Effects of a lupin protein concentrate on lipids, blood pressure and insulin resistance in moderately dyslipidaemic patients: a randomised controlled trial

Chiara Pavanello[1], Carmen Lammi,[2] Massimiliano Ruscica[3], Raffaella Bosisio[3], Giuliana Mombelli[4], Chiara Zanoni[2], Laura Calabresi[1,3], Cesare R. Sirtori[1,3], Paolo Magni[3*], Anna Arnoldi[2*]

[1] Centro Grossi Paoletti, Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, Italy.
[2] Dipartimento di Scienze Farmaceutiche, Università degli Studi di Milano, Milan, Italy.
[3] Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, Italy
[4] Centro Dislipidemie, A.S.S.T. Grande Ospedale Metropolitano Niguarda, Milan, Italy

Address correspondence to:
prof. Anna Arnoldi, Dipartimento di Scienze Farmaceutiche, Università degli Studi di Milano, via Mangiagalli 25, 20133 Milano, Italy, e-mail anna.arnoldi@unimi.it, tel +39 0250319372, fax +39 0250319343, or Dr. Paolo Magni, Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, via Balzaretti 9, 20133 Milano, Italy, e-mail paolo.magni@unimi.it, tel +39 0250318229, fax +39 0250318204.

Conflict of interest

The authors declare no conflict of interest.

Abstract

The study had the objective of evaluating the effects of a lupin protein concentrate on plasma lipids and other cardiovascular risk factors, e.g. blood pressure and insulin resistance, compared to a skimmed milk powder. Fifty subjects followed a randomised, parallel, double-blind, single-centre study, consisting in a 12-week intervention: half of the participants consumed a lupin protein concentrate (30 g/day of protein), the other half a lactose-free skimmed milk powder (30 g/day of protein), both integrated into a mixed low-lipid diet. At the end of intervention, both groups showed similar reductions of total cholesterol concentrations *versus* baseline (-6.7%, and -7.2%, respectively), but the

reductions of LDL-cholesterol (-8.0%), non-HDL-cholesterol (-7.5%), and proprotein convertase subtilisin/kexin type 9 (PCSK9) (-12.7%) levels were statistically significant only after the lupin diet. A significant reduction of HDL-cholesterol concentration was observed only after the milk diet. The differences between the two groups, however, were not statistically significant.

Keywords:
lupin protein, lipids, PCSK9, blood pressure, glycaemic control

Abbreviations: *ACE,* angiotensin converting enzyme; *ApoA-I,* apolipoprotein A-I; *ApoB,* apolipoprotein B; *BMI,* body mass index; *BP,* blood pressure; CK, creatine kinase; *CRP,* C-reactive protein; *CV,* cardiovascular; DBP, diastolic blood pressure; *EGF-A*, first epidermal growth factor-like repeat; *ELISA,* enzyme-linked immunosorbent assay; *F,* females; *FG,* fasting glucose; *HDL-C,* high-density lipoprotein cholesterol; *HC,* hip circumference; *HOMA-IR,* homeostasis model assessment for insulin resistence; *LDL-C,* low-density lipoprotein cholesterol; *Lp(a),* lipoprotein(a); *M,* males; *NCEP,* National Cholesterol Education Program; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *SBP,* systolic blood pressure; *SD,* standard deviation; *sICAM-1*, soluble intercellular adhesion molecule-1; *TG,* triglycerides; *WC,* waist circumference

1. Introduction

A partial replacement of animal foods with grain legumes may represent a first approach to the prevention of cardiovascular disease (Arnoldi, Zanoni, Lammi, & Boschin, 2015; Bazzano, Thompson, Tees, Nguyen, & Winham, 2011), as indicated by some recent meta-analyses that have underlined numerous positive effects induced by a regular intake of these seeds. A meta-analysis focused on lipids considered 26 controlled randomised trials (CRTs): one serving per day (median dose 130 g/d) significantly lowered LDL cholesterol levels compared with the control diets, with a mean change equal to -0.17 mmol/L (Ha, Sievenpiper, de Souza, Jayalath, Mirrahimi, Agarwal, et al., 2014). Another considered 8 CRTs including subjects with and without hypertension and concluded that a regular consumption of grain legumes significantly lowered systolic blood pressure (BP) (-2.25 mmHg) and mean arterial BP (-0.75 mmHg), whereas the change of the diastolic BP was non-significant (Jayalath, Souza, Sievenpiper, Ha, Chiavaroli, Mirrahimi, et al., 2014). A third meta-analysis evaluated the glycaemic control in individuals with diabetes, including 13 RCTs. A 35% daily replacement of animal with plant protein significantly lowered HbA(1c) (mean = -0.15%), fasting glucose (mean = -0.53 mmol/L), and fasting insulin (mean = -10.09 pmol/L) compared with control arms, indicating modest improvements in glycaemic control (Viguiliouk, Stewart, Jayalath, Ng, Mirrahimi, de Souza, et al., 2015).

