UNIVERSITÀ DEGLI STUDI DI MILANO

Graduate School in Pharmacological Sciences

Department of Pharmacological and Biomolecular Sciences

PhD Course in Pharmacotoxicological, Pharmacognostic Sciences and Pharmacological Biotechnology

XXVII Ciclo

PhD THESIS

LEGUME PROTEINS FOR THE MANAGEMENT OF CHRONIC DISEASES: HYPERLIPIDEMIA AND DIABETES

BIO/14

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Anno Accademico 2013/2014

GRADUATE SCHOOL OF PHARMACEUTICAL SCIENCES
XXVII CICLES

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INTRODUCTION

Replacing of animal by plant protein in foods is currently an important topic of discussion due to the ecological and physiological benefits associated with vegetable sources of proteins. In view of the growing global population, as well as, the limited availability of agricultural lands, there is an urgent need for high quality proteins from sustainable plant sources such as legumes (e.g., soy, pea, and lupin). Animal proteins are expensive in terms of market price, land requirement and environmental impact, on the other hand, vegetarian sources of protein are almost always incomplete proteins not getting all nine essential amino acids from foods. However is established as eating a variety of vegetable source proteins one can obtain the complete range of amino acids and the recommended daily protein requirement. Vegetable proteins also supply essential fats, complex carbohydrates, and fibers. Ultimately vegetable proteins are economic and versatile alternative to animal proteins as functional ingredients in food formulations and among plant proteins, legumes represent, together with cereals, the main plant source of proteins in human diet.

1.1 Legumes Proteins

1.1.1 Nutritional importance of legumes

Legumes have played a vital part in many ancient civilizations. The use of legumes as a basic dietary staple can be traced back more than 20,000 years in some eastern cultures. Legumes are crop plants from the family *Fabaceae* (or *Leguminosae*). The key characteristic of legume plants is the ability, of nearly all its members, to fix atmospheric nitrogen to produce their own protein compounds thanks to the

symbiotic association with nitrogen-fixing bacteria (i.e. rhizobia) found in the root nodules.

Leguminous seeds are an important source of food proteins. The beneficial effect of dietary intake of legume seed are the basis of various health claims. Grain legumes are widely recognized as important sources of food and feed proteins. In many regions of the world, legume seeds are the unique supply of protein in the diet and very often they represent a necessary supplement to other protein sources. On the other hand in developed countries plant proteins can now be regarded as versatile functional ingredients or as biologically active components more than as essential nutrients. This evolution towards health and functionality is mainly driven by the demands of consumers and health professionals, such as the partial replacement of animal foods with legumes is claimed to improve overall nutritional status and, the needs of the food industry, respectively (Duranti M., 1997). Legume seeds accumulate large amounts of proteins during their development. They are stored in membrane bound organelles (protein bodies) in the cotyledonary parenchyma cells, survive desiccation on seed maturation and undergo hydrolysis at germination, thus providing ammonia and carbon skeletons to the developing seedlings. Seed proteins that behave in this way are termed 'storage proteins'. However, because of their insolubility in water and solubility in salt solutions, the storage proteins are also named globulins and the two terms are commonly used interchangeably. Besides storage proteins, legume seeds contain several comparatively minor proteins including trypsin inhibitors, lectins, lipoxygenase and urease, which are relevant to the nutritional quality of the seed.

Some of them, like urease from jack-bean, also seem to have adopted a storage role by virtue of their amount in the seed (Casey R., 1986).

1.1.2 Nutritional value of legume seed proteins

1.1.2.1 Nutritional Value

All legume seed proteins are relatively low in sulphur-containing amino acids and tryptophan, but the amounts of another essential amino acid, lysine, are much greater than in cereal grains (Ampe et al., 1986; Rockland L.B., 1981). Therefore, with respect to lysine and sulphur amino acid contents, cereal and legume proteins are nutritionally complementary. The degree of mutual supplementation may also depend, however, on the contents of second limiting amino acids, i.e. threonine in cereals and tryptophan in legumes (Duranti M., 1997).

Lupin flours can be an excellent choice for improving the nutritional value of food products. The high lysine, low methionine content complements that of wheat flour proteins, which are poor in lysine and relatively higher in the sulphur-containing amino acids. In lupins, the main limiting amino acids are methionine and cystine, followed by valine and then tryptophan. Since lupins are legumes, the lack of sulphur-containing amino acids is not surprising, valine seems to be adequate in L. albus (Aguilera J. M., 1978). Lupin protein isolates, prepared on a bench scale, have been shown to have good nutritional properties when supplemented with methionine or mixed with cereals (Ruiz, 1976). Studies have shown that lupin flour can be successfully incorporated into products at up to 20% inclusion, to produce products that rate higher in terms of color, texture, taste and overall acceptability

than the control (Kohajdová Z. ;Karovičová, 2011). A number of pasta products containing lupin flour are currently available on the domestic market (Kyle, 1994). Soybean flours are increasingly being used in many countries because they are a good source of vegetable proteins, with a low fat content. The nutritional value of the soybean is not the only factor enhancing its consumption, as it plays an important role in health (Jacques et al., 1992a, b). All nine of the essential amino acids required by humans can be found in the amino acid composition of soybean. A digestibility of 95–100% has been found in isolated soybean protein in evaluations of animals and humans (Jacques et al., 1992b). Moreover, the essential amino acid content in soybean exceeds the amino acid requirements of children and adults, which confirms the protein quality of this vegetable.

1.1.2.2 Legume seed antinutritional compounds

Legume seeds contain several antinutritional protein and non-protein compounds. The presence of anti-nutritional compounds in crop plants is the result of an evolutionary adaptation which enables the plant to survive and complete its life cycle under natural conditions, regardless of the negative consequences on the quality and safety of the food products. Indeed, due to their anti-nutritional or even toxic properties, various potentially harmful compounds have been shown to play a protective role against insects, fungi, predators and a number of stress conditions (Peumans and Vandamme, 1995).

1.1.2.3 Protein antinutritional compounds

To this class belong seed hydrolase inhibitors, which are important in determining the quality of legume seeds. Their antinutritional effect in the irreversible inhibition of various digestive enzyme is well documented (Leterme et al., 1992). If inactivate, the protein inhibitors may even play a positive nutritional role, due to their high content of sulfphur-containing amino acids relative to the majority of the seed proteins. The most important protein inhibitors are Trypsin Inhibitors (Tis) of both the Bowman-Birk type in *Pisum sativum* and Kunitz type in *Glicine max* and α-amylase inhibitors. It has been shown that the Tis, have been proven to act as protective agents against insect attack (Hilder et al., 1990; Johnson et al., 1989; Liener, 1986; Moreno et al., 1990). Seed lectins are sugar-binding proteins, as well as hemagglutinins, which are able to agglutinate red blood cells. Some lectins can cause agglutination of the red blood cells followed by hemolysis and, in extreme cases, death. Allergies to legume seeds are relatively uncommon in humans due to the low allergenic capacity of storage proteins (Lallès, 1996), but they could develop with increased consumption.

1.1.2.4 Non-Protein antinutritional compounds

Legume seeds contain a number of non-protein antinutritional compounds with significantly different structures and effects. To this class belong: alkaloids, phytic acid, phenolic compounds, saponins, vicine and convicine. Alkaloids limit the acceptance of various legume seeds, such as lupin, both for their strong bitter taste and toxicity (Cuadrado, 1992). Due to the water solubility of alkaloids and their

low size, it is possible to remove them from the seeds by soaking and cooking in water. A probable role of alkaloids can also be to provide the seeds pest protection. In addition, alkaloids extracted from lupin seeds can be used for pharmacological and other biomedical purposes. Phytic acid, is present in legume seeds, making up the major portion of the total phosphorous in the seed. Phytic acid is responsible for the reduction of the bioavailability of essential minerals, forming insoluble complexes which are less available for digestion and absorption in the small intestine. Phenolic compounds, such as tannins, can crosslink with proteins by reacting with lysine or methionine residues, making them unavailable during digestion (Davis, 1981). Vicine and convicine are confined to *Vicia* and are known to be responsible for a type of hemolytic anemia, known as favism. Legumes are well known inducers of intestinal gasses (flatulence), due to the presence of α-D-galactopyranosyl residues bound to the glucose moiety of sucrose. Animals and humans are not able to digest such oligosaccharides, because of the absence of α-galactosidase in their intestinal mucose, consequently the α−galactosides pass into the colon and are fermented by the intestinal bacteria with production of gas (Fleming, 1981). Saponins are a diverse group of compounds commonly found in legumes; their general structure consists of a steroid or triterpene group linked to one or more sugar molecules. The presences of both polar and non-polar groups provide saponins with strong surface-active properties, which are responsible for their adverse biological effects. A well-known toxic effect of saponins is their ability to lyse erythrocytes, as well as other cells, such as those found in the intestinal mucose, thus affecting nutrient absorption.

1.2 The seed proteins

Legume seeds accumulate large amounts of proteins during their development. Despite wide variation in their detailed structures, all seed storage proteins have a number of common properties. First, they are synthesized at high levels in specific tissues and at certain stages of development, in fact, their synthesis is regulated by nutrition, and they act as a sink for surplus nitrogen. However, most also contain cysteine and methionine, and adequate sulfur is therefore also required for their synthesis. Many seeds contain separate groups of storage proteins, some of which are rich in sulfur amino acids and others of which are poor in them. The presence of these groups may allow the plant to maintain high levels of storage protein synthesis despite variations in sulfur availability. Storage proteins are stored in membrane bound organelles (protein bodies) in the cotyledonary parenchyma cells, survive desiccation on seed maturation and undergo hydrolysis at germination, thus providing ammonia and carbon skeletons to the developing seedlings. Osborne (1924) classified them into groups on the basis of their extraction and solubility in water (albumins), dilute saline (globulins), alcohol-ether mixtures (prolamins), and dilute acid or alkali (glutelins). The major seed storage proteins include albumins, globulins, and prolamins.

1.2.1 Classification of seed storage proteins

Legume seeds accumulate proteins during their development, most are bereft of catalytic activity and play no structural role in the cotyledonary tissue. For the

insolubility in water and solubility in salt solutions, the storage proteins are also called globulins.

