1 **Lupin protein exerts cholesterol-lowering effects targeting PCSK9: from clinical evidences to**
2 **elucidation of the *in vitro* molecular mechanism using HepG2 cells**

3 Carmen Lammi,[1] Chiara Zanoni,[2] Laura Calabresi,[1,2] Anna Arnoldi[1]*
4
5 [1]Department of Pharmaceutical Sciences, University of Milan, Milan, Italy
6 [2]Dipartimento Cardiotoracovascolare, A.O. Ospedale Niguarda Cà Granda, Milan, Italy
7 [3]Department of Pharmacological and Biomolecular Sciences, Università di Milano, Italy
8
9 *Anna Arnoldi, Department of Pharmaceutical Sciences, University of Milan, via Mangiagalli 25,
10 20133 Milan, Italy. E-Mail: anna.arnoldi@unimi.it, tel.: +390250319372, fax: +390250319343.
11
12 **Abbreviations:** FBS, foetal bovine serum; HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A
13 reductase; HNF1-alpha, hepatocyte nuclear factor 1 alpha; LDL-C, LDL cholesterol; PCSK9,
14 proprotein convertase subtilisin/kexintype 9; SREBP-2, regulatory element binding proteins 2.
15
16 **Keywords**: bioactive peptides, HepG2 cells, HNF1-alpha, *Lupinus*, PCSK9, plant proteins
17
18
19

20 **Abstract**

21 PCSK9 inhibition is a novel approach for cholesterol reduction, for its crucial pathophysiological role
22 in cholesterol metabolism. This work aimed at evaluating whether lupin protein/peptides may
23 modulate PCSK9 production. Mild hypercholesterolaemic subjects consumed lupin protein or casein
24 (30 g/day) for 4 weeks. The final level of circulating PCSK9, measured by ELISA, was reduced by
25 8.5% ($p = 0.0454$) versus baseline value, whereas it remained unchanged in the control group (casein).
26 For investigating the mechanism of action, HepG2 cells were treated with peptic and tryptic peptides
27 from lupin protein: reductions of PCSK9 production and secretion were observed as well as a decrease
28 of hepatic nuclear factor 1-alpha (HNF1-alpha). For the first time, this work provides evidences that
29 lupin protein/peptides may modulate the PCSK9 protein level production and secretion, contributing
30 to explain the beneficial effects observed in animal and human studies and opening a completely new
31 area of investigation on plant proteins.

32 ## 1. Introduction

33 Proprotein convertase (PC) subtilisin/kexintype 9 (PCSK9) is primarily synthesised in the liver and
34 small intestine (Benjannet et al., 2004). After intracellular autocatalytic cleavage of its prodomain,
35 mature PCSK9 is secreted from liver cells (McNutt, Lagace, & Horton, 2007). Animal studies have
36 shown that the PCSK9 binding to the low density lipoprotein (LDL) receptor (LDLR) targets the
37 receptor for lysosomal degradation, thereby providing a possible mechanism through which PCSK9
38 may affect cholesterol metabolism (Lo Surdo et al., 2011). In the absence of PCSK9, the hepatic
39 LDLR is shuttled back to the plasma membrane after cholesterol delivery to the lysosome for
40 degradation. PCSK9 binding, instead, prevents this LDLR shuttling and targets it for degradation
41 (Lagace et al., 2006). PCSK9 primarily acts on the LDLR as a circulating plasma protein and several
42 small-scale studies have shown a positive relationship between circulating PCSK9 and LDL
43 cholesterol (LDL-C) levels (Lambert, Sjouke, Choque, Kastelein, & Hovingh, 2012).

44 High levels of circulating LDL-C accelerate the progression of atherosclerosis and LDL-C lowering
45 is currently considered the most effective strategy in preventing coronary artery disease. Several
46 recent studies have correlated the novel target PCSK9 with parameters directly related to
47 atherosclerosis progression (Giunzioni & Tavori, 2015; Gu & Zhang, 2015; Stein & Raal, 2015;
48 Zhang et al., 2015). The role of circulating PCSK9 in promoting hypercholesterolaemia is beyond
49 dispute and strongly supported by preclinical experiments and clinical trials, where antibodies
50 directed against the LDLR binding site in PCSK9 have effectively reduced LDL-C levels (Roth,
51 McKenney, Hanotin, Asset, & Stein, 2012; Stein et al., 2012).

52 Although specific drugs, such as statin, are the main solution for the treatment of
53 hypercholesterolaemia, a number of new pharmacological approaches based on the inhibition of
54 PCSK9 function are currently under development (Lambert, Sjouke, Choque, Kastelein, & Hovingh,
55 2012; Verbeek, Stoekenbroek, & Hovingh, 2015). In this scenario, also natural compounds may have
56 an important preventive role. An example is berberine (Kong et al., 2004), an isoquinoline alkaloid

57 found in the leaves of some species of the genus *Berberis,* which inhibits the progression of
58 hypercholesterolaemia targeting PCSK9 (Cameron, Ranheim, Kulseth, Leren, & Berge, 2008).

59 Lupin is a grain legume cultivated as a sustainable crop in many countries either for animal or human
60 nutrition. The interest for this seed relies mostly on its high protein content (35-40%). Besides its
61 useful nutritional features, in the last decade numerous investigations have shown that lupin provides
62 interesting health benefits (Arnoldi, Boschin, Zanoni, & Lammi, 2015), particularly in the area of
63 hyperglycaemia control (Bertoglio et al., 2011; Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008;
64 Lovati, Manzoni, Castiglioni, Parolari, Magni, & Duranti, 2012), hypertension prevention (Lee et al.,
65 2009), and cholesterol reduction (Bähr, Fechner, Kiehntopf, & Jahreis, 2015; Marchesi et al., 2008;
66 Sirtori et al., 2004; Sirtori et al., 2012; Weisse et al., 2010). The protein seems to be relevant in these
67 beneficial effects (Arnoldi, Boschin, Zanoni, & Lammi, 2015).

68 In the framework of the European project Bioprofibre, some years ago we have performed a double-
69 blind randomised clinical trial for evaluating the potential hypocholesterolaemic effects of some
70 legume proteins, including lupin protein, versus casein as control protein in mild
71 hypercholesterolaemic patients (Sirtori et al., 2012). This intervention study had a parallel design and
72 was 4 weeks long. The consumption of the dietary bar containing lupin protein (30 g/day) resulted in
73 a significant reduction of total cholesterol (-11.6 mg/dl = -4.2%, $p<0.05$), whereas no significant
74 cholesterol changes were observed in the subjects consuming the control bars containing casein.

75 Further studies were dedicated to elucidate the mechanism of action, mainly working on peptides. In
76 particular, we have demonstrated that tryptic and peptic peptides derived from lupin protein are able
77 to interfere with 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) activity, up-
78 regulating the LDLR and sterol regulatory element binding proteins 2 (SREBP-2), and increasing the
79 LDL-uptake in HepG2 cells (Lammi, Zanoni, Scigliuolo, D'Amato, & Arnoldi, 2014).

80 These results suggested the possibility that lupin protein may also influence the expression of PCSK9.
81 In order to achieve a rapid confirmation of this hypothesis, we decided to measure the level of

82 circulating PCSK9 in the plasma of the subjects of the clinical study described above (Sirtori et al.,
83 2012). The positive results of this preliminary experiment prompted us to undertake an extensive *in*
84 *vitro* investigation on the mechanism of action through which peptic and tryptic peptides from lupin
85 protein modulate PCSK9 production by targeting its intracellular processing. This mechanistic study
86 was performed using human hepatic HepG2 cells as model system, since hepatocytes are the main
87 source of circulating PCSK9 (Lagace et al., 2006). The same cellular model had been already used
88 by us to characterise the hypocholesterolaemic effects of lupin peptides (Lammi, et al., 2014), since
89 it also expresses high levels of LDLR (Tavori et al., 2013).

90

91 **2. Materials and methods**

92 **2.1. Chemicals**

93 Dulbecco's modified Eagle's medium (DMEM), L-glutamine, foetal bovine serum (FBS), phosphate
94 buffered saline (PBS), penicillin/streptomycin, chemiluminescent reagent, and 96-well plates were
95 purchased from Euroclone (Milan, Italy). Bovine serum albumin (BSA), RIPA buffer, and the
96 antibody against β-actin were bought from Sigma-Aldrich (St. Louis, MO, USA). The antibody
97 against PCSK9 and HNF1-alpha were bought from GeneTex (Irvine, CA, USA). The antibodies
98 against, rabbit Ig-HRP, mouse Ig-HRP, phenylmethanesulfonyl fluoride (PMSF), Na-orthovanadate
99 inhibitors were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); the inhibitor
100 cocktail Complete Midi from Roche (Basel, Swiss). Mini protean TGX pre-cast gel 7.5% and Mini
101 nitrocellulose Transfer Packs were purchased from BioRad (Hercules, CA, USA). The Human
102 Proprotein Convertase 9 Immunoassay (Quantikine ELISA) was bought from R&D System
103 (Minneapolis, MN, USA).

104

105 **2.2. Brief description of protocol of Bioprofibre clinical study and quantification of PCSK9 by**
106 **ELISA in the subject plasma**

107 The complete protocol of the clinical study and details of the ethical approval are described in a
108 previous paper (Sirtori et al., 2012). Briefly, participants were recruited at the University Center for
109 Dyslipidaemias of the Niguarda Hospital (Milano, Italy), according to the following criteria: (i) males
110 and postmenopausal females; (ii) participants in primary prevention, non diabetics; (iii) total
111 cholesterol > 220 mg/dL and triglycerides < 200 mg/dL. A randomised, double-blind, parallel group
112 design was followed, with total cholesterol as main endpoint. All the candidates underwent a
113 stabilisation period on a hypolipidaemic dietary regimen for 4 weeks. The selected subjects were then
114 randomised into the dietary treatment groups: each group consumed for 4 weeks two bars per day
115 containing 30 g the specific protein source, i.e. casein (control) or a lupin protein isolate prepared by
116 the Fraunhofer Institute IVV (Freising, Germany). At the starting and final visits, blood samples were
117 collected after an overnight fast. Both serum and ethylenediaminetetraacetic acid (EDTA) plasma
118 were prepared by low-speed centrifugation at 4 °C and stored at -80 °C. Plasma total cholesterol,
119 triacylglycerols, and HDL-cholesterol (HDL-C) were determined with standard enzymatic techniques
120 (Roche Diagnostics) on a Roche Diagnostics Cobas 400 Analyzer. Plasma LDL-C was calculated
121 with the Friedewald's formula. For measuring PCSK9, aliquots of the plasma samples were 20-fold
122 diluted with Calibrator diluent RD5P (provided by the ELISA kit of R&D System, Minneapolis, MN,
123 USA). The experiments were carried out at 37 °C following the manufacturer's instructions. Before
124 starting the assay, human PCSK9 standards (20.0, 10.0, 5.0, 2.5, 1.25, and 0.625 ng/mL) were
125 prepared from the stock solution PCSK9 Standard (40 ng/mL) with serial dilutions (for building the
126 standard curve) and meanwhile 100 μL the Assay Diluent RD1-9 (provided into the kit) were added
127 to each wells. Afterward, standards and samples (50 μL) were pipetted into the wells and the ELISA
128 plate was allowed to incubate for 2 h at RT. Subsequently, wells were washed 4 times with Wash
129 Buffer, and 200 μL of human PCSK9 Conjugate (HRP-labeled anti-PCSK9) was added to each wells
130 for a 2 h incubation at RT. Following aspiration, wells were washed 4 times with Wash Buffer
131 provided by the kit. After the last wash, 200 μL of Substrate Solution were added to the wells and
132 allowed to incubate for 30 min at RT. The reaction was stopped with 50 μL of Stop Solution (2 N

133 sulfuric acid) and the absorbance at 450 nm was measured using Synergy H1 (Biotek, Bad
134 Friedrichshall, Germany). The lower limit of detection was 0.096 ng/mL.

135

136 **2.3. Preparation and analysis of the pepsin and trypsin peptide mixtures**

137 White lupin seeds (*Lupinus albus* cultivar Ares) were provided by Terrena (Matrignè-Ferchaud,
138 France). The total protein extract was obtained and analysed as previously reported (Lammi et al,
139 2014). In brief, proteins were extracted from defatted flour with 100 mM Tris-HCl/0.5 M NaCl buffer,
140 pH 8.2 for 2 h at 4 °C. After centrifugation at 6,500 *g*, for 20 min at 4 °C, the supernatant was dialysed
141 against 100 mM Tris-HCl buffer pH 8.2 for 24 h at 4 °C. After assessing the protein concentration by
142 Bradford assay, the total protein extract was dissolved in Tris-HCl buffer 100 mM at pH 8, then the
143 pH was adjusted to the optimal hydrolysis conditions for each enzyme (pH = 2 for pepsin and = 8 for
144 trypsin) by adding 1 M NaOH or 1 M HCl. After 18 h incubation and enzyme inactivation, the
145 mixtures were ultra-filtered through 3,000 Da cut-off centrifuge filters (Amicon Ultra-0.5, Millipore,
146 Billerica, MA, USA) at 12,000 *g* for 30 min at 4 °C. The analyses were conducted on a nano
147 chromatographic system, UltiMate 3000 RSLC nano-System (Thermo Scientific, Waltham, MA,
148 USA). The peptide mixtures were loaded on a reversed-phase trap column (Acclaim PepMap100,
149 C18, 100 Å, 100 μm i.d. × 2 cm, Thermo Scientific) for the cleanup and pre-concentration, then
150 separated on fused silica reverse-phase column (picoFrit column, C18, 2.7 μm, New Objective),
151 eluting with a 30 min gradient from 4% buffer A (2% acetonitrile and 0.1% formic acid in water) to
152 60% buffer B (2% water and 0.1% formic acid in acetonitrile) at a constant flow rate of 300 nL/min.
153 The chromatographic column was connected to a LTQ-XL mass spectrometer (Thermo Scientific)
154 equipped with a nano-spray ion source. Full scan mass spectra were acquired in the mass range from
155 m/z 350 to 2000 Da and the five most intense ions were automatically selected and fragmented in the
156 ion trap. The results of the analysis are reported in detail in a previous paper (Lammi et al, 2014).

157

158 **2.4. Cell line culture**

159 HepG2 cell line was bought from ATCC (HB-8065, ATCC from LGC Standards, Milan, Italy). The
160 HepG2 cell line was cultured in DMEM high glucose with stable L-glutamine supplemented with
161 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin (complete growth medium) and incubated
162 at 37 °C under 5% CO_2 atmosphere. HepG2 cells were used for no more than 20 passages after
163 thawing, because the increase of the number of passages may change the cell characteristics and
164 impair assay results.

165

166 **2.5. Western blot analysis**

167 1.5 x 10^5 HepG2 cells/well (24-well plate) were treated with 1.0 and 2.5 mg/mL of peptic and 0.5 and
168 1.0 mg/mL of tryptic peptides for 24 h. After each treatment, the medium of each well was collected
169 in an ice-cold microcentrifuge tube and processed for the PCSK9 immunoassay. Meanwhile the cells
170 were scraped in 40 µL ice-cold lysis buffer (RIPA buffer + inhibitor cocktail + 1:100 PMSF + 1:100
171 Na-orthovanadate) and transferred in an ice-cold microcentrifuge tube. After centrifugation at 16,060
172 g for 15 min at 4 °C, the supernatant was recovered and transferred in a new ice-cold tube. Total
173 proteins were quantified by the Bradford method and 50 µg of total proteins loaded on a pre-cast
174 7.5% sodium dodecyl sulfate - polyacrylamide (SDS-PAGE) gel at 130 V for 45 min. Subsequently,
175 the gel was transferred to a nitrocellulose membrane (Mini nitrocellulose Transfer Packs), using a
176 Trans-blot Turbo at 1.3 A, 25 V for 7 min. Target proteins, on milk blocked membrane, were detected
177 by primary antibodies as follows: anti-PCSK9, anti-HNF1-alpha, and anti-β-actin. Secondary
178 antibodies conjugated with HRP and a chemiluminescent reagent were used to visualise target
179 proteins and their signal was quantified using the Image Lab Software (Biorad). The internal control
180 β-actin was used to normalise loading variations.

181

182 **2.6. Quantification of excreted PCSK9 in cell culture experiments by ELISA**

183 The supernatants collected from HepG2 cells were centrifuged at 600 g for 10 min at 4 °C. They were
184 recovered and diluted with the ratio 1:10 with DMEM in a new ice-cold tube. PCSK9 was quantified

185 by ELISA (R&D System, Minneapolis, MN, USA) using the same kit and methodology described
186 above for the quantification in the plasma of the subjects of the clinical study (section 2.2).
187

188 **2.7. Statistically Analysis**

189 Statistical analyses were carried out by one-way ANOVA using the software Prism 6 (Graphpad, La
190 Jolla, CA, USA) followed by Dunnett's test. Values were expressed as means ± SEM; P-values $<$
191 0.05 were considered to be significant.
192

193 **3. Results**

194 **3.1. Lupin protein reduce the plasma levels of PCSK9 in mild hypercholesterolaemic subjects**

195 The control group (casein bar) and treatment group (lupin protein bar) of the clinical trial (Sirtori, et
196 al., 2012) were composed by 19 and 20 individuals, respectively. For 4 weeks, they consumed two
197 dietary bars corresponding to a total amount of 30 g protein per day. The average baseline and final
198 values of the lipids and PCSK9 plasma levels are reported in Table 1: whereas HDL-cholesterol and
199 total triglyceride levels remained essentially unchanged in both groups, a significant total cholesterol
200 reduction (-11.6 mg/dl = -4.2%, $p<0.05$) and a small non-significant LDL-cholesterol one were
201 observed in the lupin group, but not in the control group. Additionally, a significant reduction of
202 PCSK9 plasma level was detected in the treatment group, whereas only a very small non-statistically
203 significant change was observed in the control group. In particular, when the data were compared by
204 ANOVA with the Dunnett's test, followed by adjustment for baseline values, the reduction of the
205 plasma PCSK9 levels of the lupin group was equal to 8.5% ($p = 0.0454$) *versus* the control group.
206

207 **3.2. Preparation and analysis of the peptide mixtures**

208 A total protein extract from lupin seed was hydrolysed separately with pepsin and trypsin to produce
209 peptic peptides and tryptic peptides. Analysis by nano LC-MS/MS permitted to identify more than
210 2000 peptides in the pepsin digested mixture and about 3000 in the trypsin digested one (Lammi et
211 al, 2014). By consulting the Uniprot_viridiplantae database using the Mascot software, it was possible
212 to assign only a small number of peptides to known lupin proteins, probably due to the very
213 incomplete sequencing of lupin proteins. Most peptides belong to the main lupin storage proteins, by
214 far the most abundant in the total protein extract as shown by SDS-PAGE.
215 In the tryptic sample (Table 2a), 12 peptides were assigned to *L. albus* vicilin-like protein (Q53HY0),
216 10 peptides to *L. albus* beta-conglutin precursor (Q6EBC1), 4 peptides to *Lupinus angustifolius*
217 conglutin beta (B0YJF8)], 4 peptides to *L. angustifolius* conglutin-beta (Q53I55)], and 2 to *L. albus*
218 conglutin-delta seed storage protein precursor (Q99235). Moreover, two peptides were assigned to
219 another plant protein, i.e. *Zea mays* actin partial (ADF3). In the peptic hydrolysate (Table 2b), 21
220 peptides were assigned to *L. albus* vicilin-like protein (Q53HY0), 18 peptides to *L. albus* beta-
221 conglutin (Q6EBC1), 7 peptides to *L. angustifolius* conglutin-alpha 3 (F5B8V8], and 8 peptides to *L.*
222 *albus* conglutin-gamma (Q9FSH9).
223

224 **3.3. Lupin peptides reduce the protein levels of PCSK9 and hepatocyte nuclear factor 1 alpha**
225 **(HNF1-alpha)**

226 HepG2 cells were treated for 24 h with peptic and tryptic peptides at the concentrations of 1.0-2.5
227 mg/mL and 0.5-1.0 mg/mL, respectively. Using immunoblotting technique, two bands were detected:
228 the former corresponded to the precursor ~75 kDa PCSK9 (PCSK9-P), the latter to the mature ~62
229 kDa PCSK9 (PCSK9-M). Immunoblotting experiments showed that the treatments with both lupin
230 peptide mixtures reduced the PCSK9 protein levels (Figure 1). In particular, lupin peptic peptides
231 (1.0 and 2.5 mg/mL) reduce the precursor PCSK9 protein level by 66% and 58% *versus* the untreated

232 sample, while tryptic lupin peptides (0.5 and 1.0 mg/mL) reduce the precursor PCSK9 protein levels
233 by 61% and 53% *versus* the control, respectively, at each treatment concentration (Figure 1B).
234 In the same experiments, also the protein levels of mature PCSK9-M were measured by
235 immunoblotting. Figure 1C shows that both peptic and tryptic peptides are able to decrease the protein
236 level of PCSK9-M *versus* the untreated sample. In particular, at 1.0 and 2.5 mg/mL, respectively,
237 peptic peptides mediate a 52% and 58% reduction of the PCSK9-M protein; whereas at 0.5 and 1.0
238 mg/mL tryptic peptides mediate a 61% and 44% decrease *versus* the untreated sample (Figure 1C).
239 Both peptic and tryptic peptides affected also the protein levels of HNF-1 alpha (Figure 2): peptic
240 peptides decreased it by 28% at 1 mg/mL and 25% at 2.5 mg/mL *versus* the untreated sample,
241 whereas, the treatment with tryptic peptides led to a significant 30% reduction of the HNF1-alpha
242 protein production at 1.0 mg/mL, but not at 0.5 mg/mL.

243

244 3.4. Lupin peptides reduce the secretion of mature PCSK9 by human hepatic HepG2 cells

245 The same cells were treated with peptic peptides (1.0 and 2.5 mg/mL) and tryptic peptides (0.5 and
246 1.0 mg/mL) for 24 h. The second day, the cell culture medium was collected and the effects of lupin
247 peptide treatments on the capacity of HepG2 cells to secrete PCSK9-M were assessed using the
248 ELISA kit. Figure 3 clearly indicates that both peptic and tryptic lupin peptides significantly reduced
249 the secretion of PCSK9-M by about one third *vs.* the untreated samples. More in detail, untreated
250 HepG2 cells secreted 141 ng/mL of PCSK9-M, whereas, after treatment with peptic lupin peptides
251 (1.0 and 2.5 mg/mL), they secreted 94.3 and 97.3 ng/mL of PCSK9 and, after treatment with tryptic
252 lupin peptides (1.0 and 0.5 mg/mL), they secreted 94.3 and 93.6 ng/mL PCSK9-M, respectively.

253

254 4. Discussion

255 In recent years, many studies have extensively improved our understanding of the
256 (patho)physiological role of PCSK9 in human biology (Giunzioni & Tavori, 2015; Gu & Zhang,
257 2015; Lambert, Sjouke, Choque, Kastelein, & Hovingh, 2012). PCSK9 plays a pivotal role in the
258 regulation of the LDLR activity on the hepatic cell surface, because it promotes its degradation and
259 prevents its recycling to the cell membrane. Consequently, it has become a novel target for lipid-
260 lowering therapy and numerous pharmacological approaches to inhibit PCSK9 function are currently
261 under investigation. One possibility is the development of agents that interfere with LDLR binding
262 by targeting PCSK9 in the circulation, as shown with several monoclonal antibodies (Koren et al.,
263 2012), small peptides (Shan, Pang, Zhang, Murgolo, Lan, & Hedrick, 2008; Zhang et al., 2014), and
264 adnectins. Very recently, the American Food and Drug Administration has approved the monoclonal
265 antibody evolocumab (Shantha & Robinson, 2015), for the treatment of patients who are unable to
266 keep their LDL-C under control with current treatment options. A second approach is to reduce
267 hepatic PCSK9 synthesis through gene silencing with small interfering RNA (siRNA) or antisense
268 oligonucleotides (Fitzgerald et al., 2014; Lindholm et al., 2012). The third approach, which has not
269 reached clinical development yet, involves inhibition of PCSK9 production by targeting its
270 intracellular processing (Lambert et al., 2012).

271 Berberine is a natural occurring alkaloid with cholesterol-lowering properties, present in the
272 formulation of Armolipid and Armolipid Plus (Ruscica et al., 2014), two commercial dietary
273 supplements. Experimental evidences indicate that berberine decreases the PCSK9 mRNA expression
274 and increases LDLR *in vitro* and in animal studies (Cameron, Ranheim, Kulseth, Leren, & Berge,
275 2008; Kong et al., 2004), through the down-regulation of the HNF1-alpha protein expression (Dong,
276 Li, Singh, Cao, & Liu, 2015). In a similar way, curcumin suppression of PCSK9 expression is
277 associated with increases in cell-surface LDLR expression and activity in HepG2 cells as well as a
278 reduction of nuclear abundance of HNF1-alpha, with a distinct molecular mechanism compared to
279 statins (Tai, Chen, Chen, Wu, Ho, & Yen, 2014).

280 Up-to-now, the most common pharmacological strategy for the treatment of hypercholesterolaemia
281 is based on statins, which function by inhibiting HMGCoAR, the rate-limiting enzyme in cholesterol
282 synthesis, thereby elevating the LDLR expression to increase the LDL particle uptake from the
283 circulation (Goldstein & Brown, 2009). However, some patients are insensitive to statin treatment or
284 experience serious adverse effects (Pirillo & Catapano, 2015). Furthermore, statins increase the
285 expression of PCSK9 (Awan et al., 2012), thereby counteracting their beneficial effects.

286 Using HepG2 cells, we have recently demonstrated that the hypocholesterolaemic effects of peptides
287 deriving from the hydrolysis of lupin protein are based on the inhibition of HMGCoAR activity
288 (Lammi et al., 2014). Similarly to statins, the consequent reduction of intracellular cholesterol levels
289 lead to SREBP-2 activation, which in turn increases the expression of LDLR. The consequent
290 improved LDLR activity leads to an enhanced ability of HepG2 cells to uptake extracellular LDL
291 with a final hypocholesterolemic effect. In this context, this study represents a major innovation, since
292 it provides either clinical indications that a lupin diet decreases the plasma levels of PCSK9 or *in*
293 *vitro* evidences that lupin peptides may positively influence intracellular PCSK9 processing in
294 hepatocytes.
295 To the best of our knowledge, this is the first study showing that the consumption of lupin protein (30
296 g/day) decreases the plasma PCSK9 levels in moderately hypercholesterolaemic individuals. This
297 means that the potential health benefits of consuming lupin protein may derive not only from the
298 reduction of total cholesterol and LDL-cholesterol (Bähr, Fechner, Kiehntopf, & Jahreis, 2015; Sirtori
299 et al., 2012), but also from the improvement of the PCSK9 plasma levels. This fact is very relevant,
300 since PCSK9 plasma levels correlates with the incidence of cardiovascular disease (CVD) events in
301 humans (Shantha & Robinson, 2015) and are predictive of recurrent clinical events in patients with
302 stable CVD treated with low-dose atorvastatin (Ridker, Rifai, Bradwin, & Rose, 2015).
303 Furthermore, this work has elucidated some relevant differences between the mechanism of action of
304 lupin and statins. In fact, although lupin peptides apparently inhibit the activity of HMGCoAR as

305 statins (Lammi et al., 2014), surprisingly they do not increase the plasma level of PCSK9 (Table 1),
306 a main drawback of statins.
307 An important aspect of the PCSK9-LDLR pathway in mediating LDL clearance is that their
308 transcription is coordinately regulated by sterols through a common SRE motif embedded in their
309 gene promoters and is co-induced by current cholesterol lowering drugs, such as statins, through
310 activation of SREBPs (Dubuc et al., 2004; Horton et al., 2003; Jeong, Lee, Kim, Kim, Yoon, & Park,
311 2008). Statin treatment increases the transcription of both LDLR and PCSK9 (Dubuc et al., 2004).
312 This undesirable inducing effect of statins on PCSK9 transcription is increasingly recognised as a
313 major limitation to their therapeutic efficacy in further lowering plasma LDL-C. While in a preceding
314 paper (Lammi et al., 2014), we have demonstrated that either peptic or tryptic peptides from lupin
315 protein increase the LDLR protein levels and activity through the up-regulation of SREBP-2, here we
316 show that the same peptides also reduce the 692-amino acid precursor (~75 kDa) PCSK9 protein
317 levels with the consequence of a reduction of the mature enzyme (~62 kDa) (Figure 1).
318 A general picture of the molecular mechanism through which lupin peptides may induce the
319 cholesterol-lowering effect in hepatocytes is schematically shown in Figure 4. Pro-PCSK9 undergoes
320 autocatalytic intramolecular processing between the Q152 and S153 residues in the endoplasmic
321 reticulum to form the mature enzyme (Benjannet et al., 2004). The cleavage of the prodomain is
322 required for PCSK9 maturation and secretion (Li et al., 2007; McNutt, Lagace, & Horton, 2007). In
323 agreement with this consideration and with our clinical results, our findings suggest that the lupin
324 peptide treatment affects the secretion of mature PCSK9 in the culture medium of HepG2 cells
325 (Figure 3). In order to elucidate the mechanism of action through which lupin peptides positively
326 affect the LDLR pathway reducing the PCSK9 protein levels and secretion, a crucial aspect is the
327 regulation of PCSK9 transcription. In particular, several transcription factors, such as SREBPs and
328 HNF-1, have been identified as transcriptional activators of PCSK9 gene expression (Horton et al.,
329 2003; Li, Dong, Park, Lee, Chen, & Liu, 2009; Dong et al., 2010). PCSK9 and LDLR both contain
330 functional sterol regulatory elements (SREs) in their promoters that respond to change in intracellular

331 cholesterol levels through the activation of the SREBP pathway (Dubuc et al., 2004; Maxwell, Soccio,
332 Duncan, Sehayek, & Breslow, 2003). However, since the HNF1 binding site is unique to the PCSK9
333 promoter and is not present in the LDLR promoter, modulations of PCSK9 transcription through
334 HNF1 sequence will not affect LDLR gene expression. Thus, the HNF1 binding site represents a
335 divergent point to disconnect the co-regulation of PCSK9 with LDLR and other SREBP target genes
336 (Dong, Li, Singh, Cao, & Liu, 2015). Indeed, in this study, we have shown that cholesterol-lowering
337 lupin peptides decrease hepatic PCSK9 and secreted PCSK9 concentrations without affecting LDLR
338 protein levels and other SREBP-2 target genes (such as HMGCoAR) and also the down-regulation
339 of HNF1-alpha protein content in HepG2 cells has been observed (Figure 2).
340 For the first time, this investigation provides evidence that the inhibition of PCSK9 production and
341 secretion is a key effect of lupin protein, which contribute to its hypocholesterolaemic property.
342 Furthermore, in the framework of a research aimed at a deep comprehension of the
343 hypocholesterolaemic mechanism of action of lupin protein, these findings help explaining the
344 beneficial effects observed in the human study, since also a significant reduction of plasma PCSK9
345 levels have been observed (Sirtori at al., 2012). Our results provide new scientific evidences
346 supporting the use of lupin protein as an ingredient for developing innovative functional foods and
347 open a new area of research on plant proteins in general (Braithwaite, Tyagi, Tomar, Kumar,
348 Choonara, & Pillay, 2014; Girgih, He, Malomo, Offengenden, Wu, & Aluko, 2014; Patten,
349 Abeywardena, Head, & Bennett, 2012).
350
351

352 **Author contribution**

353 CL: ideation, experiment design, manuscript writing. CZ: experimentation & figure preparation. LC:
354 supervision of the clinical study. AA: manuscript writing & grant retrieval.
355

356 **Acknowledgment**

357 Research funded by the European Union Seventh Framework Program (FP7/2007-2013), under grant
358 agreement n. 285819. We are indebted to Alpro Foundation for a postdoc fellowship to CZ and to the
359 Carlo Sirtori Foundation (Milan, Italy) for having provided part of equipment used in this
360 experimentation.

361

362 The authors declare non conflict of interest

363

364 **5. References**

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

367 Awan, Z., Seidah, N. G., MacFadyen, J. G., Benjannet, S., Chasman, D. I., Ridker, P. M., & Genest,
368 J. (2012). Rosuvastatin, proprotein convertase subtilisin/kexin type 9 concentrations, and LDL
369 cholesterol response: the JUPITER trial. *Clinical Chemistry, 58*(1), 183-189.

370 Benjannet, S., Rhainds, D., Essalmani, R., Mayne, J., Wickham, L., Jin, W., Asselin, M. C., Hamelin,
371 J., Varret, M., Allard, D., Trillard, M., Abifadel, M., Tebon, A., Attie, A. D., Rader, D. J.,
372 Boileau, C., Brissette, L., Chrétien, M., Prat, A., & Seidah, N. G. (2004). NARC-1/PCSK9
373 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL)
374 receptor and LDL cholesterol. *Journal of Biological Chemistry, 279*(47), 48865-48875.

375 Bertoglio, J. C., Calvo, M. A., Hancke, J. L., Burgos, R. A., Riva, A., Morazzoni, P., Ponzone, C.,
376 Magni, C., & Duranti, M. (2011). Hypoglycemic effect of lupin seed gamma-conglutin in
377 experimental animals and healthy human subjects. *Fitoterapia, 82*(7), 933-938.

378 Boschin, G., Scigliuolo, G. M., Resta, D., & Arnoldi, A. (2014). Optimization of the Enzymatic
379 Hydrolysis of Lupin (Lupinus) Proteins for Producing ACE-Inhibitory Peptides. *Journal of
380 Agricultural and Food Chemistry, 62*(8), 1846-1851.

381 Braithwaite, M. C., Tyagi, C., Tomar, L. K., Kumar, P., Choonara, Y. E., & Pillay, V. (2014).
382 Nutraceutical-based therapeutics and formulation strategies augmenting their efficiency to
383 complement modern medicine: An overview. *Journal of Functional Foods, 6*, 82-99.

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

387 Cameron, J., Ranheim, T., Kulseth, M. A., Leren, T. P., & Berge, K. E. (2008). Berberine decreases
388 PCSK9 expression in HepG2 cells. *Atherosclerosis, 201*(2), 266-273.

389 Dong, B., Li, H., Singh, A. B., Cao, A., & Liu, J. (2015). Inhibition of PCSK9 transcription by
390 berberine involves down-regulation of hepatic HNF1α protein expression through the
391 ubiquitin-proteasome degradation pathway. *Journal of Biological Chemistry, 290*(7), 4047-
392 4058.

393 Dong, B., Wu, M., Li, H., Kraemer, F. B., Adeli, K., Seidah, N. G., Park, S. W., & Liu, J. (2010).
394 Strong induction of PCSK9 gene expression through HNF1alpha and SREBP2: mechanism
395 for the resistance to LDL-cholesterol lowering effect of statins in dyslipidemic hamsters.
396 *Journal of Lipid Research, 51*(6), 1486-1495.

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

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

404 Fitzgerald, K., Frank-Kamenetsky, M., Shulga-Morskaya, S., Liebow, A., Bettencourt, B. R.,
405 Sutherland, J. E., Hutabarat, R. M., Clausen, V. A., Karsten, V., Cehelsky, J., Nochur, S. V.,
406 Kotelianski, V., Horton, J., Mant, T., Chiesa, J., Ritter, J., Munisamy, M., Vaishnaw, A. K.,
407 Gollob, J. A., & Simon, A. (2014). Effect of an RNA interference drug on the synthesis of
408 proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL
409 cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1
410 trial. *Lancet, 383*(9911), 60-68.

411 Girgih, A. T., He, R., Malomo, S., Offengenden, M., Wu, J., & Aluko, R. E. (2014). Structural and
412 functional characterization of hemp seed (*Cannabis sativa* L.) protein-derived antioxidant and
413 antihypertensive peptides. *Journal of Functional Foods, 6*, 384-394.

414 Giunzioni, I., & Tavori, H. (2015). New developments in atherosclerosis: clinical potential of PCSK9
415 inhibition. *Vascular Health Risk Management, 11*, 493-501.

416 Goldstein, J. L., & Brown, M. S. (2009). The LDL receptor. *Arteriosclerosis, Thrombosis and*
417 *Vascular Biology, 29*(4), 431-438.

418 Gu, H. M., & Zhang, D. W. (2015). Hypercholesterolemia, low density lipoprotein receptor and
419 proprotein convertase subtilisin/kexin-type 9. *Journal of Biomedical Research, 29*(5), 356-
420 361.

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

425 Jeong, H. J., Lee, H. S., Kim, K. S., Kim, Y. K., Yoon, D., & Park, S. W. (2008). Sterol-dependent
426 regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory
427 element binding protein-2. *Journal of Lipid Research, 49*(2), 399-409.

428 Kong, W., Wei, J., Abidi, P., Lin, M., Inaba, S., Li, C., Wang, Y., Wang, Z., Si, S., Pan, H., Wang,
429 S., Wu, J., Li, Z., Liu, J., & Jiang, J. D. (2004). Berberine is a novel cholesterol-lowering drug

430 working through a unique mechanism distinct from statins. *Nature Medicine, 10*(12), 1344-
431 1351.

432 Koren, M. J., Scott, R., Kim, J. B., Knusel, B., Liu, T., Lei, L., Bolognese, M., & Wasserman, S. M.
433 (2012). Efficacy, safety, and tolerability of a monoclonal antibody to proprotein convertase
434 subtilisin/kexin type 9 as monotherapy in patients with hypercholesterolaemia (MENDEL): a
435 randomised, double-blind, placebo-controlled, phase 2 study. *Lancet, 380*(9858), 1995-2006.

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

440 Lambert, G., Sjouke, B., Choque, B., Kastelein, J. J., & Hovingh, G. K. (2012). The PCSK9 decade.
441 *Journal of Lipid Research, 53*(12), 2515-2524.

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

446 Lee, Y. P., Mori, T. A., Puddey, I. B., Sipsas, S., Ackland, T. R., Beilin, L. J., & Hodgson, J. M.
447 (2009). Effects of lupin kernel flour-enriched bread on blood pressure: a controlled
448 intervention study. *American Journal of Clinical Nutrition, 89*(3), 766-772.

449 Li, H., Dong, B., Park, S. W., Lee, H. S., Chen, W., & Liu, J. (2009). Hepatocyte nuclear factor 1alpha
450 plays a critical role in PCSK9 gene transcription and regulation by the natural
451 hypocholesterolemic compound berberine. *Journal of Biological Chemistry, 284*(42), 28885-
452 28895.

453 Li, J., Tumanut, C., Gavigan, J. A., Huang, W. J., Hampton, E. N., Tumanut, R., Suen, K. F., Trauger,
454 J. W., Spraggon, G., Lesley, S. A., Liau, G., Yowe, D., & Harris, J. L. (2007). Secreted PCSK9
455 promotes LDL receptor degradation independently of proteolytic activity. *Biochemistry*
456 *Journal, 406*(2), 203-207.

457 Lindholm, M. W., Elmén, J., Fisker, N., Hansen, H. F., Persson, R., Møller, M. R., Rosenbohm, C.,
458 Ørum, H., Straarup, E. M., & Koch, T. (2012). PCSK9 LNA antisense oligonucleotides induce
459 sustained reduction of LDL cholesterol in nonhuman primates. *Molecular Therapy, 20*(2),
460 376-381.

461 Lo Surdo, P., Bottomley, M. J., Calzetta, A., Settembre, E. C., Cirillo, A., Pandit, S., Ni, Y. G.,
462 Hubbard, B., Sitlani, A., & Carfí, A. (2011). Mechanistic implications for LDL receptor
463 degradation from the PCSK9/LDLR structure at neutral pH. *EMBO Reports, 12*(12), 1300-
464 1305.

465 Lovati, M. R., Manzoni, C., Castiglioni, S., Parolari, A., Magni, C., & Duranti, M. (2012). Lupin seed
466 gamma-conglutin lowers blood glucose in hyperglycaemic rats and increases glucose
467 consumption of HepG2 cells. *British Journal of Nutrition, 107*(1), 67-73.

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

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

474 McNutt, M. C., Lagace, T. A., & Horton, J. D. (2007). Catalytic activity is not required for secreted
475 PCSK9 to reduce low density lipoprotein receptors in HepG2 cells. *Journal of Biological*
476 *Chemistry, 282*(29), 20799-20803.

477 Patten, G. S., Abeywardena, M. Y., Head, R. J., & Bennett, L. E. (2012). Processed dietary plants
478 demonstrate broad capacity for angiotensin converting enzyme and angiotensin II receptor
479 binding inhibition in vitro. *Journal of Functional Foods, 4*(4), 851-863.

480 Pirillo, A., & Catapano, A. L. (2015). Statin intolerance: diagnosis and remedies. *Current*
481 *Cardiological Reports, 17*(5), 27.

482 Ridker, P. M., Rifai, N., Bradwin, G., & Rose, L. (2015). Plasma proprotein convertase
483 subtilisin/kexin type 9 levels and the risk of first cardiovascular events. *European Heart*
484 *Journal,* Oct 27. pii: ehv568.

485 Roth, E. M., McKenney, J. M., Hanotin, C., Asset, G., & Stein, E. A. (2012). Atorvastatin with or
486 without an antibody to PCSK9 in primary hypercholesterolemia. *New England Journal of*
487 *Medicine, 367*(20), 1891-1900.

488 Ruscica, M., Gomaraschi, M., Mombelli, G., Macchi, C., Bosisio, R., Pazzucconi, F., Pavanello, C.,
489 Calabresi, L., Arnoldi, A., Sirtori, C. R., & Magni, P. (2014). Nutraceutical approach to
490 moderate cardiometabolic risk: results of a randomized, double-blind and crossover study
491 with Armolipid Plus. *Journal of Clinical Lipidology, 8*(1), 61-68.

492 Shan, L., Pang, L., Zhang, R., Murgolo, N. J., Lan, H., & Hedrick, J. A. (2008). PCSK9 binds to
493 multiple receptors and can be functionally inhibited by an EGF-A peptide. *Biochemical and*
494 *Biophysical Research Communications, 375*(1), 69-73.

495 Shantha, G. P., & Robinson, J. G. (2015). Emerging innovative therapeutic approaches targeting
496 PCSK9 to lower lipids. *Clinical Pharmacology and Therapeutics,* Oct 22. doi:
497 10.1002/cpt.281.

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

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

506 Stein, E. A., Mellis, S., Yancopoulos, G. D., Stahl, N., Logan, D., Smith, W. B., Lisbon, E., Gutierrez,
507 M., Webb, C., Wu, R., Du, Y., Kranz, T., Gasparino, E., & Swergold, G. D. (2012). Effect of
508 a monoclonal antibody to PCSK9 on LDL cholesterol. *New England Journal of Medicine,*
509 *366*(12), 1108-1118.

510 Stein, E. A., & Raal, F. J. (2015). Lipid-Lowering Drug Therapy for CVD Prevention: Looking into
511 the Future. *Current Cardiological Reports, 17*(11), 104.

512 Tai, M. H., Chen, P. K., Chen, P. Y., Wu, M. J., Ho, C. T., & Yen, J. H. (2014). Curcumin enhances
513 cell-surface LDLR level and promotes LDL uptake through downregulation of PCSK9 gene
514 expression in HepG2 cells. *Molecular Nutrition and Food Research, 58*(11), 2133-2145.

515 Tavori, H., Fan, D., Blakemore, J. L., Yancey, P. G., Ding, L., Linton, M. F., & Fazio, S. (2013).
516 Serum proprotein convertase subtilisin/kexin type 9 and cell surface low-density lipoprotein
517 receptor: evidence for a reciprocal regulation. *Circulation, 127*(24), 2403-2413.

518 Verbeek, R., Stoekenbroek, R. M., & Hovingh, G. K. (2015). PCSK9 inhibitors: Novel therapeutic
519 agents for the treatment of hypercholesterolemia. *European Journal of Pharmacology, 763*(Pt
520 A), 38-47.

521 Weisse, K., Brandsch, C., Zernsdorf, B., Nkengfack Nembongwe, G. S., Hofmann, K., Eder, K., &
522 Stangl, G. I. (2010). Lupin protein compared to casein lowers the LDL cholesterol:HDL
523 cholesterol-ratio of hypercholesterolemic adults. *European Journal of Nutrition, 49*(2), 65-
524 71.

525 Zhang, L., Song, K., Zhu, M., Shi, J., Zhang, H., Xu, L., & Chen, Y. (2015). Proprotein convertase
526 subtilisin/kexin type 9 (PCSK9) in lipid metabolism, atherosclerosis and ischemic stroke. *Int*
527 *Journal of Neuroscience,* doi: 10.3109/00207454.2015.1057636.

528 Zhang, Y., Eigenbrot, C., Zhou, L., Shia, S., Li, W., Quan, C., Tom, J., Moran, P., Di Lello, P.,
529 Skelton, N. J., Kong-Beltran, M., Peterson, A., & Kirchhofer, D. (2014). Identification of a
530 small peptide that inhibits PCSK9 protein binding to the low density lipoprotein receptor.
531 *Journal of Biological Chemistry, 289*(2), 942-955.

532

533

534 **Captions of figures**

535 **Figure 1. Effects of peptic and tryptic lupin peptides on PCSK9 protein levels.** HepG2 cells (1.5
536 $\times 10^5$) were treated with 1.0 and 2.5 mg/mL of peptic peptides (P) and 0.5 and 1.0 mg/mL of tryptic
537 peptides (T) for 24 h. PCSK9 and β-actin immunoblotting signals were detected using specific anti-
538 PCSK9 and anti-β-actin primary antibodies, respectively. PCSK9-P and PCSK9-M represent the
539 proprotein and the cleaved mature form of PCSK9, respectively (A). PCSK9-P (B) and PCSK9-M
540 (C) signals were quantified by ImageLab software (Biorad) and normalised with β-actin signals. Bars
541 represent averages of duplicate samples ± SEM of three independent experiments. (*) $P < 0.05$, (**)
542 $P < 0.001$ and (***) $P < 0.0001$ *versus* untreated sample (C).

543 **Figure 2. Effects of peptic (P) and tryptic (T) lupin peptides on HNF1-alpha protein levels**.
544 HepG2 cells (1.5×10^5) were treated with 1.0 and 2.5 mg/mL of peptic peptides (P) and 0.5 and 1
545 mg/mL of tryptic peptides (T) for 24 h. HNF1-alpha and β-actin immunoblotting signals were
546 detected using specific anti-HNF1-alpha and anti-β-actin primary antibodies, respectively. HNF1-
547 alpha signals (A) were quantified by ImageLab software (Biorad) and normalised with β-actin signals.
548 Bars represent averages of duplicate samples ± SEM of three independent experiments (B). (*) P <
549 0.05 *versus* untreated sample (C).

550 **Figure 3. Analysis of secreted PCSK9 levels by HepG2 cells after peptic and tryptic lupin**
551 **peptide treatments**. HepG2 cells (1.5×10^5) were treated with 1.0 and 2.5 mg/mL of peptic peptides
552 (P) and 0.5 and 1.0 mg/mL of tryptic peptides (T) for 24 h. After each treatment, the medium was
553 collected and the secreted PCSK9 levels were measured by ELISA. This assay employs the
554 quantitative sandwich enzyme immunoassay techniques. A calibration curve was built using a
555 recombinant human PCSK9. The absorbance of each reaction was measured at 450 nm using the
556 Synergy H1 fluorescent plate reader from Biotek. Data points represent averages ± SEM of three
557 independent experiments in duplicate. (***) $P < 0.0001$ versus untreated sample (C).

558

559

560 **Figure 4. Hypocholesterolaemic mechanism of action mediated by lupin peptides in human**
561 **hepatic HepG2 cells**. Upon cell penetration, peptic and tryptic lupin peptides act as competitive
562 inhibitor of HMGCoAR leading to a reduction of intracellular cholesterol synthesis. When
563 intracellular cholesterol level decreases, the transcription factor SREBP2 is activated and LDLR and
564 HMGCoAR genes are transcripted with subsequent increase of LDLR and HMGCoAR protein levels
565 and localization of LDLR in plasma membrane (**a**). In parallel, in a synergic way, lupin peptides
566 reduce the PCSK9 protein level production and secretion. In particular, through the reduction of
567 HNF1-alpha protein level, they lead to a decrease of intracellular precursor and mature PCSK9
568 protein levels. In agreement, the PCSK9 down-regulation is translated in a reduction of HepG2 cell
569 ability to secrete mature PCSK9 in the extracellular medium, with the consequent stabilisation of
570 active LDLR on hepatic cellular membrane (**b**). For this reason, the distinct modulation of the two
571 pathways leads to hypocholesterolaemic effects through an improved and synergic activity of LDLR,
572 which can bind and carry the extracellular LDL in HepG2 cells.

573

574

575

576 **Table 1. Effect of lupin protein consumption on lipid parameters and PCSK9 plasma levels in**
577 **mild hypercholesterolaemic patients: data are expressed as mean ± SD.**

Parameter		Casein	Lupin protein
Total cholesterol (mg/dL)[a]	Baseline	272.4±34.4	274.0±40.0
	4 weeks	275.2±26.9	262.4±40.8*
LDL-cholesterol (mg/dL)[a]	Baseline	188.8±36.5	188.2±35.4
	4 weeks	193.7±26.7	182.6±40.6
HDL-cholesterol (mg/dL)[a]	Baseline	57.0±12.4	56.0±13.9
	4 weeks	57.6±12.8	54.7±15.7
Triglycerides (mg/dL)[a]	Baseline	126.1±45.4	145.6±70.9
	4 weeks	115.2±34.6	126.5±59.6
PCSK9 (ng/mL)	Baseline	78.8±17.3	82.9±22.2
	4 weeks	75.3±14.0	75.9±22.4*

578 *$p<0.05$ *vs.* baseline

579 [a] Selected data from a previous paper (Sirtori et al., 2012)

580

Table 2a. Lupin peptides identified by mass spectrometry in the tryptic hydrolysate

Accession number	Protein name	Mascot protein score	Protein mass	N. peptides	m/z	z	Mr	Peptide delta	ion score	peptide sequence
Q53HY0	**vicilin-like protein [*Lupinus albus*]**	**969**	**61994**	**12**	419,35	2	836,40	0,27	40	YEEIQR
					1051,58	1	1050,50	0,07	32	SNEPIYSNK
					526,62	2	1050,50	0,73	27	SNEPIYSNK
					688,15	2	1373,63	0,66	47	YGNFYEITPDR
					1463,62	1	1462,69	-0,08	47	ELTFPGSAEDIER
					732,62	2	1462,69	0,54	63	ELTFPGSAEDIER
					762,62	2	1522,66	0,56	76	EQEQQQGSPSYSR
					1557,63	1	1556,79	-0,17	102	HSDADYVLVVLNGR
					779,47	2	1556,79	0,12	63	HSDADYVLVVLNGR
					791,66	2	1580,75	0,56	61	GQEQSHQDEGVIVR
					823,03	2	1643,69	0,35	76	QQDEQEEEPEEVR
					863,99	2	1726,00	-0,04	28	IVEFQSKPNTLILPK
					576,40	3	1726,00	0,18	48	IVEFQSKPNTLILPK
					932,71	2	1863,85	-0,45	94	ILLGNEDEQEYEEQR
					840,60	3	2518,31	0,46	33	LSEGDIFVIPAGYPISVNASSNLR
					1090,84	3	3268,66	0,83	71	NPQVQDLDISLTFTEINEGALLLPHYNSK
Q6EBC1	**beta-conglutin precursor [*Lupinus albus*]**	**917**	**62092**	**10**	419,35	2	836,40	0,27	40	YEEIQR
					1051,58	1	1050,50	0,07	32	SNEPIYSNK
					526,62	2	1050,50	0,73	27	SNEPIYSNK
					688,15	2	1373,63	0,66	47	YGNFYEITPDR
					762,62	2	1522,66	0,56	76	EQEQQQGSPSYSR
					1557,63	1	1556,79	-0,17	102	HSDADYVLVVLNGR
					779,47	2	1556,79	0,12	63	HSDADYVLVVLNGR
					1568,69	1	1567,84	-0,16	64	INEGALLLPHYNSK
					784,95	2	1567,84	0,05	59	INEGALLLPHYNSK
					863,99	2	1726,00	-0,04	28	IVEFQSKPNTLILPK
					576,40	3	1726,00	0,18	48	IVEFQSKPNTLILPK

					874,70	2	1746,84	0,55	65	AVNELTFPGSAEDIER
					932,71	2	1863,85	-0,45	94	IILGNEDEQEYEEQR
					1034,78	3	3100,55	0,77	72	YGNFYEITPDRNPQVQDLNISLTYIK
Q99235	**conglutin-delta seed storage protein precursor [*Lupinus albus*]**	**573**	**17641**	**2**	687,93	2	1373,61	0,24	60	IQQQEEEEEGR
					934,22	2	1866,93	-0,51	56	QEEQLLEQELENLPR
Q53I55	**legumin-like protein [*Lupinus albus*]**	**399**	**58830**	**4**	638,61	3	1912,87	-0,06	49	HNIGESTSPDAYNPQAGR
					957,68	2	1912,87	0,47	78	HNIGESTSPDAYNPQAGR
					966,12	2	1929,75	0,48	102	HGREEEEEEEEEDER
					1264,26	2	2526,22	0,29	66	LNALEPDNTVQSEAGTIETWNPK
					843,21	3	2526,22	0,37	21	LNALEPDNTVQSEAGTIETWNPK
					1591,41	2	3180,45	0,35	100	EGGQGQQQEGGNVLSGFDDEFLEEALSVNK
					1061,44	3	3180,45	0,84	27	EGGQGQQQEGGNVLSGFDDEFLEEALSVNK
B0YJF8	**conglutin-beta [*Lupinus angustifolius*]**	**115**	**61490**	**4**	419,35	2	836,40	0,27	40	YEEIQR
					611,40	3	1831,78	-0,61	21	QQDEQEEEEEEVRR
					840,60	3	2518,31	0,46	33	LSEGDIFVIPAGYPISVNASSNLR
					1090,84	3	3268,66	0,83	71	NPQVQDLDISLTFTEINEGALLLPHYNSK
ADF3	**actin, partial [*Zea mays*]**	**90**	**37282**	**2**	874,37	2	1746,88	-0,16	90	SYELPDGQVITIGAER
					652,78	3	1955,04	0,28	27	VAPEEHPTLLTEAPLNPK

Table 2b. Lupin peptides identified by mass spectrometry in the peptic hydrolysate

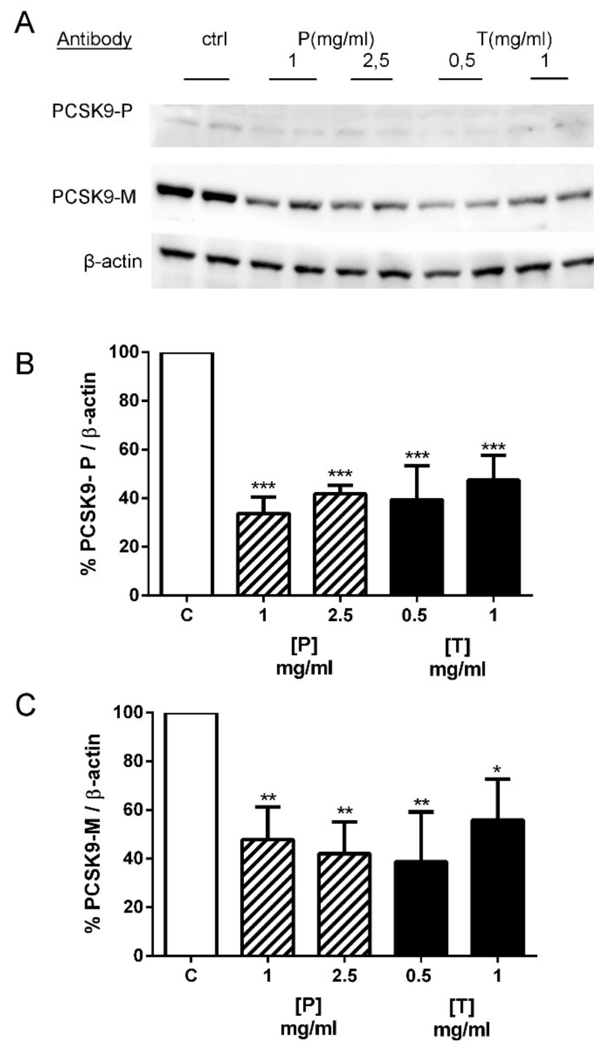
Accession number	**Protein name**	**Mascot protein score**	**Protein mass**	**N. peptides**	**m/z**	**z**	**Mr**	**Peptide delta**	**ion score**	**peptide sequence**
Q53HY0	**Vicilin-like protein [*Lupinus albus*]**	**434**	**61994**	**21**	667,23	1	666,29	-0,07	39	SEGDIF
					695,29	1	694,35	-0,07	32	DISLTF
					415,56	2	828,38	0,73	44	SNKYGNF
					958,32	1	957,49	-0,18	21	AIPINNPGY
					505,05	2	1007,50	0,59	32	IKNQQQSY
					1105,36	1	1104,56	-0,21	31	AIPINNPGYF

					553,34	2	1104,56	0,11	49	AIPINNPGYF
					578,54	2	1154,57	0,50	32	IKNQQQSYF
					1169,52	1	1168,61	-0,10	36	ILNPDDNQKL
					595,27	2	1188,63	-0,10	25	LPHYNSKAIF
					1189,58	1	1188,63	-0,06	38	LPHYNSKAIF
					651,77	2	1301,71	-0,19	27	LLPHYNSKAIF
					652,33	2	1302,57	0,08	62	YPSSTKDQQSY
					454,46	3	1359,69	0,66	35	SRRQRNPYHF
					681,19	2	1359,69	0,67	32	SRRQRNPYHF
					688,58	2	1375,73	-0,60	56	IVVVGEGNGKYEL
					725,88	2	1449,64	0,10	44	YPSSTKDQQSYF
					763,10	2	1523,76	0,44	43	EITPDRNPQVQDL
					518,59	3	1552,91	-0,17	23	RVVKLAIPINNPGY
					844,31	2	1686,82	-0,21	32	YEITPDRNPQVQDL
					844,72	2	1687,80	-0,38	24	RSNEPIYSNKYGNF
					851,24	2	1699,98	0,49	61	RVVKLAIPINNPGYF
					865,24	2	1727,73	0,74	47	YDFYPSSTKDQQSY
					1114,25	2	2226,09	0,39	120	TKYAQSSSGKDKPSQSGPFNL
Q6EBC1	**Beta-conglutin [Lupinus albus]**	**141**	**62092**	**18**	667,23	1	666,29	-0,07	39	SEGDIF
					415,56	2	828,38	0,73	44	SNKYGNF
					429,48	2	856,50	0,44	54	IKINEGAL
					958,32	1	957,49	-0,18	21	AIPINNPGY
					505,05	2	1007,50	0,59	32	IKNQQQSY
					1105,36	1	1104,56	-0,21	31	AIPINNPGYF
					578,54	2	1154,57	0,50	32	IKNQQQSYF
					1169,52	1	1168,61	-0,10	36	ILNPDDNQKL
					603,69	2	1204,62	0,73	22	LPHYNSKAIY
					652,33	2	1302,57	0,08	62	YPSSTKDQQSY
					454,46	3	1359,69	0,66	35	SRRQRNPYHF
					725,88	2	1449,64	0,10	44	YPSSTKDQQSYF
					763,10	2	1523,76	0,44	43	EITPDRNPQVQDL

					518,59	3	1552,91	-0,17	23	RVVKLAIPINNPGY
					844,31	2	1686,82	-0,21	32	YEITPDRNPQVQDL
					844,72	2	1687,80	-0,38	24	RSNEPIYSNKYGNF
					851,24	2	1699,98	0,49	61	RVVKLAIPINNPGYF
					865,24	2	1727,73	0,74	47	YDFYPSSTKDQQSY
F5B8V8	**Conglutin-alpha [*Lupinus angustifolius*]**	**73**	**67683**	**7**	703,36	1	702,37	-0,02	28	VVPQNF
					797,22	1	796,34	-0,13	33	SGFDPQF
					816,38	1	815,45	-0,09	22	LVVPQNF
					854,49	1	853,50	-0,02	21	RGIPAEVL
					1029,57	1	1028,53	0,03	23	VIPPGTPYW
					639,13	2	1275,61	0,64	24	YRNGIYAPHW
					647,15	2	1292,64	-0,36	62	VIPPGTPYWTY
Q9FSH9	**Conglutin-gamma [*Lupinus albus*]**	**50**	**49872**	**8**	354,39	2	706,34	0,42	36	FSHFGL
					719,40	1	718,33	0,07	28	FDLNNP
					422,88	2	843,52	0,21	44	VKIPQFL
					619,18	2	1235,64	0,70	31	TPLTISKQGEY
					633,73	2	1264,69	0,76	35	VDGGVHTRAGIAL
					708,72	2	1414,77	0,66	50	DTKKISGGVPSVDL
					726,97	2	1451,81	0,11	46	LTQKGLPNNVQGAL
					678,14	3	2032,11	-0,71	24	VLPIQQDASTKLHWGNIL

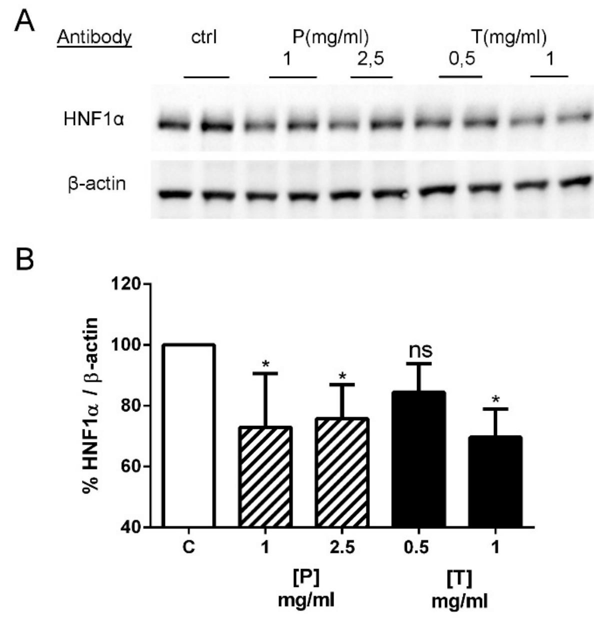
581

582



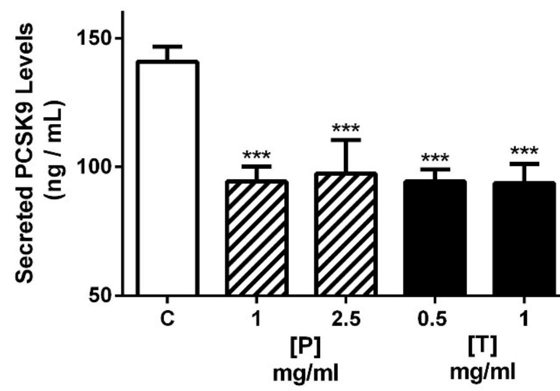
583

584 Figure 1



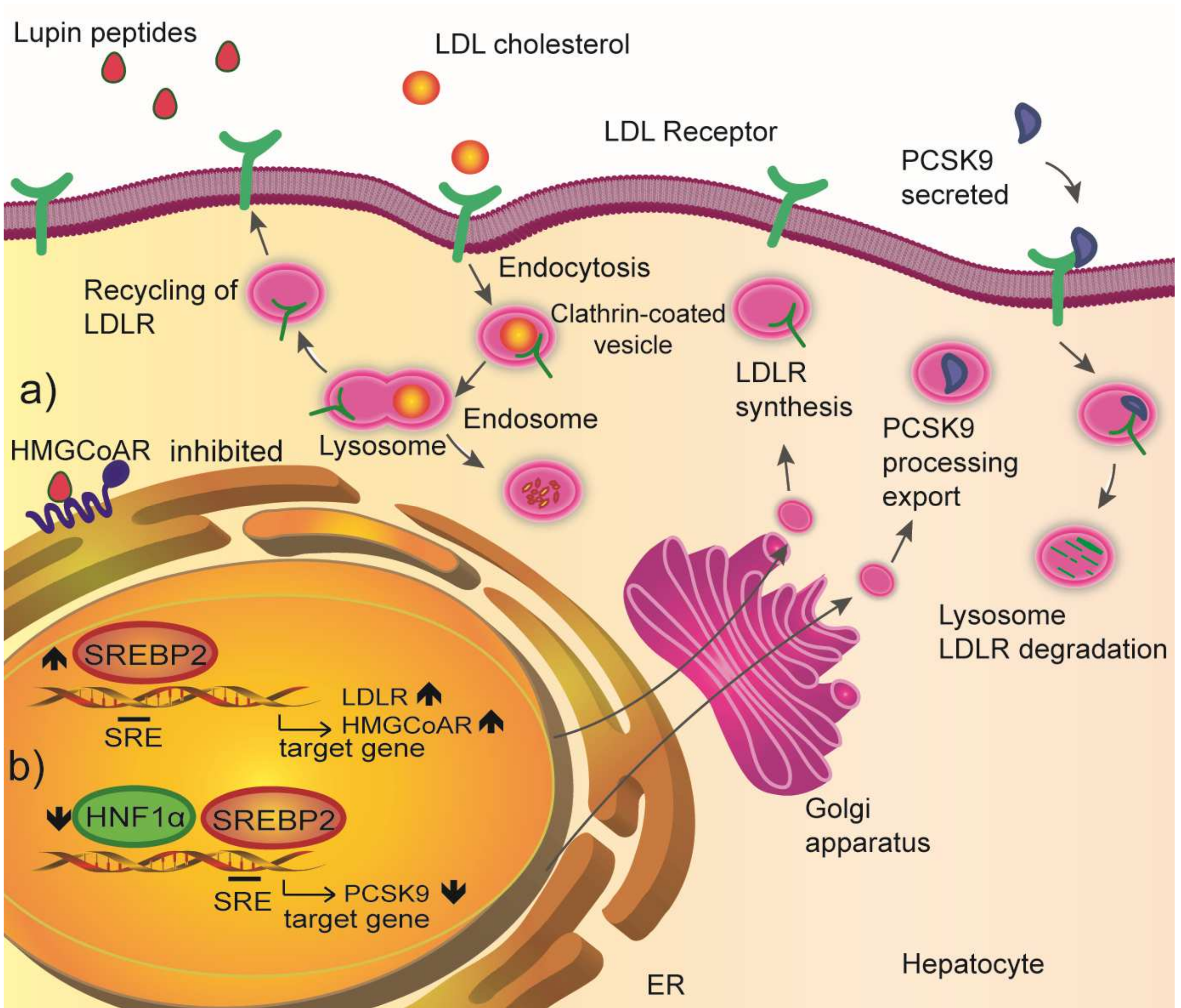
585

586 Figure 2



587

588 Figure 3



589

590 Figure 4