Lupin is gaining a lot of attention in recent years, since the protein content is similar to soybean, but phytoestrogens are negligible (Arnoldi, Boschin, Zanoni, & Lammi, 2015). In the Mediterranean countries, this grain legume has been cultivated and consumed as human food or animal feed for centuries. Beside the protein abundance (Sujak, Kotlarz, & Strobel, 2006), the nutritional interest for lupin is linked also to its content of unsaturated fatty acids, fibres, minerals, tocopherols (Boschin & Arnoldi, 2011), and polyphenols (Siger, Czubinski, Kachlicki, Dwiecki, Lampart-Szczapa, & Nogala-Kalucka, 2012).

Clinical studies have shown that lupin consumption can provide useful health benefits particularly in hyperglycaemia prevention (Bertoglio, Calvo, Hancke, Burgos, Riva, Morazzoni, et al., 2011; Dove, Mori, Chew, Barden, Woodman, Puddey, et al., 2011; Hall, Thomas, & Johnson, 2005) and in plasma total cholesterol (TC) and low-density lipoprotein (LDL) cholesterol (LDL-C) reduction (Bähr, Fechner, Kiehntopf, & Jahreis, 2015; Bähr, Fechner, Kramer, Kiehntopf, & Jahreis, 2013; Sirtori, Triolo, Bosisio, Bondioli, Calabresi, De Vergori, et al., 2012). Different seed components may be responsible of these effects, in particular the fibre (Fechner, Kiehntopf, & Jahreis, 2014; Hall, Johnson, Baxter, & Ball, 2005) and the protein (Bahr, Fechner, Kiehntopf, & Jahreis, 2015; Sirtori et

al., 2012; Weisse, Brandsch, Zernsdorf, Nembongwe, Hofmann, Eder, et al., 2010). The mechanism at the basis of the LDL-C reduction induced by the protein seems to be linked to the inhibition of the activity of 3-hydroxymethylglutarylcoenzyme A reductase (HMGCoAR) and the up-regulation of LDL-receptors (LDLR) in liver cells with a statin-like mechanism (Lammi, Zanoni, Scigliuolo, D'Amato, & Arnoldi, 2014), as already observed in the case of soybean peptides (Lammi, Zanoni, Arnoldi, & Vistoli, 2015). The effects of the fibre are probably linked to the formation of short chain fatty acids (Fechner, Kiehntopf, & Jahreis, 2014).

Other studies have shown that a lupin diet is also useful for a moderate control of hypertension (Lee, Mori, Puddey, Sipsas, Ackland, Beilin, et al., 2009; Wu & Ding, 2001). This activity may be linked to the inhibition of the activity of angiotensin converting enzyme (ACE) by peptides generated through cleavage of lupin protein by digestion and absorbed (Boschin, Scigliuolo, Resta, & Arnoldi, 2014).

In view of the increasing interest for plant proteins, the present study had the objective of comparing the effects of including a lupin protein concentrate or a lactose-free milk powder, both commercially available, in the diet of moderately hypercholesterolaemic subjects. The primary end-point of the trial was LDL-C, whereas secondary endpoints were the complete lipoprotein profile, anthropometric parameters, adipokine levels, inflammation markers, and markers related to the glycaemic control. The specific features of this study in respect with those already published in literature are: a) the use of commercially available ingredients, instead of model ingredients/foods, b) the broad set of parameters considered here.

2. Materials and Methods

2.1 Study design and population

The study was performed at the Centro Dislipidemie (ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy) and was designed as a randomised, parallel group, double-blind single-centre study. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the local ethics committee (date of approval: July 27, 2012; approval number: 296_07/2012). Each subject signed a written informed consent form.

Inclusion criteria were: (i) males and postmenopausal females; (ii) age between 45 and 75 years; (iii) body mass index (BMI) within the range 25-32 kg/m^2; (iv) LDL-C levels in the

range 130 and 190 mg/dL. Exclusion criteria were: any type of food allergy; chronic liver or renal disease; diabetes mellitus; uncontrolled arterial hypertension; past history of cerebrovascular accident or coronary events; myocardial infarction; percutaneous transluminal coronary angioplasty or coronary artery bypass graft; any concomitant therapy known to alter any of the parameters to be assessed; any current alcohol or drug abuse; any clinically significant medical condition that could interfere with the study; inability or unwillingness to comply with the protocol requirements, as deemed by the investigators. All patients had no history of CV events and did not consumed any drug affecting lipid/lipoproteins or glycaemic profile. The scheme of the trial design is reported in Figure 1. After a run-in period of 4 weeks on a balanced low-lipid diet, only subjects who had shown changes in total cholesterol smaller that 10% during run-in were recruited for the study.

2.2 Tested lupin and milk products

The tested products were commercially available food grade materials and did not represent any safety risk. Both lupin (*Lupinus angustifolius*) protein concentrate (FRALU-CON, L.I. Frank, Twello, The Netherland) and lactose-free skimmed milk powder (Eila® lactose free skimmed milk powder, Valio, Helsinki, Finland) were in powder form and were specifically for the study packed in small opaque bags, each containing a daily dose corresponding to 30 g of protein. FRALU-CON composition (on 100 g): moisture 11%, protein 55.5%, fats 9.8%, carbohydrates 7.6%, dietary fibre 14.6%, ash 2.5-3.5%. Eila® lactose free skimmed milk powder composition: moisture 4%, protein 47%, fats 0.9%, carbohydrates 38%, dietary fibre 0%, ash 4.6%. The lupin and milk bags were identical and identified only by a letter (A or B), in order to maintain double-blindness. Only one person (A.A.), who had organised the packaging and was not directly involved in clinical intervention, knew the significance of the letters that was disclosed only at the end of study.

2.3 Dietary intervention

A registered dietician was responsible for the design and follow-up of the provided diet. Subjects were instructed to follow a balance normocaloric/low-lipid diet, planned in accordance to the Mediterranean diet criteria (Estruch, Ros, Salas-Salvado, Covas, Corella, Aros, et al., 2013), with three main meals and two snacks, adapted to individual preferences in order to improve patient compliance. Extra virgin olive oil in moderate quantity was suggested as topping. Dietary plans were defined with the aid of a dedicated

157 software (Dietosystem, DS Medica srl, Milan, Italy) specifically tailored for the Italian food
158 items. Dietary composition was designed differently for male and female subjects,
159 according to the diverse daily requirements (Table 1).
160 Subjects were randomly assigned to receive either the lupin diet or the milk diet for 12
161 weeks. They received all bags necessary for one-month treatment during their visits to the
162 clinical centre and were instructed to add the powders to their normal foods, avoiding
163 extensive cooking. Dietary planning ensured to keep the major nutrient intake equal
164 between the groups (Table 1) and both diets were planned and monitored in order to avoid
165 any nutrient excess or deficiency. In order to have a constant total energy intake over the
166 entire study period, participants were asked to complete a diary, recording three non-
167 consecutive days to assess their habitual diet and the investigators continuously provided
168 personalised recommendation to the participants during the whole study. Food diaries
169 were analysed with the above-mentioned software.
170
171 *2.4 Clinical and biochemical evaluations*
172 Clinical and biochemical evaluations were performed at screening, baseline, and every 4
173 weeks until the end of treatment. Subjects were then followed-up after 4 additional weeks
174 to assess potential late side-effects. At all visits, patients underwent a fasting venous
175 blood sampling and a full clinical examination, including the evaluation of height, body
176 weight, heart rate, and arterial BP. Waist circumference (WC) was measured by means of
177 a non-stretchable tape at the umbilical level (standing position). Hip circumference (HC)
178 was measured by tape at the largest point. All visits were performed by the same
179 investigator (PM). Plasma samples were prepared by low-speed centrifugation and
180 aliquots were immediately stored at −20 °C for subsequent assays. Safety and compliance
181 information were collected at each visit, also by means of a food diary relative to 3 days,
182 freely selected by the patient over each month of nutritional intervention.
183 On each blood sample, total cholesterol (TC), TG, HDL-C, Lp(a), apolipoprotein A-I
184 (ApoAI), apolipoprotein B (ApoB), C-reactive protein (CRP), fasting glucose (FG) were
185 measured according to standard clinical procedures. LDL-C was calculated according to
186 the Friedewald equation (Friedewald, Levy, & Fredrickson, 1972). Non-HDL-C was
187 calculated as TC – HDL-C (Sniderman, Williams, & de Graaf, 2010). Proprotein
188 convertase subtilisin/kexin type 9 (PCSK9) was quantified using a specific kit (R&D
189 System, Minneapolis, MN), following a procedure described in a previous paper (Lammi,
190 Zanoni, Calabresi, & Arnoldi, 2016). Commercial enzyme-linked immunosorbent assay

(ELISA) kits were used according to manufacturer's specifications and previously published protocols (Ruscica, Macchi, Gandini, Morlotti, Erzegovesi, Bellodi, et al., 2016) to quantify plasma adiponectin, soluble intercellular adhesion molecule-1 (sICAM-1) (R&D System, Minneapolis, MN) and insulin (Mercodia, Uppsala, Sweden). The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) index was calculated as glucose x insulin divided by 22.5 (Matthews, Hosker, Rudenski, Naylor, Treacher, & Turner, 1985).

2.5 Statistical analyses

The randomisation table was obtained by computer-generated random numbers. Basal values were indicated as median (interquartile range) in Table 2, whereas basal values and treatment effects were indicated as means ± standard deviation (SD) in Table 3. The effects of the lupin or milk diets on the different biomarkers were calculated as follows: mean of values at the end of treatment (after 12 weeks) minus the baseline value (after 4 weeks run-in), and the differences (expressed as Δ) were then compared. All differences were assessed by paired Student's t test, whereas comparisons between arms were evaluated by 2-sample t test. For non-normally distributed data, Wilcoxon signed rank test and Kruskal Wallis test were, respectively, used. Treatment effectiveness was considered achieved if statistical significance was demonstrated at the prespecified nominal α-level (0.05) for all the endpoints. No adjustment for multiple endpoints is necessary under this scenario (Sankoh, D'Agostino, & Huque, 2003).

All tests are two-sided and p values <0.05 were considered as statistically significant. Statistical analysis was performed by using the SPSS 19.0 software (SPSS Inc., Chicago, USA). All collected data were tested for normal distribution and for homogeneity of variances applying the Kolmogorov-Smirnov test and the Levene's test, respectively. Correlations between normally distributed variables were examined by calculating Pearson's correlation coefficients; otherwise Spearman´s correlation was used.

Power analysis on the 41 subjects who concluded the study revealed > 80% power for the present study to detect a 8% difference in the primary outcome measure, i.e. LDL-C (mg/dL).

3. Results

3.1 Patients

Fifty-six subjects [26 male (M) and 30 female (F)] were assessed for eligibility, 6 were excluded, and 50 (25 M, 25 F) were enrolled into the study and randomly allocated to either the lupin diet (*n* = 25; 12 M, 13 F) or the milk diet (*n* = 25; 13 M, 12 F), for a total duration of 12 weeks. Six patients were excluded showing lipids out of the values defined for the inclusion. The lupin diet arm was completed by 19/25 patients, while 22/25 completed the milk diet arm (Figure 2). The two participating groups had almost identical basal characteristics (Table 2). Concomitant medications were the following: angiotensin-converting enzyme inhibitor / angiotensin receptor blockers / calcium antagonists / beta-blockers 26.4% of subjects; thienopyridines 5.6%; beta-blockers 1.9%; proton-pump inhibitors 9.4%; antidepressant 9.4%; other drugs 11.3%.

3.2 Effects of treatments

Table 3 shows the effects induced by the 12-weeks treatment with the lupin diet as well as the milk one on anthropometric parameters, lipids, adipokine levels, and inflammation markers. Significant changes in body weight (-1.3 kg, -1.7%, *p* 0.036) and BMI (-0.4 kg/m^2, -1.5%, *p* 0.023) were found with the milk diet, whereas a decrease in WC/HC was recorded only with the lupin diet (-0.02, -2%, *p* 0.047).

The glucose concentrations were not influenced by the dietary treatments and adiponectin levels remained unchanged in both groups. The HOMA index was slightly raised by the milk diet, whereas the opposite was found in the lupin diet, however, both changes were not statistically significant. Mild non-significant reductions of BPs were observed in both treatments.

Both groups showed similar reductions of TC concentrations compared to baseline (-17.3 mg/dL, -6.7%, *p* 0.037; -19.3 mg/dL, -7.2%, *p* 0.007, respectively), but the reduction of LDL-C was statistically significant only after the lupin diet (-14.3 mg/dL, -8.0% *p* 0.010), that induced also a more favourable change of non-HDL-C (-15.5 mg/dL, -7.5%, *p* 0.006). A significant reduction of HDL-C concentration was also found after the milk diet, whereas the small decrease after the lupin diet was not significant. An increase of Lp(a) levels (+11.9%, *p* 0.006) was recorded after the lupin diet, whereas the small decrease in the milk diet was not significant. A significant decrease of the PCSK9 level (-11.6 mg/dL, -12.7%, *p* 0.001) was observed only with the lupin diet, with the additional consequence of a significant lowering of the ApoB:PCSK9 ratio (p 0.036). However, it is important to observe that for all these parameters the differences between the two groups were not significant.

4. Discussion

The interest for including lupin foods in human nutrition is continuously increasing stimulated by its technological flexibility and the growing knowledge of the health benefits they can provide (Arnoldi, Boschin, Zanoni, & Lammi, 2015). The present investigation had been encouraged by the results of some clinical studies that have pointed out, albeit not constantly, that a diet including lupin products may positively affect either TC cholesterol or LDL-C (Bähr, Fechner, Kiehntopf, & Jahreis, 2015; Bähr, Fechner, Kramer, Kiehntopf, & Jahreis, 2013; Hall, Johnson, Baxter, & Ball, 2005; Sirtori, et al., 2012). Besides the standard lipidic profile, other features known to increase the risk of CV events were investigated, in particular parameters linked to overweight as well as glucose metabolism and BP.

Although significant in some cases, the changes of the anthropometric parameters (weight, BMI, WC, HC, and WC/HC ratio) were always too small to have any favourable health consequence on these subjects either with the lupin or the milk diet.

In the same way, no evidence of glycemia regulation following the lupin diet was observed. This was unexpected, since post-prandial experiments on healthy volunteers have shown that the consumption of a lupin flour enriched bread ameliorates blood glucose and insulin values (Hall, Thomas, & Johnson, 2005; Keogh, Atkinson, Eisenhauer, Inamdar, & Brand-Miller, 2011). These Authors have attributed the observed changes to the characteristics of lupin fibre that, however, is not the only hypoglycaemic factor in lupin seed. In fact, another group has attributed the hypoglycaemic activity to γ-conglutin, a specific sulphur-rich protein fraction corresponding to about 4% of the total lupin protein (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008). This hypothesis has been confirmed by a clinical trial performed on a purified γ-conglutin sample (Bertoglio, et al., 2011) and experimental studies in liver cells (Capraro, Magni, Faoro, Maffi, Scarafoni, Tedeschi, et al., 2013) as well as in animals (Magni, Sessa, Accardo, Vanoni, Morazzoni, Scarafoni, et al., 2004), which have suggested an insulin-mimetic mechanism. Thus, it seems possible to affirm that either lupin fibre or γ-conglutin might be responsible of the hypoglycaemic activity observed in literature. The fact that the present study was performed on a lupin protein concentrate containing only a reduced amount of fibre and γ-conglutin may possibly explain the scarce activity detected here. It is important to underline, however, that a recent paper has shown that also β-conglutin, another protein fraction, which is abundant in the tested lupin concentrate, may be important for the hypoglycaemic activity of lupin

(Lima-Cabello, Alche, Foley, Andrikopoulos, Morahan, Singh, et al., 2016). These Authors have produced five different purified recombinant isoforms of β-conglutin and shown that three out of these isoforms bind to insulin. Then, treating peripheral blood mononuclear cell cultures from type 2 diabetes subjects and healthy controls, they have investigated how these conglutins influence insulin-signalling pathway. These experiments have been performed on intact proteins that certainly *in vivo* would undergo cleavage by digestion.

Triglyceride changes did not appear to occur either in the present study or in earlier trials (Bähr, Fechner, Kramer, Kiehntopf, & Jahreis, 2013; Sirtori, et al., 2012; Weisse, Brandsch, Zernsdorf, Nkengfack Nembongwe, Hofmann, Eder, et al., 2010). On the contrary, experiments on rodents have shown a triglyceride reduction in adult rats after a diet including a lupin protein isolate, apparently related to the expression of liver genes involved in fatty acid synthesis and triglyceride hydrolysis (Spielmann, Shukla, Brandsch, Hirche, Stangl, & Eder, 2007).

Significant decreases of total cholesterol (-7.2%), LDL-C (-8%) and non-HDL-C (-7.5%) were observed only with the lupin diet, although the changes were not significant when compared to the milk diet. These variations are in line with those observed by other authors that fall in the range 3-9% depending on the kind of model foods containing lupin protein given to the subjects (Bähr, Fechner, Kiehntopf, & Jahreis, 2015; Bähr, Fechner, Kramer, Kiehntopf, & Jahreis, 2013; Sirtori, et al., 2012; Weisse, Brandsch, Zernsdorf, Nkengfack Nembongwe, et al., 2010). Those studies have been performed on model foods prepared including an experimental lupin protein isolate, whereas here we used a commercial protein concentrate added to the normal diet of the participating subjects. The diverse composition of the foods involved may possibly explain the differences observed.

We have recently demonstrated that tryptic and peptic peptides derived from lupin protein are able to interfere with HMGCoAR activity, up-regulating the LDLR and sterol regulatory element binding proteins 2 (SREBP-2), and increasing the LDL-uptake in HepG2 cells (Lammi, Zanoni, Scigliuolo, D'Amato, & Arnoldi, 2014). A following paper (Lammi, Aiello, Vistoli, Zanoni, Arnoldi, Sambuy, et al., 2016) had the goal of assessing whether these lupin peptides are absorbed by human intestinal Caco-2 cells. Cells were differentiated for 15 days and transport experiments were performed by incubating each lupin peptide mixture from the apical side. After 4 h, basolateral solutions were collected and analysed by HPLC-Chip-MS/MS. Eleven tryptic and eight peptic peptides were identified in the basolateral samples and an *in vitro* assay showed that basolateral peptides maintain their capacity to inhibit HMGCoAR activity.

Considering that proteins are digested in the gastrointestinal system producing simple amino acids as well as short peptides, it is possible to formulate the hypothesis that the hypocholesterolaemic activity observed in the clinical studies is linked to specific peptides that are present in the lupin protein sequences, delivered by digestion, and absorbed.

A further result of this study is that only the lupin diet decreased in a significant way (-12.7%) the levels of PCSK9. This is in line with the change (-8.5%) observed in another clinical study performed on a lupin protein isolate (Lammi, Zanoni, Calabresi, & Arnoldi, 2016). Figure 3 shows the relationship between the change and the baseline values of PCSK9 in both groups. Whereas the data of the subjects on the milk diet appear to be scattered, there is a clear linear dependence in case of the lupin diet: this is one of the first dietary studies in which this kind of dependence has been observed. Recently, some of us have proposed that the activity of lupin protein on PCSK9 may be due to specific peptides produced by cleavage of lupin protein by digestion. The main experimental evidences of this hypothesis are the following: 1) peptic and tryptic peptides, obtained digesting a total protein extract from lupin seed with pepsin and trypsin, are able to inhibit *in vitro* the interaction of PCSK9 with the LDLR (Lammi, Zanoni, Calabresi, & Arnoldi, 2016); 2) some of these peptides are absorbed in a Caco-2 model of the small intestine (Lammi, et al., 2016); 3) the absorbed peptides maintain their capacity to inhibit the PCSK9/LDLR protein-protein interaction (Lammi, Zanoni, Aiello, Arnoldi, & Grazioso, 2016).

Conclusion

The treatment of individuals with a moderate dyslipidaemia with a commercial lupin protein concentrate leads to a reduction of TC, LDL-C, non-HDL-C, PCSK9, and ApoB:PCSK9 ratio, although the observed changes were not significant in comparison with those induced by a lactose-free skimmed milk powder. These findings confirm data from other authors, indicative of improvement of the lipid profile, and are of interest in the general overview of lupin as a dietary component for reducing CV risk. Further studies would be necessary to understand in detail the effects of these treatments. In any case, the results of this study underline the importance of replacing at least in part animal with plant foods.

Funding

Research funded by the European Union Seventh Framework Program (FP7/2007-2013), under grant agreement PF7-SME-2011-GA-285819.

Acknowledgements

None

366 References

367 Arnoldi, A., Boschin, G., Zanoni, C., & Lammi, C. (2015). The health benefits of sweet lupin seed
368 flours and isolated proteins. *Journal of Functional Foods, 18*, 550-563.
369 Arnoldi, A., Zanoni, C., Lammi, C., & Boschin, G. (2015). The role of grain legumes in the
370 prevention of hypercholesterolemia and hypertension. *Critical Reviews in Plant Sciences,*
371 *34*(1-3), 144-168.
372 Bazzano, L. A., Thompson, A. M., Tees, M. T., Nguyen, C. H., & Winham, D. M. (2011). Non-soy
373 legume consumption lowers cholesterol levels: a meta-analysis of randomized controlled
374 trials. *Nutrition, Metabolism & Cardiovascular Diseases, 21*(2), 94-103.
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., & Arnoldi, A. (2011). Legumes are valuable sources of tocopherols. *Food Chemistry,*
379 *127*(3), 1199-1203.
380 Boschin, G., Scigliuolo, G. M., Resta, D., & Arnoldi, A. (2014). Optimization of the enzymatic
381 hydrolysis of lupin (*Lupinus*) proteins for producing ACE-inhibitory peptides. *Journal of*
382 *Agricultural and Food Chemistry, 62*(8), 1846-1851.
383 Bähr, M., Fechner, A., Kiehntopf, M., & Jahreis, G. (2015). Consuming a mixed diet enriched with
384 lupin protein beneficially affects plasma lipids in hypercholesterolemic subjects: a
385 randomized controlled trial. *Clinical Nutrition, 34*(1), 7-14.
386 Bähr, M., Fechner, A., Kramer, J., Kiehntopf, M., & Jahreis, G. (2013). Lupin protein positively
387 affects plasma LDL cholesterol and LDL:HDL cholesterol ratio in hypercholesterolemic
388 adults after four weeks of supplementation: a randomized, controlled crossover study.
389 *Nutrition Journal, 12*, 107.
390 Capraro, J., Magni, C., Faoro, F., Maffi, D., Scarafoni, A., Tedeschi, G., Maffioli, E., Parolari, A.,
391 Manzoni, C., Lovati, M. R., & Duranti, M. (2013). Internalisation and multiple
392 phosphorylation of gamma-Conglutin, the lupin seed glycaemia-lowering protein, in HepG2
393 cells. *Biochemical and Biophysical Research Communications, 437*(4), 648-652.
394 Dove, E. R., Mori, T. A., Chew, G. T., Barden, A. E., Woodman, R. J., Puddey, I. B., Sipsas, S., &
395 Hodgson, J. M. (2011). Lupin and soya reduce glycaemia acutely in type 2 diabetes. *British*
396 *Journal of Nutrition, 106*(7), 1045-1051.
397 Duranti, M., Consonni, A., Magni, C., Sessa, F., & Scarafoni, A. (2008). The major proteins of lupin
398 seed: characterisation and molecular properties for use as functional and nutraceutical
399 ingredients. *Trends in Food Science & Technology, 19*(12), 624-633.
400 Estruch, R., Ros, E., Salas-Salvado, J., Covas, M. I., Corella, D., Aros, F., Gomez-Gracia, E.,
401 Ruiz-Gutierrez, V., Fiol, M., Lapetra, J., Lamuela-Raventos, R. M., Serra-Majem, L., Pinto,
402 X., Basora, J., Munoz, M. A., Sorli, J. V., Martinez, J. A., Martinez-Gonzalez, M. A., &

Investigators, P. S. (2013). Primary prevention of cardiovascular disease with a Mediterranean diet. *New England Journal of Medicine, 368*(14), 1279-1290.

Fechner, A., Kiehntopf, M., & Jahreis, G. (2014). The formation of short-chain fatty acids is positively associated with the blood lipid-lowering effect of lupin kernel fiber in moderately hypercholesterolemic adults. *New England Journal of Medicine, 144*(5), 599-607.

Friedewald, W. T., Levy, R. I., & Fredrickson, D. S. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical Chemistry, 18*(6), 499-502.

Ha, V., Sievenpiper, J. L., de Souza, R. J., Jayalath, V. H., Mirrahimi, A., Agarwal, A., Chiavaroli, L., Mejia, S. B., Sacks, F. M., Di Buono, M., Bernstein, A. M., Leiter, L. A., Kris-Etherton, P. M., Vuksan, V., Bazinet, R. P., Josse, R. G., Beyene, J., Kendall, C. W. C., & Jenkins, D. J. A. (2014). Effect of dietary pulse intake on established therapeutic lipid targets for cardiovascular risk reduction: a systematic review and meta-analysis of randomized controlled trials. *Canadian Medical Association Journal, 186*(8), E252-E262.

Hall, R., Thomas, S., & Johnson, S. (2005). Australian sweet lupin flour addition reduces the glycaemic index of a white bread breakfast without affecting palatability in healthy human volunteers. *Asia Pacific Journal of Clinical Nutrition, 14*(1), 91-97.

Hall, R. S., Johnson, S. K., Baxter, A. L., & Ball, M. J. (2005). Lupin kernel fibre-enriched foods beneficially modify serum lipids in men. *Eur J Clin Nutr, 59*(3), 325-333.

Horton, J. D., Cohen, J. C., & Hobbs, H. H. (2007). Molecular biology of PCSK9: its role in LDL metabolism. *Trends in Biochemical Sciences, 32*(2), 71-77.

Jayalath, V. H., Souza, R. J., Sievenpiper, J. L., Ha, V., Chiavaroli, L., Mirrahimi, A., Di Buono, M., Bernstein, A. M., Leiter, L. A., Kris-Etherton, P. M., Vuksan, V., Beyene, J., Kendall, C. W., & Jenkins, D. J. (2014). Effect of Dietary Pulses on Blood Pressure: A Systematic Review and Meta-analysis of Controlled Feeding Trials. *American Journal of Hypertension, 27*(1), 56-64.

Keogh, J., Atkinson, F., Eisenhauer, B., Inamdar, A., & Brand-Miller, J. (2011). Food intake, postprandial glucose, insulin and subjective satiety responses to three different bread-based test meals. *Appetite, 57*(3), 707-710.

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

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

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

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

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

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

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

Lima-Cabello, E., Alche, V., Foley, R. C., Andrikopoulos, S., Morahan, G., Singh, K. B., Alche, J. D., & Jimenez-Lopez, J. C. (2017). Narrow-leafed lupin (*Lupinus angustifolius* L.) β-conglutin proteins modulate the insulin signaling pathway as potential type 2 diabetes treatment and inflammatory-related disease amelioration. *Molecular Nutrition & Food Research*. 61 (5), DOI 10.1002/mnfr.201600819

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

Magni, C., Sessa, F., Accardo, E., Vanoni, M., Morazzoni, P., Scarafoni, A., & Duranti, M. (2004). Conglutin gamma, a lupin seed protein, binds insulin in vitro and reduces plasma glucose levels of hyperglycemic rats. *Journal of Nutritional Biochemistry, 15*(11), 646-650.

Matthews, D., Hosker, J., Rudenski, A., Naylor, B., Treacher, D., & Turner, R. (1985). Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia, 28*(7), 412-419.

Ruscica, M., Macchi, C., Gandini, S., Morlotti, B., Erzegovesi, S., Bellodi, L., & Magni, P. (2016). Free and bound plasma leptin in anorexia nervosa patients during a refeeding program. *Endocrine, 51*(2), 380-383.

Sankoh, A. J., D'Agostino, R. B., & Huque, M. F. (2003). Efficacy endpoint selection and multiplicity adjustment methods in clinical trials with inherent multiple endpoint issues. *Statistics in Medicine, 22*(20), 3133-3150.

476 Siger, A., Czubinski, J., Kachlicki, P., Dwiecki, K., Lampart-Szczapa, E., & Nogala-Kalucka, M.
477 (2012). Antioxidant activity and phenolic content in three lupin species. *Journal of Food*
478 *Composition and Analysis, 25*(2), 190-197.
479 Sirtori, C. R., Triolo, M., Bosisio, R., Bondioli, A., Calabresi, L., De Vergori, V., Gomaraschi, M.,
480 Mombelli, G., Pazzucconi, F., Zacherl, C., & Arnoldi, A. (2012). Hypocholesterolaemic
481 effects of lupin protein and pea protein/fibre combinations in moderately
482 hypercholesterolaemic individuals. *British Journal of Nutrition, 107*(8), 1176-1183.
483 Sniderman, A., Williams, K., & de Graaf, J. (2010). Non-HDL C equals apolipoprotein B: except
484 when it does not! *Current Opinion in Lipidology, 21*(6), 518-524.
485 Spielmann, J., Shukla, A., Brandsch, C., Hirche, F., Stangl, G. I., & Eder, K. (2007). Dietary lupin
486 protein lowers triglyceride concentrations in liver and plasma in rats by reducing hepatic
487 gene expression of sterol regulatory element-binding protein-1c. *Annals of Nutrition and*
488 *Metabolism, 51*(4), 387-392.
489 Sujak, A., Kotlarz, A., & Strobel, W. (2006). Compositional and nutritional evaluation of several
490 lupin seeds. *Food Chemistry, 98*(4), 711-719.
491 Viguiliouk, E., Stewart, S. E., Jayalath, V. H., Ng, A. P., Mirrahimi, A., de Souza, R. J., Hanley, A.
492 J., Bazinet, R. P., Mejia, S. B., Leiter, L. A., Josse, R. G., Kendall, C. W. C., Jenkins, D. J.
493 A., & Sievenpiper, J. L. (2015). Effect of Replacing Animal Protein with Plant Protein on
494 Glycemic Control in Diabetes: A Systematic Review and Meta-Analysis of Randomized
495 Controlled Trials. *Nutrients, 7*(12), 9804-9824.
496 Weisse, K., Brandsch, C., Zernsdorf, B., Nembongwe, G. S. N., Hofmann, K., Eder, K., & Stangl, G.
497 I. (2010). Lupin protein compared to casein lowers the LDL cholesterol:HDL cholesterol-
498 ratio of hypercholesterolemic adults. *European Journal of Nutrition, 49*(2), 65-71.
499 Wu, J., & Ding, X. (2001). Hypotensive and physiological effect of angiotensin converting enzyme
500 inhibitory peptides derived from soy protein on spontaneously hypertensive rats. *Journal of*
501 *Agricultural and Food Chemistry, 49*(1), 501-506.
502
503
504

505 **Figure Legends**

506

507 **Figure 1.** Schematic representation of the trial design and schedule of assessments

508

509 **Figure 2.** CONSORT statement flow diagram

510

511 **Figure 3.** Correlation between PCSK9 reduction and baseline