1.2.2 The globulins

The globulins are the most widely distributed group of storage proteins; they are present not only in dicots but also in monocots (including cereals and palms) and fern spores. They can be divided into two groups based on their sedimentation coefficients (SV20): the 7S vicilin-type globulins and the 11S legumin-type globulins. Both groups show considerable variation in their structures, which result partly from post-translational processing. In addition, both are deficient in cysteine and methionine, although 11S globulins generally contain slightly higher levels of these amino acids.

1.2.3 The 11S globulins

The 11S legumins are the major storage proteins not only in most legumes but also in many other dicots and some cereals (oats and rice). The mature proteins consist of six subunit pairs that interact with non covalent bond. Each of these subunit pairs consists in turn of an acidic subunit of MW, ~40,000 Da and a basic subunit of MW, ~20,000 Da, linked by a single disulfide bond. Each subunits pair is synthesized as a precursor protein that is proteolytically cleaved after disulfide bond formation. Legumins are not usually glycosylated, an exception being the 12S globulin of lupin (Duranti, 1988).

1.2.4 The 7S globulins

7S globulins are typically trimeric proteins of MW, ~150,000 to 190,000 Da that lack cysteine residues and hence cannot form disulfide bonds. Their detailed subunit compositions vary considerably, mainly because of differences in the extent of post-translational processing (proteolysis and glycosylation). For example, the vicilin subunits of pea are initially synthesized as groups of polypeptides of MW, ~47,000 and ~50,000 Da, but post-translational proteolysis and glycosylation then give rise to subunits with MW values between 12,500 and 33,000 Da. These subunits are difficult to purify and characterize, but molecular cloning allowed their origins and the sites of proteolytic cleavage and glycosylation to be identified. The globulins of soybean and common bean are extensively glycosylated, while in pea and the fava bean virtually no glycosylation occurs, but processing by proteolytic cleavage is common (Shewry P.R.; Napier, 1995).

1.3 Functional food: soybean and lupin proteins for hyperlipidemia and glucose control

1.3.1 Functional foods

A close relationship exists between diet and major diseases of the industrialized world such as hyperlipidemia, obesity, diabetes, metabolic syndrome, and tumor development.

However, the only dietary intervention or, alternatively, the use of specific drugs may not be sufficient to prevent and treat these diseases, because there may be

contraindications to their use. Therefore, is necessary to identify innovative approaches for their prevention and treatment involving the use of compounds effective but non-toxic, and possibly present in the foods themselves.

The functional foods are a large and heterogeneous group of food products, which are characterized by having specific beneficial properties. In fact, although there is still no universally accepted definition for the term "functional food", there is a general consensus that functional foods are special foods that can confer specific health effects to the diet beyond the simple nutrition value. It is extremely important to emphasize that the fundamental characteristic of a functional food is just in his nature to be a food and not a product in the form of pills, tablets or capsules. The market for functional foods, although still " niche" has increased rapidly over the past few years. The reasons for the growth of the market for functional foods lies in the increased awareness of the role of diet in maintaining a level of optimum health and prevention of specific diseases related to sedentary lifestyle and unbalanced diet (obesity, hypertension, osteoporosis, diabetes, cardiovascular diseases, etc.).

How easily derived, is rather difficult to establish a precise and universal definition of the term “functional food” given the large and diverse group of food products to which it refers. In other words, we cannot yet have an official definition, common to all states that produce and trade for functional foods.

Today we have experimental evidence that peptides from soybean and lupin can play function of nutraceutical compounds able to reduce plasma cholesterol, triglycerides and glucose concentrations. However, the molecular determinants

responsible for these activities and their mechanism/s of action are actually under study.

1.3.2 Biological activity of legume proteins and their impact on the nutraceutical and supplements industry

The business for nutraceutical ingredients in the world is worth € 50 billion and continues to grow (source Cordis - Information Service of the European Commission in the field of research and development for science). Currently the leader in the industry are the United States and Japan, with an ever-increasing progress of EU countries (eg; France). In this business field numerous products with hypocholesterolemic action (for example, products based on phytosterols) are present which base their mechanism of action on preventing the absorption of dietary cholesterol and the reabsorption of endogenous cholesterol in the gastrointestinal tract. The molecules that are discussed in this regard instead with their innovative mechanism of action (after confirmation following on human beings test), will be open to the sector of prevention, generating a strong impact on businesses as well as the health of the consumer.

1.3.3 Consumer Health care: cost, risk prevention and drug consumption

The diseases that have the greatest impact on the healthcare and pharmaceutical industries are: metabolic syndrome, coronary heart disease and tumors.

In the coming years, health care spending is set to increase, since there is a higher risk of developing cardiovascular disease. Dyslipidemia is an important

cardiovascular risk factor which can be modified through targeted actions for its prevention.

The cardiovascular system drugs are the drugs most commonly used (32.7%) with a coverage of 94% of the Italian health system (SSN), although there are regional variations. The National Report 2007 shows the expenditure per capita in Lombardia amounted to EUR 197.5.

Functional foods may be defined differently in function of the context in which they are considered: the public, the food industry or agriculture. The most commonly accepted definition is the one that treats them as "products formulated with natural chemical components with the function to provide health benefits, lowering the risk of some diseases, participate in specific biological processes or correct some diseases caused by nutritional deficiencies". Nutritional foods are often referred to as nutraceuticals. Some of them are similar to conventional foods, are used as components of a usual diet and are able to produce health benefits and/or reduction of chronic diseases. Others are obtained from the same foods but are marketed as dietary or medicinal formulations; they are also able to exercise beneficial physiological activity or protective activity against chronic diseases (Stephen, 1998).

Functional foods may be obtained in different ways, and today, the techniques used in this field are constantly changing. The most common modifications are designed to the production processes of the food which allow more retention of some components that would otherwise be lost, selection of plants which enable for obtaining of new cultivars with modified composition of the food product.

Application of genetic interventions that allow to obtain foods with characteristics, sometimes very specific depending on the requirements is the general improvement of the techniques of industrial production.

The beneficial effect of functional foods may be determined by the presence of components characterized by varied chemical nature including alkaloids, saponins, isoflavonoids, phytates and proteins of various nature. In the latter case, the biological activity may be due to peptides released from food proteins after enzymatic digestion. The hydrolysis can take place during the process of gastrointestinal digestion or result from processes of germination, fermentation (the proteolytic system of bacteria is able to contribute to the liberation of peptides) and food handling.

1.4 Soy protein

Evidence of the beneficial properties of legume seed proteins in the prevention and even therapy of various diseases, many of which typical of the affluent countries, is currently accumulating. In particular, pathologies such as diabetes, cancer, cardiovascular disease (CVD), hypertension and obesity appear to be involved. A health claim related to legume seed protein dietary intake as a mean to prevent heart attack and other CVD-related syndromes has already been published. More recently, other scientists' boards have claimed that "grain legumes effectively contribute to a balanced diet and can prevent widely diffused diseases, including type II diabetes and cardiovascular diseases" (Alissa, 2011)

Soybean storage proteins have already been shown to play a major role in the plasma lipid homeostasis control. This biological activity was the basis of a USA-Food and Drug Administration (USA-FDA) health claim in 1999. In particular, soy proteins have been defined by the USA-FDA as a powerful tool in reducing the risk of cardiovascular disease. A daily intake of 25 gr of soy protein may reduce the value of LDL cholesterol by 10-20% of in hypercholesterolemic patients (FDA, 1999)

Fig.1 Glycine max (soy)

1.4.1 Classification

The components present in greater quantities in soy are proteins, which on average constitute up to 40% of the total dry substance (Liu, 1997) .

Storage globulins comprise two main protein constituents in soybean seeds that can be differentiated by their sedimentation coefficients, the 7S protein or β-conglycinin and the 11S protein or glycinin. The 11S fraction is a non-glycosylated

protein characterized by 12 polypeptide chains divided into 6 basic and 6 acid subunits with an overall molecular weight around 350,000 Da. The fraction 7S, molecular weight around 150,000 Da, is a glycoprotein formed by three different subunits, α, α' and β, associated in various combinations. Within the protein, α, α' and β are present in ratio 2:1.7:1 and have molecular weights of 67, 71 and 50 kDa respectively. Taking advantage of the different isoelectric point its possible separate the two globulins.

1.4.2 β-Conglycinin

1.4.2.1 Different purified fractions of β-conglycinin

The proteins of the soybeans are represented by two major components, glycinin and β- conglycinin, which amounted to 40 % and 30 % compared to total protein (Utsumi, 1997a). In the literature there are references about α– , β– , τ–conglycinin. These terms were defined by Catsimpoolas and Ekenstam (1969), referring to the three distinctive separated cractions from a crude preparation of conglycinin. Following further studies Catsimpoolas states that in α-conglycinin has enzymatic activity typical of the 2S subunit. β- and τ-Conglycinin not have enzymatic activity and differ from the other according to the ability to polymerize reversibly at neutral pH following a decreasing ionic strength from 0.5 to 0.1 M. β-conglycinin has a coefficient of sedimentation of about 7S at high values of ionic strength and 9-10 S for smaller values of ionic strength; this property is not found for τ-conglycinin . Because of its high proportion in the 7S

fraction, β-conglycinin was studied more intensely than α- and τ-conglycinin (Liu, 1997).

1.4.2.2 The composition of β-conglycinin subunit

β-conglycinin has a trimeric structure analogous to that of other 7S globulins (Utsumi, 1997a) and is composed of three kinds of subunit namely α, α' and β. The α and α’ subunit of β-conglycinin are composed of the extension regions (α, 125 residues, α’, 141 residues) and by “core” region (418 residues for the α and α’) (Maruyama et al., 1998). β subunit consists only of the “core” region (416 residues), as presented in the **Figure 2**. All subunits are glycosylated (Utsumi, 1997b).

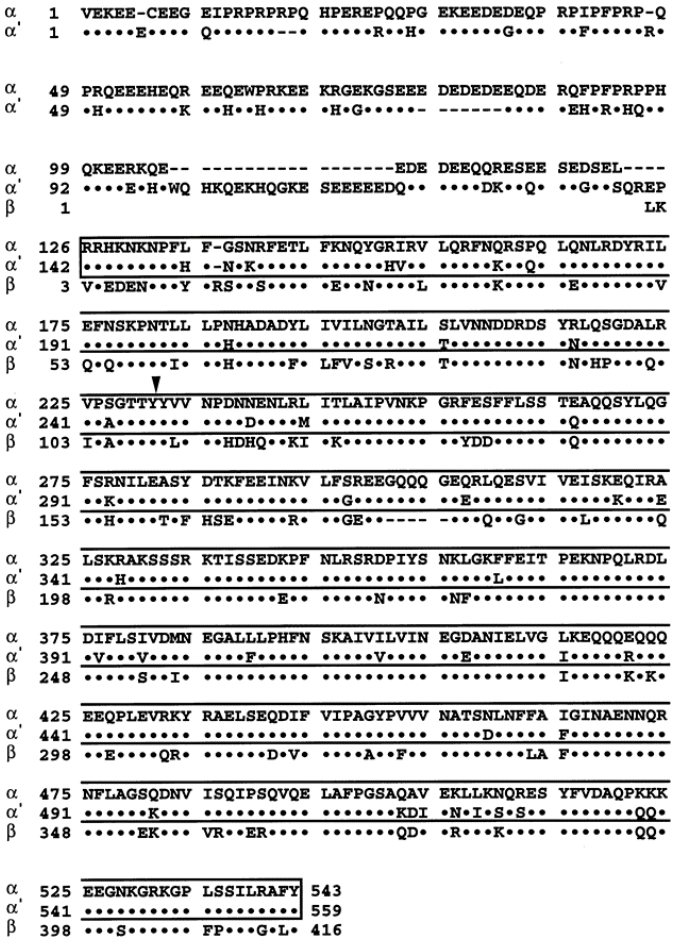


Fig.2

Fig.2: The amino acid sequences of the α' and β subunits are shown by dots (**.**) when they are identical to those of the α subunit and by letters when non-identical. Dashes (-) are introduced for maximal alignment of the sequences. Arrowhead indicates the position where the insert sequence reported by Doyle et al. occurred. The regions corresponding to the deletion mutants α_c (position 126-543 of α subunit) and α'_c (position 142-559 of α'subunit) are surrounded by a box.

1.4.2.3 Different molecular species of β-conglycinin

In the presence of denaturing agents such as urea and SDS, the proteins of leguminous 11S and 7S release their constituent polypeptide chains (Duranti M., 1997) that can be solved using a ion exchange chromatography (Liu, 1997).

These polypeptides are naturally heterogeneous, (Pusztai and Stewart, 1980) the heterogeneity is evident both in size and in the charge levels (Brown et al., 1981; Horstmann et al., 1993; Tucci et al., 1991) and resulting from a combination of two factors, the multi gene origin of each globulin and post-translational modifications (Wright, 1986). The relative contribution of these factors varies significantly between different genes and inside of the same genus. (Duranti M., 1997). It is evident that the mechanisms for the assembly of the native proteins 7S and 11S from their heterogeneous subunits are not clearly defined that the random association can occur in many cases (Gatehouse et al., 1981), this exponentially increases the number of molecular species in both classes. The situation is further complicated by the fact that many of these proteins, especially the 7S, are glycosylated in irregular way (Duranti M., 1997). Among the ten species of β-conglycinin theoretically possible, six (from B_1 to B_6) were isolated (Thanh, 1976) (**Table 1**).

B_1	1α' and 2β
B_2	1α and 2β
B_3	1α 1α' and 1β
B_4	2α and 1β
B_5	2α 1α'
B_6	2α

Table 1. Compositions in the subunits of the six molecular species of β–conglycinin

The six form of β-conglycinin are able to dimerize in a reversible manner to low levels of ionic force or to a pH between 4.8 and 11.0.

1.4.2.4 Structure-function relationships at the molecular level: the properties of β-conglycinin

The functional properties of the three subunits α, α', and β, of β–conglycinin have been described using recombinant proteins obtained by means of an expression system with *E. coli*. (Maruyama et al., 1999). The use of recombinant proteins is necessary for the presence of different molecular species of β–conglycinin in the seeds of *Glycine max*. The expression systems used by Utsumi and Maruyama have provided homogeneous molecular species of β–conglycinin and α and α' subunits without regions of the extension.

Solubility: The properties of gelation and the formation of foams and emulsions, are based on the solubility of the β–conglycinin in the solution medium. β–conglycinin soluble in water but not in saline solutions (Osborne, 1924). The isoelectric point of β–conglycinin is between 4.8 and 4 (Koshiyama, 1968) . Like the native protein, at a value of ionic strength equal to 0.5 M, the α and α'

recombinant subunits are soluble. To a value of 0.08 M ionic strength the two recombinants subunits are insoluble at pH value greater than 4.8. While the native protein is insoluble in a more restricted range of pH. It is evident that the oligosaccharide residues of the native protein and the of extension regions of the α and α' subunit will greatly affect the solubility.

Thermal stability: At pH 7.6 and 0.5 ionic strength of the thermal stability of the individual subunits β, α' and α are equal to 90.8 °C, 81.7 °C and 78.6 °C respectively. Under equal conditions, to the regions of the "core" of α and α' values are equal to 77.3 °C and 83.3 °C respectively (Maruyama et al., 1998); for the β-conglycinin native will have two different values, 79.0 °C and 83.1°C. These experimental results suggest that the oligosaccharide residues do not contribute to thermal stability of the native protein and that, the stability of the different molecular species is conferred by the subunit having the lower denaturation temperature between the subunits constituting the protein.

1.4.3 The cholesterol-lowering activity of β-conglycinin

The hypocholesterolemic property of purified proteins from soy has been clearly demonstrated in animal models and in humans (Carroll, 1991). The reduction of serum cholesterol is apparently associated with the activation of the receptors for the catabolism of the major transporters of cholesterol in plasma, the low density lipoproteins (Lovati et al., 1987; Sirtori et al., 1997). In this regard two hypotheses have been proposed; a direct effect of the protein (mainly 7S and 11S globulins or fragments thereof), or an effect of soy isoflavones: daidzein and geneistein. Both

of these assumptions are based on experimental findings and clinical outcomes (Anderson et al., 1995; Erdman, 1995).

1.4.3.1 Increased expression of the LDL receptor-cholesterol by β-conglycinin

The exposure of 7S soy globulin in HepG2 cells at different concentrations was associated with a dose-dependent increase in the uptake and degradation of LDL cholesterol (Lovati et al., 1992; Lovati et al., 1996). Further evidence showed that the expression of LDL receptors on blood type system cells, lymphocytes, increases following the consumption of soy protein (Lovati et al., 1987). Subsequently, the metabolic fate of the individual subunits of β-conglycinin was studied in HepG2 cells. In the first place it has been demonstrated that the 7S globulin interacts at the cellular level with the binding sites sensitive to heparin-sensitive binding sites with little competition from the more abundant proteins present in the incubation medium. A study based on laser fluorescence showed that the globulin 7S is picked up by HepG2 and subjected to proteolysis only in the extra-nuclear compartment(Manzoni et al., 2003). It was also observed that in HepG2 cells, after 96 hour exposure to 7S soy globulin the the α and α'subunits were not in their original primary structure while the β subunit resulted essentially indigested. Finally, while β subunit did not increase the expression of LDL receptor, α and α' subunits showed powerful LDL receptor induction (Lovati, 1998). In the system used to evaluate the cholesterol-lowering property of β-conglycinin, the soy isoflavones contenent was present in very low concentrations. In order to confirm that the LDL receptor activity of soy may reside in the protein

we explored, in the same experimental system, the LDL receptor of proteins isolated from a commercial isoflavones-free soy protein concentrate (Croksoy). Previous studies from our group had indicated indirectly, between the subunits constituting the 7S globulin (α, α' and β) that the α' subunit as likely responsible for increased LDL receptor activity. This hypothesis was formulated on the basis of the very clear lack of LDL receptor activation by a mutant soybean variety "Kebury" lacking the α' subunit of β-conglicinin. These conclusions have been confirmed directly thanks to the availability of purificated α'subunit separated from 7S globulin by affinity chromatography on a matrix containing Zn^{2+}. This technique was adopted starting from the observation that the α' contains, in the region of extension, a number of histidines higher than that of the α subunit which are able to bind several transition metals, including Zn^{2+}. The exposure of HepG2 cells in the condition previously described, to purified, α' subunit has determined the activation of LDL receptor and, in parallel the increase of LDL receptor promoter activity (Manzoni et al., 2003).

This confirms that the LDL receptor activating-cholesterol lowering properties of soy proteins, was due to the α' subunit of the 7S soy. These results suggest that the activation properties of the receptors for LDL cholesterol can be traced to a stretch of 37 amino acids that are found in the N-terminal region of the subunit α', but not α subunit (Wright, 1998). An additional hypotheses about the activity of α' lies in the possibility of an interaction with sterol regulatory element protein, such as SREBP-2, able to increase the expression of the LDL receptors (Manzoni et al., 2003).

1.4.3.2 Interaction of β-conglycinin with thiredoxin 1 (Trx1) and cyclophilin B (CyPB)

The interaction of the 7S globulin with cell membranes occurs through specific binding with proteins from the relatively low molecular weight (Lovati et al., 1996). Analysis of the N-terminal amino acid sequence of the proteins involved in these interactions have identified two specific protein ligands: Trx 1 and CYPB (Manzoni et al., 2003). The fundamental role of these two proteins in cellular homeostasis is widely recognized and confirmed by their relevant conservation during evolution (Arner and Holmgren, 2000). Cyclophilin B, belonging to the family of peptidyl-proline cis-trans isomerase (Bergsma et al., 1991), is a cyclosporine binding-protein (Cacalano et al., 1992); is primarily associated to the cell secretory pathway and is essential for the folding structure of proteins in vivo (Hoffmann and Schiene-Fischer, 2014; Steinmann et al., 1991)

In particular, CyPB is stored both in the endoplasmic reticulum both in complexes on the plasma membrane (Allain et al., 1994). Thioredoxin 1 is a small multifunctional protein, with a complex redox in the conserved sequence of the active site (-Cys-Gly-Pro-Cys) (Lillig and Holmgren, 2007) that plays a variety of roles from elimination of the reactive species of oxygen to the regulation of the proliferation of some cell types. It has been observed an increase of the expression of Trx and glutaredoxin 1 in samples of human coronary arteries as a result of intake of soy protein; this suggests a possible involvement of dithiol-disulfide oxidoreductase complex in the protection of human coronary (Okuda et al., 2001).

The involvement of these proteins main help to understand the complex mechanisms involved in reducing the progression of the atheromatous plaques observed after administration of a diet with soy proteins in an animal model of human soft plaques (Castiglioni et al., 2003)

Has been observed "*in vitro*" interaction of 7S globulin with Trx 1, this suggests that Trx 1 could to act as a carrier of the 7 S globulin in the cells, where it acts by regulating the expression of the receptors for LDL cholesterol (Manzoni et al., 2003). The interaction of 7S globulin with CyPB, however, suggests the involvement of this vegetable protein in the cholesterol transport between the endoplasmic reticulum and the cavity of the cell surface of the arteries.

1.5 Cholesterol and Atherosclerosis

1.5.1 The functions of cholesterol in the human body

Cholesterol is the typical sterol of animal cells. In vertebrates, the most amount of cholesterol is produced in the liver. A small part of cholesterol is incorporated into the membranes of the hepatocyte, while a large portion is exported under one of the two possible forms: bile acids or cholesterol esters. Bile acids and their salts are relatively hydrophilic derivatives of cholesterol synthesized in the liver to promote the digestion of lipids. Cholesterol esters are formed in the liver by the action of cholesterol acyl transferase. This enzyme catalyzes the transfer of a fatty acid from acetyl coenzyme A to the hydroxyl group of cholesterol, converting the cholesterol in a form even more hydrophobic. Cholesterol esters are stored in the liver or are transported in those tissues that use cholesterol.

All animal tissues during growth need cholesterol for the synthesis of membranes and some organ (for example, the adrenal cortex and the gonads) since it is used as precursor for the production of steroid hormones. Cholesterol is also the precursor of vitamin D. (Cox, 1993).

1.5.2 The transport of cholesterol

Cholesterol esters that must be transported to peripheral tissues, are secreted into the blood as constituents of lipoprotein complexes called very low density lipoproteins (VLDL).

During the movement of VLDL, triglycerides, and most of their apolipoprotein are removed in the capillaries of muscle and adipose tissue, converting sequentially VLDL into intermediate density lipoproteins (IDL), and then in low density lipoprotein (LDL). The peripheral tissues, normally, derive the majority of their exogenous cholesterol from LDL by receptor-mediated endocytosis. Inside the cell, cholesterol esters are hydrolyzed by a lysosomal lipase in free cholesterol, used by cells or which is esterified by acyl transferase cholesterol to be stored as droplets of esters from cholesterol. Cholesterol, cholesterol esters and triglycerides from food are transported in the blood by lipoprotein complexes synthesized in the intestine and are called chylomicrons. After removal of triglycerides from the peripheral tissues, chylomicrons residues bind to specific receptors of the liver cells and are internalized via receptor-mediated endocytosis in a manner similar to that of LDL. In the liver, the dietary cholesterol is used in the biosynthesis of bile acids or compacted into VLDL to be excreted. Cholesterol circulates constantly in

the blood, between the liver and the peripheral tissues. While LDL transport cholesterol from the liver, cholesterol is transported back to the liver by the high density lipoproteins (HDL) (Voet, 1992).When the amount of cholesterol synthesized and obtained from the diet exceeds the amount necessary for the synthesis of cells membranes, bile salts and sterols, the pathological accumulation of cholesterol in the blood vessels can lead to the formation of atherosclerotic plaques capable to obstruct the vessels. The atherosclerosis is related to high levels of cholesterol in the blood and in particular to the levels of cholesterol bound to LDL; there is in fact, an indirect correlation between HDL levels and coronary damage (Cox, 1993).

According to the National Institute of Health deaths caused by atherosclerosis in Italy in 2008 amounted to 6808; 64% of these deaths belong to the female population. Cardiovascular diseases (CHD), the deadline of a slow process of atherosclerosis of the blood vessels are the leading cause of death in Western countries, especially the United States of America and northern Europe. Many factors contribute to their development, and between them a fundamental role is played by the recruitment of a diet rich in animal fats and low in protein of plant origin. It should be remembered that only 5% of the population atherosclerosis is genetic origin, while in the remaining 95% is related to etiology of eating habits and behavior of individuals, which are crucial in particular for plasma cholesterol levels. The increase in plasma lipids, particularly cholesterol and low density lipoproteins (LDL), is one of the key factors in the onset and development of atherosclerosis. Numerous studies conducted both in animal models and humans,

have shown how this increase is caused not only by the presence in the diet of cholesterol and saturated fatty acids, but also by proteins of animal origin.

1.5.3 The LDL receptor

The LDL-R gene provides instructions for making a protein called a low density lipoprotein receptor (Kong et al., 2006). This receptor binds to particles called low density lipoproteins (LDLs), which are the primary carriers of cholesterol in the blood (Jeon and Blacklow, 2005). Cholesterol is a waxy, fat-like substance that is produce in the body and obtained from foods that come from animals.

Low density lipoprotein receptors sit on the outer surface of many types of cells, were they pick up low density lipoprotein circulating in the bloodstream and transport them into the cell. Once inside the cell, the low density lipoprotein is broken down to release cholesterol. After low density lipoprotein receptors drop off their cargo, they are recycled back to the cell surface to pick up more low density lipoproteins (Goldstein and Brown, 2001).

Low density lipoprotein receptor plays a critical role in regulating the amount of cholesterol in the blood. They are particularly abundant in the liver, which is the organ responsible for removing most excess cholesterol from the body.

The LDL-derived cholesterol acts at several levels, including suppression of transcription of HMGCoA reductase gene though the sterol regulatory element binding proteins (SREBPs) (Osbornet, 2000) pathway and acceleration of the degradation of the enzyme protein and suppresses transcription of the LDL receptor gene. The LDL derived cholesterol also regulates other processes in a

coordinated action that stabilizes the cell's cholesterol contenent. It activates a cholesterol-esterifying enzyme, acyl CoA, cholesterol acyltransferase (ACAT) (Yamashita et al., 2014), so that excess cholesterol can be stored as cholesteryl ester droplets in the cytoplasm. The internalization of LDL is also regulated by PCSK9 protein (pro-protein convertase subtilisin/kexin type 9) (Rashid et al., 2005) whose function is to induce the degradation of the LDL-R on the cell surface. The sterol regulatory element binding proteins, are localized at the membrane of the smooth endoplasmic reticulum and regulate the expression of over 30 genes involved in lipid metabolism. SREBPs consist of about 150 amino acids organized into three domains: an NH_2 terminal domain, containing the binding site to the DNA, two hydrophobic trans-membrane segments and a COOH terminal domain, member of the link with SREBP cleavage-activating protein (SCAP), which acts as a sensor of the level of sterols. In the absence af sterols, the SCAP-SREBP escort him from the endoplasmatic reticulum (ER) to the Golgi apparatus, where they are located two different protease, SP1 (serine protease) and SP2 (zinc protease). The action of these two enzyme determines the release from the end of the $–NH_2$ terminal nuclear SREBP (n SREBP) a fragment between 60-70 kDa able to translocate to nucleus and interact with DNA. At nuclear level the nSREBP binds the sterol-responsive element (SRE)-1 of target genes involved in cholesterol homeostasis increasing their expression. When the cholesterol contenent in the cell increase, the SCAP changes in conformation, preventing the complex SCAP/SREBP moving to the Golgi apparatus to begin the sequence of events described above.

The number of low density lipoprotein receptors on the surface of the liver cells determines how quickly cholesterol (in form of low density lipoproteins) is removed from the bloodstream (Defesche, 2004). Mutation in the LDLR gene cause an inherited form of high cholesterol called familial hypercholesterolemia. More than 1.000 mutation have been identified in this gene. Some of these genetic changes reduce the number of low density lipoprotein receptors produced within cells. Other mutation distrupt the receptor's ability to remove low density lipoproteins from the blood. As a result, people with mutation in the LDLR gene have very high blood cholesterol levels. The excess of cholesterol circulating through the bloodstream, it is deposited abnormally in the tissues such as skin, tendons, and coronary arteries, inducing a great increase in the risk to have an heart attack (Ueda, 2005).

Most people with familial hypercholesterolemia inherit one altered copy of LDL-R gene from an affect parent and one normal copy of the gene from the other parent. These case are associate with an increased risk of early heart disease, typically beginning in a person's forties or fifties. Rarely, a person with familial hypercholesterolemia is born with two mutated copies of the LDL-R genes. This situation occurs when the person has two affect parents, each of whom passes on one altered copy of gene. The presence of two LDL-R mutations results in a more severe form of hypercholesterolemia that usually appears in childhood (Marais et al., 2004; van Aalst-Cohen et al., 2004)

1.6 Lupin Protein

Legume seeds are an abundant source of proteins and lupin is one of the richest. Lupin seed deserves largest interest as a result of its chemical composition and augmented availability in many countries in recent years. Lupin is a non-starch leguminous seed with a high protein content, almost as high as that of soybean (about 35% of the dry weight), and a relatively low oil content (Duranti et al., 2008).

Lupin is an economically and agriculturally valuable plant which is able to grow in many and different soils and climates. Interest in lupin production is increasing, due to its potential as a source of protein, or for pharmaceutical purposes, a green manure or, due to the high alkaloid content, as a natural component of plant pesticides (Sujak et al., 2006). Apart from the high protein content, lupin has a strong capability for nitrogen fixation and organic phosphorus release from soil and can be used in crop rotation during intensive grain production (Fan et al., 2002; Honeycutt, 1998). The best utilization of this plant can be extended to the production of protein concentrates, which, "when added to other food products or fodder", can enrich their nutritional values, thus giving functional food (Archer et al., 2004; Batterham et al., 1986; Dijkstra et al., 2003; Guillaume et al., 1987; Linnemann and Dijkstra, 2002). Lupin seeds may also be a potential source of alimentary cellulose for the production of dietetic food. Among the lupin seed species, the most cultivated, primarily in Australia, is blue lupin (*Lupinus angustifolius* L.), while the typical European and South American varieties are yellow (*Lupinus luteus* L.) and white (*Lupinus albus* L. **Fig.3**) lupins. Blue and

yellow lupin seeds are mostly used for feed, while the white lupins are primarily grown for food uses.

Fig. 3 (*Lupinus albus* L.) white lupin.

1.6.1 Classification

Most proteins of legume seeds are located in the storage vacuoles of the cotyledonary tissues and mostly, but not exclusively, belong to the family of the storage proteins which serve as nitrogen and carbon skeleton sources for the emerging plantlet (Duranti et al., 2008). White lupin seeds contain two classes of proteins, which according to Osborne's classification, correspond to the albumin and globulin fractions. Seeds of white lupin have a protein content ranging from 33% to 47%, according to genotype and location (Dervas et al., 1999). Oil content varies from 6% to 13% with a high concentration of polyunsaturated fatty acids (Huyghe, 1997). The presence of alkaloids proves to be non-toxic at low concentrations. Since most alkaloids of lupin are water-soluble, the alkaloid level

of lupin (0.5–4%) can be decreased to 0.04% by soaking in running water, brine or scalding. Also it has been possible to grow sweet genetic varieties with low alkaloid contents ranging from 0.008% to 0.012% (Allen, 1998) (Tsaliki et al., 1999; Vasilakis and Doxastakis, 1999). Globulins are the typical salt soluble storage proteins of the seeds. This definition may not fit all cases, since both the solubility and the storage role of a protein are not always unequivocally defined, but for the sake of simplicity we will refer to the main lupin seed proteins as globulins. The first separation of lupin seed globulins dates back to the pioneering work of Blagrove and Gillespie (1975). These Authors used a cellulose acetate electrophoresis technique to separate four main fractions in L. albus and L. angustifolius. These fractions were named α-, β-, γ, δ-conglutins on the basis of their electrophoretic mobility. Further separations included isoelectric focusing, which illustrated the acidic nature of α-, β-, δ-conglutins and the basic nature of γ-conglutin, and ion exchange chromatography which showed the extreme heterogeneity of the composition of these proteins (Casero, 1983; Duranti, 1981; Restani, 1981) as compared to other legume seed proteins.

1.6.2 γ-Conglutin

1.6.2.1 Molecular characteristics

γ-Conglutin is an unusual basic 7S protein, which is equally soluble in water and salt solutions. This protein represent 4-5 % of total proteins in mature lupin seeds (Duranti, 1981). At neutral pH, the prevalent form of γ-conglutin is a tetramer (Duranti et al., 2000), or a hexamer, according to other authors (Blagrove &

Gillespie, 1975). At acidic pH, the oligomer dissociates to a monomer of about 50 kDa, with a quick transition around pH 5.0 (Duranti, 1986a, b). Each monomer is in turn composed of two disulphide linked subunits of 17 and 29 kDa (Restani, 1981). Both subunits are heterogeneous as a probable result of post-translational proteolysis. However, heterogeneity is much less pronounced than in α- and β-conglutins. Endogenous proteolysis converts the pro-polypeptide into the two mature subunits, as described for L. angustifolius γ-conglutin (Ilgoutz et al., 1997). However, the precise site of cleavage has not been identified yet, as trimming of the terminal regions of both subunits is likely to occur (Ilgoutz et al., 1997). Recently, four large and five small chains have been identified by proteomic analysis (Magni et al., 2007). The 29 kDa subunit is glycosylated with covalently linked mannose and glucosamine units. The corresponding glycosylation consensus sequence is present in both gene and resides in a cysteine-rich stretch in proximity to the N-terminal region. Two complete deduced amino acid sequences of γ-conglutin have been deposited. However, only the product of one gene (Q9FEX1_LUPAL) has been detected in the mature dry seeds (Scarafoni et al., 2001). Lupin γ-conglutin is located in the protein bodies of developing lupin seeds (Pernollet, 1978; Shewry, 1995; Weber, 1980). However, the protein has also been detected in the extracellular apoplastic regions of germinating lupin cotyledons (Duranti, 1994). The unusual stability of γ-conglutin during seed germination and in *"in vitro"* tests with various proteolytic enzymes (Duranti et al., 1995) strongly suggests that it is not a storage protein. On the other hand, if the denatured protein is submitted to proteolytic attack, degradation by trypsin and other enzymes is

complete. This suggests an ‘‘all or none’’ cleavage mechanism of γ-conglutin (Duranti, unpublished data). Whether this feature reflects some functionality of the protein has yet to be established. The unusual extra-vacuolar location of γ-conglutin adds further evidence that this protein probably plays a non storage role (Citharel, 1988; Duranti et al., 1991; Duranti, 1994). Intense research activity of our group is devoted to elucidating the physiological role of this protein. γ-Conglutin displays further unique properties of its own: it binds divalent metal ions, especially Zn^{++} and Ni^{++} (Duranti et al., 2001) which have been shown to promote the refolding of the denatured protein (Duranti et al., 2002) and, independently, insulin with a Kd of 10^{-5} M over a wide range of pH values (Magni et al., 2004).

1.6.2.2 The glucose lowering activity of γ–conglutin

Lupin γ-conglutin is a protein known to elicit a significant glucose decrease response. Although the mechanism of action of this dietary protein is still far from being understood, these experimental findings represent the rationale behind the attribution of anti-diabetic properties to lupin seeds, as claimed by traditional pharmacopoeia.

Some years ago, the ability of this protein to lower plasma glucose concentrations upon glucose overload in mice was first shown (Magni et al., 2004). Since then, experimental evidences on the peculiar biological effects of γ-conglutin have accumulated. In particular, the effect of γ-conglutin on the activation of

differentiating myocyte signaling pathway closely resembled that of insulin (Terruzzi et al., 2011).

As a matter of facts, γ-conglutin cell stimulation resulted in the persistent activation of protein synthetic pathway kinases, and increased glucose transport, GLUT4 translocation, as well as muscle-specific gene transcription regulation. Further studies on the oral administration of γ-conglutin to animal models and healthy humans confirmed its remarkable capacity of decreasing glycaemia (Bertoglio et al., 2011). In a study aimed at identifying the metabolic fate of the protein, *"in vitro"* and *"ex vivo"* approaches showed that the protein can be transcytosed through a Ca-Co2 cell monolayer and cross the intestinal barrier in an intact form (Capraro et al., 2011). Other functional *"in vitro"* studies showed the peculiar resistance of this protein to a number of proteolytic enzymes (Capraro et al., 2009). This feature supports the hypothesis that at least part of the protein may reach the intestinal lumen in an active form and thus exert systemic biological activity (Capraro et al., 2011).

γ-Conglutin was found to interact "*in vitro*" with insulin with a Kd around 7 x 10^{-5} M and, most importantly, to significantly reduce plasma glucose in rodents (Magni et al., 2004). Since insulin-binding to its own receptor causes a series of phosphorylation/dephosphorylation reactions, which lead the insulin signal from the receptor to the final metabolic and myogenic pathway. It was hypothesize, in fact, that the effect of γ-conglutin on blood glucose is due to an insulin-mimetic effect of the protein at the level of the intracellular pathway of the insulin receptor/IRS-1/PI-3-kinase, eventually leading to the recruitment and

translocation of GLUT4. Secondly, as insulin promotes muscle protein synthesis induced muscle anabolism, it was hypothesize the activation by γ-conglutin.

1.7 Diabetes Mellitus

On September 14, 2011, the International Diabetes Federation announced that 336 million people worldwide now have type 2 diabetes, and that the disease is responsible for 4.6 million deaths each year, or one death every seven seconds. It affects 12% of US adults and >25% of those over the age of 65. Diabetes is no longer restricted to the Western world, and the greatest increases in disease incidence in the next few decades are expected to be in China and India. These prevision serve to emphasize that there is currently a fast-growing diabetes pandemic. This is a major healthcare problem because diabetes increases the risk of heart disease, stroke, and microvascular complications such as blindness, renal failure, and peripheral neuropathy. Consequently, it places a severe economic burden on governments and individuals: the cost of diabetes and its complications amounts to $ 612 million per day in the USA alone. Diabetes mellitus is a chronic illness that requires continuing medical care and ongoing patient self-management education and support to prevent acute complications and to reduce the risk of long-term complications. Diabetes care is complex and requires multifactorial risk reduction strategies beyond glycemic control.

The classification of diabetes includes four clinical classes:

- Type 1 diabetes (results from β-cell destruction, usually leading to absolute insulin deficiency)

- Type 2 diabetes (results from a progressive insulin secretory defect on the background of insulin resistance)
- Other specific types of diabetes due to other causes, e.g., genetic defects in β-cell function, genetic defects in insulin action, diseases of the exocrine pancreas (such as cystic fibrosis), and drug- or chemical-induced (such as in the treatment of HIV/AIDS or after organ transplantation)
- Gestational diabetes mellitus (GDM) (diabetes diagnosed during pregnancy that is not clearly overt diabetes)

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels.

More pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia

For more time, the diagnosis of diabetes was based on plasma glucose criteria, either the fasting plasma glucose (FPG) or the 2 hours value in the 75 gr oral glucose tolerance test (OGTT) (Association, January 2010) . People with diabetes should receive medical care from a team that may include physicians, nurse practitioners, physician's assistants, nurses, dietitians, pharmacists, and mental health professionals with expertise and a special interest in diabetes. It is essential in this collaborative and integrated team approach that individuals with diabetes assume an active role in their care.

1.7.1 Insulin

Insulin is known as potent anabolic hormone and is essential for appropriate tissue development, growth, and maintenance of whole-body glucose homeostasis. Insulin is secreted by the β cells of the pancreatic islets of Langerhans in response to increased circulating levels of glucose and amino acids after a meal. This hormone regulates glucose homeostasis at many sites, reducing hepatic glucose output (via decreased gluconeogenesis and glycogenolysis) and increasing the rate of glucose uptake, primarily into striated muscle and adipose tissue. In muscle and fat cells, the clearance of circulating glucose depends on the insulin-stimulated translocation of the glucose transporter GLUT4 isoform to the cell surface. Insulin also profoundly affects lipid metabolism, increasing lipid synthesis in liver and fat cells, and attenuating fatty acid release from triglycerides in fat and muscle. Insulin resistance occurs when normal circulating concentrations of the hormone are

insufficient to regulate these processes appropriately. Thus, by definition, insulin resistance is a defect in signal transduction.

1.7.2 The insulin receptor

The insulin receptor consists of two α subunits and two β subunits that are disulfide linked into a $\alpha_2\beta_2$ heterotetrameric complex. Insulin binds to the extracellular α subunits, transmitting a signal across the plasma membrane that activates the intracellular tyrosine kinase domain of the β subunit. The insulin receptor (IR) then undergoes a series of intramolecular transphosphorylation reactions in which one β subunit phosphorylates its adjacent partner on specific tyrosine residues (Pessin and Saltiel, 2000).

1.7.3 Insulin signaling pathway

Following binding of insulin to the extracellular portion of the IR, the second messenger system involved in insulin signaling diverges into separate pathways that regulate distinct biological effects. These specific second messenger proteins are also employed in mediating the effects of a variety of other hormones. Thus, a specific and coordinated cellular response to insulin stimulation requires the integration of a full network of signaling processes (Taniguchi et al., 2006). The substrate tyrosine kinase activity of the IR initiates a cascade of cellular phosphorylation reactions that regulate protein interactions and enzymatic activities. Substrates of the IR include the insulin receptor substrates IRS-1 and IRS-2, as well as Shc (Src homology collagen) and APS (adaptor protein with a

PH and SH2 domain) (Taniguchi et al., 2006). These phosphorylated substrates then serve as docking molecules that bind to and activate cellular kinases, initiating the divergent signaling pathways that mediate cellular insulin action. The pathway of insulin signaling is that the stimulation of glucose transport and most other metabolic effects of insulin are regulated by activation of the phosphatidylinositol 3- kinase (PI3K) pathway, facilitated by binding of the regulatory subunit of PI3K to phosphotyrosine residues on IRS-1. The ultimate effector system for regulating glucose disposal is the translocation of GLUT4-containing vesicles to the plasma membrane.

1.7.4 The association of PI-3K with IRS proteins and activation of Akt/PKB

The predominant function of the insulin receptor substrate (IRS) proteins seems to be the activation and/or regulation through many pathways of the phosphoinositide 3-kinase (PI3K). PI3K is a heterodimer with two separate subunit, regulatory and catalytic subunits (p85 and p110 respectively). In its resting state, PI3K is present as an inactive p85-p110 complex. After the activation of a receptor tyrosine kinase (RTK), due to the phosphorylation of its cytoplasmic tail, the p85-p110 complex is recruited to the receptor by interaction of an SH2 domain on p85 with phosphotyrosine residues on the RTK (Fruman et al., 1998). This interaction is believed to release the inhibitory effects of p85 on the catalytic p110 (Yu et al., 1998). P110 is able to interact with its lipid substrates, the phosphatidylinositol, and convert PIP2 to PIP3. Recruitment of PI3K by RTK also puts p110 in close proximity to these lipid substrates residing in the plasma membrane. The major

exception to this reaction is that PI3K can be activated by signal adapter proteins, such as IRS-1 and IRS-2, rather than by RTKs themselves (White, 2002). It is important to note, IRS-mediated activation of PI3K requires that phosphorylated YMXM motifs occupy both SH2 domains within p85. Generation of PIP3 by activated PI3K near the plasma membrane results in interaction with, and subsequent phosphorylation of, its primary substrate Akt. Akt requires, for its full activation, the phosphorylation of the residue of Ser^{473} and the residue Thr^{308} and interacts with the phosphatidynol3, 4biphospate ($PI_{3,4}P_2$), 4,5 trisphosphate ($PI_{3,4,5}P_3$), produced by PI3K. These phospholipids are essential for its activation: their interaction with the PH domain of Akt modifies the conformation and makes that Akt is recruited to the cell membrane, where the protein complex Rictor-mTOR (Target of its mammalian rapamycin) phosphorylates at ser^{473} level and it facilitates the subsequent phosphorylation, at the level of $treonina^{308}$, by PDK1 (3-phosphoinositide dependent protein kinase-1) (Sarbassov et al., 2005). One of the major substrates of Akt is glycogen synthase kinase-3 (GSK-3), which participates in the regulation of glucose homeostasis regulating the synthesis of glycogen. The levels of glycogen (synthesized mainly in the liver and muscle) are tightly regulated by the action of the enzyme glycogen synthase (GS), which synthesizes and its antagonist, the enzyme glycogen phosphorylase. In basal conditions, GSK-3 inhibits glycogen synthesis by blocking the activity of GS inhibitory phosphorylation pathway. The activation of the insulin pathway leads to IRSs/PI3K/Akt inhibitory phosphorylation of GSK-3 at residues Ser regulators 21

(on α isoform of GSK-3) and Ser 9 (on β isoform of GSK-3). This activates the GS and the glycogen synthesis (Lee and Kim, 2007).

1.7.5 Insulin resistance

Tissue insulin resistance predisposes an individual to significant health risks in addition to its role in the pathogenesis of type 2 diabetes mellitus (Groop, 1999). In humans, insulin-stimulated muscle glucose disposal rates vary widely across the normal population, and the insulin-resistant state refers to individuals in the lower end of a normal distribution, rather than a discreet pathological condition. In "*in vivo"*, muscle insulin sensitivity is regulated on a long-term basis by factors such as obesity, and is altered in a more rapid manner by changes in dietary habits and physical activity (Barnard, 1992).Though more difficult to quantify, there is also evidence for genetic or intrinsic differences in muscle insulin sensitivity (Groop, 1999). In any individual, therefore, the degree of insulin sensitivity is determined by the interaction of numerous factors, both genetic and environmental. Both acute and chronic regulation of tissue insulin sensitivity can occur via multiple pathway through which the cellular mediators of insulin signaling can be altered by protein interactions and other modifications.

2

AIM

Food proteins are considered as source of bioactive peptides and amino acids that can exert physiological functions to promote health and prevent chronic diseases, such as lipid disorders (Lovati, 2006) diabetes (Lovati et al., 2012), hypertension (Matoba et al., 2001) cancer (Chiesa et al., 2008; De Mejia, 2010 ; Galvez et al., 2001) and obesity (Martinez-Villaluenga et al., 2010) which are typical of industrialized societies. It is known that during gastrointestinal digestion or food processing, these peptides are released from the parent protein and act as regulatory compounds with hormone-like activities (Martinez-Villaluenga et al., 2010). Soybean (*Glycine max*) and white lupin (*Lupinus angustifolium*) comprise the most widely grown legume crops in the world. In addition to being an invaluable source of oil and protein for food and feed, many papers from our group pointed out the positive effect of soybean and white lupin proteins on lipid and glucose metabolism. Despite intensive studies in this area, the protein molecules directly responsible for the observed effect have not been unequivocally identified nor with regard to the protein of soya nor with regard to those of white lupin.

The aim of the present doctorate thesis is to evaluate in "*in vitro"* and "*in vivo"* experiments the ability of polypeptides from soybean and from white lupin to interact with the molecular mechanisms involved in the regulation of plasma and tissue lipids as well as in the glucose homeostasis control.

For soybean proteins, the α' subunit of the soybean 7S globulin, the so called β-conglycinin, was shown to have a key role in the up-regulation of high affinity-

LDL receptors, in "*in vivo"* and "*in vitro"* systems (Duranti et al., 2004; Maruyama et al., 1999) suggesting that biologically active peptides, capable of modulating lipid homeostasis, are likely to be produced by cell and gastrointestinal enzymes. The native 7S globulin is a randomly assorted hetero-trimer, α, α' and β subunits , with molecular mass of 150-200 kDa (Manzoni et al., 2003). The α and α' chain consist of a core regions with a high degree of homology (87%) and extension regions (α, 125 residues; α', 141 residues) exhibiting lower homology (57%), whereas β subunit consists of only a core region that has homology with α and α' core regions (75 and 72 %, respectively) (Lovati et al., 2000). Although, the results from "*in vitro"* and "*in vivo"* experiments justify the lipid-lowering activity of α' subunit, a related concern is the biological fate of this polypeptide "*in vivo"* because it seems unlikely that it crosses the intestinal barrier with no modification. We hypothesized, in fact, that peptides, deriving from the activity of gastric/intestinal enzymes on soybean proteins, might be absorbed by the enterocytes and reach the liver through the blood stream, where they elicit the biological effect (Lovati et al., 2000; Lovati, 1998; Manzoni et al., 2003) . Our research group has been following two different approaches to identify the active peptide/s involved in the lipid regulation. The first one has been to reduce the length of the polypeptide chain of α' subunit by a biotechnological process obtaining an extension form of α' chain, roughly covering one third of the full-length polypeptide from N-terminus, which has been shown to up-regulate the LDL-R in Hep G2 cells (Consonni et al., 2011). The second approach has been to

make a screening of peptides with amino acid sequences occurring in α', α and β subunits of soy β-conglycinin, and test their biological effect "*in vitro"*. These peptides have been evaluated for their effect on the expression of LDL receptor, sterol regulatory element binding protein-2 (SREBP-2) and hydroxymethylglutaril coenzyme A reductase (HMGCoA red) in HepG2 cells. Moreover, the peptide which resulted more promising among the different compounds has been tested in "*in vivo"* experiment in order to evaluate its potential on lipid homeostasis in a rat model of human hypercholesterolemia.

Recently new data on the potential of γ-conglutin, a seed glycoprotein with a molecular weight of 47 kDa, on the glucose metabolism are emerging. Since the *"in vitro"* interaction of γ-conglutin with mammalian insulin has been described in the present study the effect of an oral dose of this protein was studied in an animal model of diabetes in order to evaluate its ability to modulate the plasma glucose rise. Moreover in *"in vitro"* experiments the ability of γ-conglutin to interact with cell compartment and to interfere in the insulin pathway has been followed in order to ascertain whether the protein was characterized by insulin-like property.

Although, the data presented in this report need to be confirmed by human studies the potential of peptides from α' subunit of soybean as well as that of lupin seed γ–conglutin to control plasma lipids and glycaemia could be considered before developing new therapeutic strategies for the prevention or regression of lipid and glucose metabolism modifications.

3

MATHERIALS

AND METHODS

3.1 Materials

3.1.1 Isolation of γ-conglutin

Dry mature seeds of white lupin (L. *albus* L, var. Multitalia) were kindly provided by Dr Massimo Fagnano, University of Naples, Naples, Italy. Type F, a γ-conglutin-enriched lupin protein isolate, prepared as described in Bez et al (Bez, 2005), was kindly supplied by Fraunhofer Institute (Munich, Germany). This preparation, the composition of which is detailed in the cited reference, contained about 80% protein and was used as such in the "*in vivo"* experiments. A laboratory-scale purified γ-conglutin was utilized in the "*in vitro*" assay. The procedure for γ-conglutin purification, as described by Duranti et al. (Duranti et al., 1994) was slightly modified to improve the homogeneity of the preparation. In particular, after conventional chromatographic steps, which included gel permeation chromatography, ion exchange chromatography on both Whatman DE52 diethylaminoethyl-cellulose and carboxymethylcellulose, a further step of metal affinity chromatography was added. For this purpose, the protein solution was loaded onto a nickel column (NiNTA-Agarose; Qiagen, Milan, Italy) equilibrated in 50 mM-Tris–HCl, pH 7.4, containing 0.5 M NaCl. The protein bound to the matrix was subsequently eluted with 50 mM sodium acetate, pH 4.5, containing 0.5 M NaCl. The purified protein was desalted by dialysis against MilliQ water and freeze dried. For the estimation of purified γ-conglutin concentrations, optical measurements at 280 nm were made. The extinction coefficient of 0.733 for a solution of 1 mg/ml was used (Capraro et al., 2010). SDS-PAGE was carried out on 12% polyacrylamide gels, according to Laemmli

(Laemmli, 1970) under reducing conditions using a mini-Protean II cell (Bio-Rad, Milan, Italy). Samples were heated at 100 °C for 5 min before loading. The gels were stained with Coomassie Blue. Densitometric scanning of the gel was carried out by ImageMaster 1D software (Amersham Pharmacia Biotech, Milan, Italy).

3.1.2 Isolation of soybean 7S globulin and α' subunit

For the isolation of the 7S globulin, a modification of a published procedure (Wu and 1999) was used. Our procedure consisted in the selective extraction of the 7S globulin with 7 mM $NaHSO_3$ at 4°C with stirring for 16 h. The flake weight to buffer volume ratio was 1:15. Centrifugation at 8000 xg for 1 h at the same temperature allowed the recovery of a supernatant, which consisted essentially of 7S globulin, and a pellet containing all insoluble materials, including the glycinin fraction and a residual amount of 7S globulin. The 7S globulin was recovered from the supernatant by 40% aqueous ethanol precipitation and centrifugation as above. The pellet was then freeze-dried. Isolation of the α' subunit*:* Urea (8 M) in 50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl (loading buffer) was used as a denaturing agent to dissociate the 7S globulin subunits. The resulting solution was applied to a MAC matrix coupled with Zn^{2+}, in a ratio of 30 g of 7S globulin/L of resin. The resin was thoroughly washed with the loading buffer to elute the unbound fraction. The bound fraction was subsequently eluted with 0.1 M imidazole in the loading buffer. The recovered 7S globulin α'subunit was then precipitated and washed with 40% aqueous ethanol before freeze drying.

This procedure was applied to 250 g of defatted soybean flakes to obtain 5 g of α' subunit. The whole procedure, has been patented (Bradford, 1976; Duranti, 2002). Total soybean proteins for SDS-PAGE analysis were directly extracted with sample denaturing buffer containing 3.75% SDS and 350 mM 2-mercaptoethanol. Protein concentrations were determined with the Bradford method (Bradford, 1976), using bovine serum albumin as the standard protein.

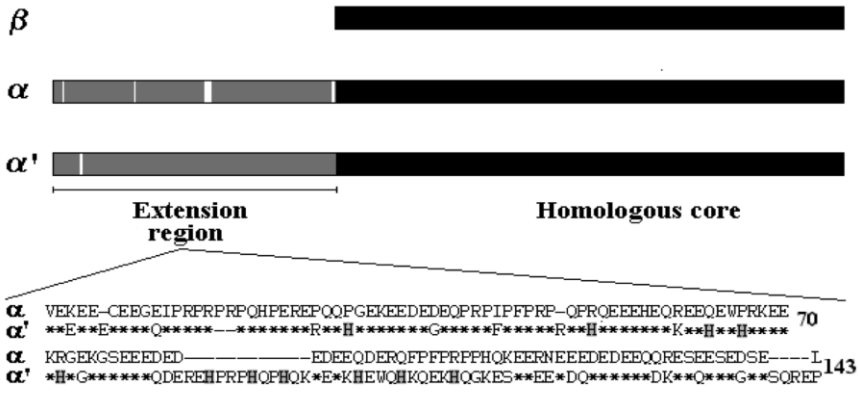


Figure.1: 7S soybean globulin subunits: homology grade

3.1.3 7S α' extension

An α'polypeptide consisting of 216 amino acid residues from the N-terminus, containing the extension region plus a stretch of the core region (Fig. 1) was cloned and over-expressed in *Pichia pastoris*. The yield of the recombinant polypeptide, which was termed α'E, was 8-fold greater than the truncated version previously obtained. The α' E polypeptide was purified by conventional biochemical techniques to make it available for biological assay. The recombinant polypeptide

was used in "*in vitro"* and "*in vivo*" experiments to evaluate its potential on lipid homeostasis**.**

3.2 Sinthetic soy peptides

From database searching (http://www.uniprot.org), we identified five peptides, belonging to α', α and β subunit of 7S soy (*Glycine max*) globulin with the aim to investigate the potential of soybean peptides on cholesterol homeostasis, and anti-inflammatory activities as well as on anti-adipogenic and anti-oxidant properties in different cell lines.

Synthetic peptides A and B (purity > 98%) were purchased by EZBiolab Inc., IN 46032, USA. Peptides C, D and E were custom synthesized by GenScript (Piscataway, NJ, USA) (Table 1).

Amino acid sequence of synthetic peptides from soybean β-conglycinin				
Peptide	*Sequence*	*MW*	*pI*	*Subunit*
A	SEEEEEDQ	994	3.4	α'-extension
B	QKEEEKHEWQ	1370	4.9	α'-extension
C	RKQEEDEDEEQQRE	1847	4.3	α'
D	EITPEKNPQLR	1324	6.2	α', β, α
E	KNPQLR	754	11.0	α', β, α

S= Ser; E= Glu; D= Asp; Q= Gln; K= Lys; H= His; W= Trp; R= Arg; I= Ile; T=Thr; N= Asn; P= Pro; L= Leu;

Table 1: Amino acid sequence of synthetic peptides from soybean β-conglycinin

3.2.1 NPDNDE synthetic peptide

A synthetic peptide corresponding to position 314-319 of α' chain 7S soy globulin was purchased by EZBiolab Inc as previously reported and used in *"in vitro"* and "*in vivo"* experiment to evaluate its potential on cholesterol homeostasis. The purity was greater than 98%.

3.3 Fluorescein isothiocyanate (FITC)-tagged proteins.

γ-Conglutin, was incubated overnight at 4°C in conjugation buffer (0.5 mM carbonate/bicarbonate, pH 9.5) in the presence of FITC (30 μg/mg protein) under stirring. Unreacted dye was removed using a PD 10 column by elution with PBS. The integrity of tagged proteins was verified by SDS gradient gel electrophoresis (7.5–17.5%).

3.4 Animal and Diet

Male Sprague–Dawley CD rats (Charles River Laboratories Italia Srl, Calco-Lecco, Italy), body weight (b.w.) 125–150 g (5-6 week), were housed in a room with controlled lighting (12 h/d), constant temperature (20-22 °C) and relative humidity (55–65%).

3.4.1 Hypercholesterolemic diet

After their arrival from shipment an adjustment period to the new home condition was allowed during which they were fed a standard diet (pelleted commercial non-

purified diet (4RF21-Mucedola, Italy). Rats were then divided into five groups of 9 rats on the basis of b.w. and transferred to a Nath's hypercholesterolemic (HC) diet (1 g/100 g cholesterol and 0.5 g/100 g cholic acid). To distinguish effects on plasma lipids as a function of altered food intake were included a group of rats pair-fed the control diet.

After 2 week of HC diet rats were allocated to treatment groups on the basis of b.w. and plasma lipids so that the distribution among the groups was similar.

3.4.2 Glucose overloading

Following a two day adaptation period, during which they were fed a standard diet (pelleted commercial non-purified diet - Mucedola 4RF21; Settimo Milanese, Milan, Italy), the animals were given 10% D-glucose in drinking-water for the entire experimental period. The rats had free access to water. The mean daily water intake was between 20 and 30 ml/rat, estimating an average daily intake of D-glucose about 2–3 g. After 1 week, the animals were divided in two groups of twelve rats according to their b.w. and plasma glucose concentrations, so that the distribution between the groups was similar.

3.5 Animal's treatment

All procedures involving animals and their care were conducted in accordance with institutional guidelines that are in compliance with national (D.L. no. 116, G.U. Suppl. 40, 18 February 1992, Circolare no. 8, G.U. July 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, 12

December 1987; Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health-NIH Publication no. 85-23, revised 1996).

3.5.1 γ-Conglutin treatment

The animals were daily treated (at 09.00 hours) by gavage for three weeks as follows: one group (D-glucose) received only the vehicle (1% carboxymethylcellulose), whereas the other group (D-glucose + γ-conglutin) received 100 mg/kg b.w. type F, corresponding to 28 mg/kg b.w. γ-conglutin in carboxymethylcellulose (CMC). A third group of rats received only the vehicle without D-glucose in drinking-water (controls). Food intake and b.w. were monitored weekly.

3.5.2 7S α' extension treatment

The animals were daily treated (at 09.00 hours) for four weeks by gavage as a suspension in a 1% (w/v) aqueous solution of CMC as follows: IDN 6442 20 mg/kg b.w. (15.42 mg of 7S-α' corresponding to 5.53 mg α'/kg b.w., IDN 6443 25 mg/kg and 50 mg/kg b.w. (5,65 mg /kg b.w. and 11,3 mg/kg b.w. of protein), clofibrate (Sigma-Aldrich, Milan, Italy) 200 mg/kg b.w.. Control groups on either the standard or the hypercholesterolemic diet received vehicle only. Food intake was monitored daily whereas variations in b.w. weekly.

3.5.3 NPDNDE treatment

The animals fed casein-cholesterol (HC) diet, divided into three groups (12 rats each) homogeneous for plasma lipids**,** were daily treated for three weeks (21 day) by gavage as a suspension of 1% (w/v) aqueous solution of CMC as follows: synthetic peptide NPDNDE 5 mg/Kg b.w., soybean 7S alfa'20 mg/kg b.w. or clofibrate (Sigma-Aldrich, Milan, Italy) 200 mg/kg b.w..

"In vivo" γ-Conglutin experiment

3.6 Glucose consumption

At the end of the experimental period, glucose loading was carried out on 10 h fasted animals. At time 0, each rat was given 2 g/kg b.w. D-glucose, administered orally. Blood samples were withdrawn from the tail vein of each rat under light diethyl ether anesthesia at 30, 60, 120 and 180 min following the carbohydrate load. Aliquots of serum were stored at – 20°C until assayed. Glucose was determined by enzymatic method (Sigma-Aldrich, Milan, Italy).

To estimate the degree of insulin resistance (IR), the homeostasis model assessment (HOMA) was used as an index of IR according to Midaoui & De Champlain (20). The HOMA-IR index was calculated using the following equation:

fasting insulin μU/ml x fasting glucose mmol/22.5

"In vivo" 7S α' experiment

3.7 Blood and liver collection

At the end of the experimental period, rats were anesthetized and whole blood was collected from the abdominal aorta in the presence of EDTA (1mg/ml). Plasma samples were kept at –20°C for subsequent determinations. Immediately after sacrifice the liver was collected, washed with ice-cold 150 mM NaCl, fast freezing in liquid nitrogen and placed at −80°C for long-term storage. A small portions of liver were quickly placed in RNAlater™ solution for tissue archiving without risk of RNA degradation. The Institutional guides for the care and use of laboratory animals were followed, and the experiments were supervised by the Laboratory Animal Welfare Service.

3.8 Lipoprotein isolation and analysis

Pool of plasma from three rats in the same experimental group were used for lipoproteins fractionation by sequential preparative ultracentrifugation. Ultracentrifugation was performed at 5°C in a Beckman Model L-5-50B ultracentrifuge (Beckman Instruments, Palo Alto, CA) with a 50 titanium rotor. Lipoproteins were separated into very low density lipoprotein (VLDL) by centrifugation for 18 h at 100,000 xg. For isolation of low density lipoprotein (LDL, 1.006<d<1.045 g/mL) and high density lipoprotein (HDL, 1.045<d<1.21 g/mL) the centrifugation was done at 100,000 xg for 20h and 24 h respectively. Isolated lipoprotein fractions were dialyzed against 0.02 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.01% Na_2EDTA and 0.02% NaN_3 (Havel et al.,

1955). Cholesterol and triglyceride concentrations in plasma and in lipoprotein fractions as well as glucose levels were determined by enzymatic methods (Horiba ABX S.A.S, Italy).

3.9 Liver lipids extraction.

Immediately after blood collection, livers were perfused in situ with ice-cold saline then removed and weighed. Liver from three rats for group were pooled and lipids were extracted with chloroform/methanol (2/1, v/v) according to the method described by Folch (Folch et al., 1957). Duplicate pieces of 0.25 g frozen liver were cut up and homogenized with 10 mL of a 2:1 chloroform/methanol solution with Ultra-Turrax T25. The homogenate was vortexed for 15 s every 5 min for a total of 30 min. Then filtered and the filtrate washed with 5 mL chloroform/methanol solution. Subsequently, 0.84 g/L KCl was added, followed by further vortexing and subsequent resting for 30 min. After removal of the top (water) layer, the bottom layer was evaporated to dryness by flushing with nitrogen.

3.10 Cholesterol and triglycerides liver measurement

Cholesterol and triglycerides content in the lipid residue was measured by enzymatic color method, after total lipids were dissolved in Triton X-100 (Carr et al., 1993). Results are expressed as milligrams triglyceride or cholesterol per gram liver (wet wt).

3.11 RT-PCR experiments.

Total RNA was extracted from frozen liver using RNeasy Mini Kit (QIAGEN S.r.l., Italy) according to instruction of the manufacturer and resuspended in 40 µl of water. All RNA samples were examined as to their concentration, purity and integrity based on absorbance ratio at 260/280 nm and at 230/260 nm. Overall sample integrity was confirmed by agarose gel electrophoresis, showing sharp and intense 18S and 28S ribosomal RNA bands with a total absence of smears. For real-time PCR determinations five micrograms of total RNA were retrotranscribed whith "iScript cDNA Synthesis Kit" (Bio-Rad Laboratories Srl, Italy) according to instruction of the manufacturer. Real-time RT-PCR assays were performed by the TaqMan technology on an ABI PRISM 7000 SDS (Applied Biosystems, Weiterstadt, Germany). Each PCR reaction contained 5 µL cDNA template (corresponding to approximately 50 ng of the total extracted RNA).

We assessed LDL-R and 18 S mRNA expression levels using the *R.norvegicus LDLR*, and Eukaryotic 18S rRNA Endogenous Control TaqMan® Gene Expression Assays (*Inventoried*) (Applied Biosystems® by Life Technologies Italia). The comparative Ct method was used to quantify the results.

γ-Conglutin experiments in cells cultures

3.12 Cells

Hepatoma cell line (HepG2) was obtained from American Type Culture Collection (Rockville, MD, USA). Cells were grown in monolayer in 75 cm^2 flasks and maintained at 37 °C in a humidified atmosphere of 95% air, 5% CO_2 in Dulbecco's

minimum essential medium (DMEM; 5.5 mM glucose) containing 10% fetal bovine serum, non-essential amino acids (1 %, v/v), penicillin (10^5 U/l) and streptomycin (0.1 g/l), and of sodium pyruvate (0.11 g/l).

3.13 Glucose consumption

To evaluate glucose consumption 2 day before the experiments HepG2 cells were plated in twenty-four-well plates (1.5x 10^5 cells/well) with some left blank. Cells were starved in serum-free DMEM supplement with 0.2% bovine serum albumin (BSA) and glucose at various concentrations (5.5, 11.1 and 16.5 mM). After 12 h, the medium was removed and the cells were exposed to 10^{-5} M γ-conglutin with or without metformin (10 nM) and/or insulin (100 nM), including the blank wells, for 24 or 48 h. At the end of incubation periods, glucose concentrations in the medium was determined by the glucose oxidase method. The amount of glucose consumption was calculated by subtracting the glucose concentration of cells treated with different compounds from the cells treated with vehicle (Li et al., 2007). In the same experimental conditions cell viability was evaluated by methyltetrazolium salts assay, essentially as described by Lovati et al. (Lovati et al., 2000).

3.14 FACS Analysis

3.14.1 Glucose uptake by HepG2 cells

The glucose uptake was measured by using the fluorescent glucose analogue 2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NDBG) at 50 μM

concentration. The assay was carried out as previously described with minor modifications (Zou et al., 2005) . Cells were incubated in DMEM containing 11.1 mM glucose in the absence/presence of 2-NBDG or 2-NBDG with 10^{-5} M γ-conglutin or 100 nM insulin for 6 h. At the end of incubation, the 2-NBDG uptake reaction was stopped by removing the incubation medium and washing the cells twice with pre-cold phosphate buffered saline (PBS), as described (Lovati et al., 2012). Cells in each well were subsequently re-suspended in PBS and maintained at 4 C° for flow cytometry analysis, which was carried out by using a FACScalibur (Becton Dickinson, Franklin Lakes, N.J., USA) flow cytometer. The fluorescence intensity of 2-NBDG present into the cells was recorded on the FL1 channel, following elimination of the dead cells labeled with propidium iodide; for each measurement the data from 5,000 single cell events were detected.

To assess cell viability, culture media from cells exposed to the different compounds were tested by methyltetrazolium salts assay, as described by Lovati *et al.* (Lovati et al., 2000).

3.14.2 Effect of endocytosis inhibitor on FITC- γ -conglutin

HepG2 cells grown in DMEM containing 11.1 mM glucose were pretreated for 30 minutes with or without different inhibitors of caveolae/lipid raft-mediated endocytosis [filipin (5μg/ml) and genistein (200 μM)], of clathrin-mediated pathway [chlorpromazine (25 μM), methyl-β-cyclodextrin (5 mM) and of macropinocytosis [amiloride (5 mM)]. The cells were then incubated with FITC-γ-conglutin (10^{-5} M) for 4 h at 37°C in the presence of inhibitors. After incubation

cells were washed with PBS 1x twice and detached by trypsinization. After centrifugation, cells were washed, resuspended in PBS and maintained at 4 C° for flow cytometry analysis, which was carried out by using a FACScalibur (Becton Dickinson, Franklin Lakes, N.J., USA) flow cytometer.

3.15 Confocal Analysis

3.15.1 Time course of γ-Conglutin uptake

HepG2 cells were grown on glass coverslips and incubated in DMEM containing 11.1 mM glucose with/without 10^{-5} M γ -conglutin for 30 min, 3, 6 and 24 h. Afterwards, they were fixed with 4% paraformaldehyde (PMF), permeabilised with Tryton X-100 0.1% and washed with PBS. Labelling was carried out overnight at 4°C with anti-γ-conglutin serum 1:100 v/v in PBS, followed by 2 h incubation at 20 °C in anti-rabbit conjugated with Alexa-Fluor 568 (1:200 v/v in PBS) and staining with DAPI (1:20.000 in PBS). Finally, coverslips were sealed with Mowiol on glass slides and examined with a video-confocal microscope (Vico-Nikon, Italy).

3.15.2 Effect of Amiloride inhibitor on FITC- γ -conglutin

HepG2 cells grown on glass coverslips were pretreated in DMEM (11.1 mM glucose) for 30 minutes with/without inhibitors of macropinocytosis (amiloride 5mM). Then, cells were incubated with FITC-γ-conglutin (50 mg/ml) for 4 h at 37°C in the continuous presence of inhibitors After cell fixation with PFM, the cells were washed with PBS pH 7.4 (Sigma) and permeablized with 0.1% Triton

X-100 (Sigma) in PBS for 20 minutes. The cells were washed and the nuclei labelled with 4′, 6′-Diamidino-2-Phenylindole (DAPI 1:20.000 in PBS) for 10 minutes. After washing with PBS the coverslips were mounted in Vectashield mounting medium for fluorescence (Vector Laboratories) and subsequently, the cells were analyzed with confocal microscope.

3.16 Mass spectrometry

For mass spectrometry analysis, each 2D-gel spot was excised and destained in 0.1% trifluoroacetic acid acetonitrile 1:1 (v/v) and dryed in a Speed Vac. Gel pieces were rehydrated with trypsin (sequence grade, Sigma Aldrich) solution (0.2 μg trypsin/spot in 50 μL 50 μM ammonium bicarbonate), and incubated overnight at 37°C. Peptides were extracted from the gel using 0.1% trifluoroacetic acid acetonitrile 1:1 (v/v). The material was dried, resuspended in 10 mL 0.1% v/v formic acid and desalted using Zip-Tip C18 (Millipore) before mass spectrometric (MS) analysis. Samples were separated by liquid chromatography using an UltiMate 3000 HPLC (Dionex, now Thermo Fisher Scientific). Buffer A was 0.1% v/v formic acid, 2% acetonitrile; buffer B was 0.1% formic acid in acetonitrile. Chromatography was performed using a PepMap C18 column (15 cm, 180 lm ID, 3 lm resin, