lammi, C. (2015). The health benefits of sweet lupin seed flours and isolated proteins. *Journal of Functional Foods, 18*, 550-563.

Bettzieche, A., Brandsch, C., Schmidt, M., Weisse, K., Eder, K., & Stangl, G. (2008). Differing effect of protein isolates from different cultivars of blue lupin on plasma lipoproteins of hypercholesterolemic rats. *Bioscience, Biotechnology, and Biochemistry, 72*(12), 3114-3121.

Bähr, M., Fechner, A., Kiehntopf, M., & Jahreis, G. (2015). Consuming a mixed diet enriched with lupin protein beneficially affects plasma lipids in hypercholesterolemic subjects: a randomized controlled trial. *Clinical Nutrition, 34*(1), 7-14.

Cameron, J., Holla, Ø., Ranheim, T., Kulseth, M. A., Berge, K. E., & Leren, T. P. (2006). Effect of mutations in the PCSK9 gene on the cell surface LDL receptors. *Human Molecular Genetics, 15*(9), 1551-1558.

Careskey, H. E., Davis, R. A., Alborn, W. E., Troutt, J. S., Cao, G., & Konrad, R. J. (2008). Atorvastatin increases human serum levels of proprotein convertase subtilisin/kexin type 9. *Journal of Lipid Research, 49*(2), 394-398.

Ching, Y. P., Davies, S. P., & Hardie, D. G. (1996). Analysis of the specificity of the AMP-activated protein kinase by site-directed mutagenesis of bacterially expressed 3-hydroxy 3-methylglutaryl-CoA reductase, using a single primer variant of the unique-site-elimination method. *European Journal of Biochemistry, 237*(3), 800-808.

Dong, B., Li, H., Singh, A. B., Cao, A., & Liu, J. (2015). Inhibition of PCSK9 transcription by berberine involves down-regulation of hepatic HNF1α protein through the ubiquitin-proteasome degradation pathway. *Journal of Biological Chemistry, 290*(7), 4047-4058.

Dubuc, G., Chamberland, A., Wassef, H., Davignon, J., Seidah, N. G., Bernier, L., & Prat, A. (2004). Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia. *Arteriosclerosis Thrombosis and Vascular Biology, 24*(8), 1454-1459.

497 Duranti, M., Consonni, A., Magni, C., Sessa, F., & Scarafoni, A. (2008). The major proteins of lupin
498   seed: characterisation and molecular properties for use as functional and nutraceutical
499   ingredients. *Trends in Food Science and Technology, 19*(12), 624-633.

500 Ferruzza, S., Rossi, C., Scarino, M. L., & Sambuy, Y. (2012). A protocol for differentiation of human
501   intestinal Caco-2 cells in asymmetric serum-containing medium. *Toxicoloy In Vitro, 26*(8),
502   1252-1255.

503 Gencer, B., Lambert, G., & Mach, F. (2015). PCSK9 inhibitors. *Swiss Medicine Weekly, 145*,
504   w14094.

505 Goldstein, J. L., & Brown, M. S. (1990). Regulation of the mevalonate pathway. *Nature, 343*(6257),
506   425-430.

507 Horton, J. D., Shah, N. A., Warrington, J. A., Anderson, N. N., Park, S. W., Brown, M. S., &
508   Goldstein, J. L. (2003). Combined analysis of oligonucleotide microarray data from
509   transgenic and knockout mice identifies direct SREBP target genes. *Proceedings of the*
510   *National Academy of Sciences of the United States of America, 100*(21), 12027-12032.

511 Istvan, E. S., Palnitkar, M., Buchanan, S. K., & Deisenhofer, J. (2000). Crystal structure of the
512   catalytic portion of human HMG-CoA reductase: insights into regulation of activity and
513   catalysis. *Embo Journal, 19*(5), 819-830.

514 Lagace, T. A., Curtis, D. E., Garuti, R., McNutt, M. C., Park, S. W., Prather, H. B., Anderson, N. N.,
515   Ho, Y. K., Hammer, R. E., & Horton, J. D. (2006). Secreted PCSK9 decreases the number of
516   LDL receptors in hepatocytes and in livers of parabiotic mice. *J of Clinica Investigation,*
517   *116*(11), 2995-3005.

518 Lammi, C., Aiello, G., Vistoli, G., Zanoni, C., Arnoldi, A., Sambuy, Y., Ferruzza, S., & Ranaldi, G.
519   (2016). A multidisciplinary investigation on the bioavailability and activity of peptides from
520   lupin protein. *Journal of Functional Foods, 24*, 297-306.

521 Lammi, C., Zanoni, C., Aiello, G., Arnoldi, A., & Grazioso, G. (2016). Lupin Peptides Modulate the
522   Protein-Protein Interaction of PCSK9 with the Low Density Lipoprotein Receptor in HepG2
523   Cells. *Scientific Reports, 6*.

524 Lammi, C., Zanoni, C., & Arnoldi, A. (2015). IAVPGEVA, IAVPTGVA, and LPYP, three peptides
525   from soy glycinin, modulate cholesterol metabolism in HepG2 cells through the activation of
526   the LDLR-SREBP2 pathway. *Journal of Functional Foods, 14*, 469-478.

527 Lammi, C., Zanoni, C., Arnoldi, A., & Vistoli, G. (2015). Two peptides from soy beta-conglycinin
528   induce a hypocholesterolemic effect in HepG2 cells by a statin-Like mechanism: comparative
529   in vitro and in silico modeling studies. *Journal of Agricultural and Food Chemistry, 63*(36),
530   7945-7951.

531 Lammi, C., Zanoni, C., Calabresi, L., & Arnoldi, A. (2016). Lupin protein exerts cholesterol-lowering
532   effects targeting PCSK9: From clinical evidences to elucidation of the in vitro molecular
533   mechanism using HepG2 cells. *Journal of Functional Foods, 23*, 230-240.

534 Lammi, C., Zanoni, C., Ferruzza, S., Ranaldi, G., Sambuy, Y., & Arnoldi, A. (2016).
535   Hypocholesterolaemic Activity of Lupin Peptides: Investigation on the Crosstalk between
536   Human Enterocytes and Hepatocytes Using a Co-Culture System Including Caco-2 and
537   HepG2 Cells. *Nutrients, 8*(7).

538 Lammi, C., Zanoni, C., Scigliuolo, G. M., D'Amato, A., & Arnoldi, A. (2014). Lupin peptides lower
539   low-density lipoprotein (LDL) cholesterol through an up-regulation of the LDL
540   receptor/sterol regulatory element binding protein 2 (SREBP2) pathway at HepG2 cell line.
541   *Journal of Agricultural and Food Chemistry, 62*(29), 7151-7159.

542 Levy, E., Ben Djoudi Ouadda, A., Spahis, S., Sane, A. T., Garofalo, C., Grenier, É., Emonnot, L.,
543   Yara, S., Couture, P., Beaulieu, J. F., Ménard, D., Seidah, N. G., & Elchebly, M. (2013).
544   PCSK9 plays a significant role in cholesterol homeostasis and lipid transport in intestinal
545   epithelial cells. *Atherosclerosis, 227*(2), 297-306.

546 Li, H., Dong, B., Park, S. W., Lee, H. S., Chen, W., & Liu, J. (2009). Hepatocyte nuclear factor 1alpha
547   plays a critical role in PCSK9 gene transcription and regulation by the natural
548   hypocholesterolemic compound berberine. *Journal of Biological Chemistry, 284*(42), 28885-
549   28895.

550 Lovati, M. R., Manzoni, C., Gianazza, E., Arnoldi, A., Kurowska, E., Carroll, K. K., & Sirtori, C. R.
551   (2000). Soy protein peptides regulate cholesterol homeostasis in Hep G2 cells. *Journal of
552   Nutrition, 130*(10), 2543-2549.

553 Marchesi, M., Parolini, C., Diani, E., Rigamonti, E., Cornelli, L., Arnoldi, A., Sirtori, C. R., & Chiesa,
554   G. (2008). Hypolipidaemic and anti-atherosclerotic effects of lupin proteins in a rabbit model.
555   *British Journal of Nutrition, 100*(4), 707-710.

556 Maxwell, K. N., Soccio, R. E., Duncan, E. M., Sehayek, E., & Breslow, J. L. (2003). Novel putative
557   SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed
558   mice. *Journal of Lipid Research, 44*(11), 2109-2119.

Oliaro-Bosso, S., Calcio Gaudino, E., Mantegna, S., Giraudo, E., Meda, C., Viola, F., & Cravotto, G. (2009). Regulation of HMGCoA reductase activity by policosanol and octacosadienol, a new synthetic analogue of octacosanol. *Lipids, 44*(10), 907-916.

Pallottini, V., Martini, C., Pascolini, A., Cavallini, G., Gori, Z., Bergamini, E., Incerpi, S., & Trentalance, A. (2005). 3-Hydroxy-3-methylglutaryl coenzyme A reductase deregulation and age-related hypercholesterolemia: A new role for ROS. *Mechanisms of Ageing and Development, 126*(8), 845-851.

Parolini, C., Rigamonti, E., Marchesi, M., Busnelli, M., Cinquanta, P., Manzini, S., Sirtori, C., & Chiesa, G. (2012). Cholesterol-lowering effect of dietary Lupinus angustifolius proteins in adult rats through regulation of genes involved in cholesterol homeostasis. *Food Chemistry, 132*(3), 1475-1479.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A., & Zucco, F. (2005). The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology, 21*(1), 1-26.

Seidah, N. G., & Prat, A. (2007). The proprotein convertases are potential targets in the treatment of dyslipidemia. *Journal of Molecular Medicine (Berlin), 85*(7), 685-696.

Seidah, N. G., & Prat, A. (2012). The biology and therapeutic targeting of the proprotein convertases. *Nature reviews. Drug discovery, 11*(5), 367-383.

Sirtori, C. R., Lovati, M. R., Manzoni, C., Castiglioni, S., Duranti, M., Magni, C., Morandi, S., D'Agostina, A., & Arnoldi, A. (2004). Proteins of white lupin seed, a naturally isoflavone-poor legume, reduce cholesterolemia in rats and increase LDL receptor activity in HepG2 cells. *Journal of Nutrition, 134*(1), 18-23.

Sirtori, C. R., Triolo, M., Bosisio, R., Bondioli, A., Calabresi, L., De Vergori, V., Gomaraschi, M., Mombelli, G., Pazzucconi, F., Zacherl, C., & Arnoldi, A. (2012). Hypocholesterolaemic effects of lupin protein and pea protein/fibre combinations in moderately hypercholesterolaemic individuals. *British Journal of Nutrition, 107*(8), 1176-1183.

Sun, W., Lee, T. S., Zhu, M., Gu, C., Wang, Y., Zhu, Y., & Shyy, J. Y. (2006). Statins activate AMP-activated protein kinase in vitro and in vivo. *Circulation, 114*(24), 2655-2662.

Welder, G., Zineh, I., Pacanowski, M. A., Troutt, J. S., Cao, G., & Konrad, R. J. (2010). High-dose atorvastatin causes a rapid sustained increase in human serum PCSK9 and disrupts its correlation with LDL cholesterol. *Journal of Lipid Research, 51*(9), 2714-2721.

590   Zhang, D. W., Lagace, T. A., Garuti, R., Zhao, Z., McDonald, M., Horton, J. D., Cohen, J. C., &

591   Hobbs, H. H. (2007). Binding of proprotein convertase subtilisin/kexin type 9 to epidermal

592   growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling

593   and increases degradation. *Journal of Biological Chemistry, 282*(25), 18602-18612.

594   Zhang, Y., Eigenbrot, C., Zhou, L., Shia, S., Li, W., Quan, C., Tom, J., Moran, P., Di Lello, P.,

595   Skelton, N. J., Kong-Beltran, M., Peterson, A., & Kirchhofer, D. (2014). Identification of a

596   small peptide that inhibits PCSK9 protein binding to the low density lipoprotein receptor.

597   *Journal of Biological Chemistry, 289*(2), 942-955.

598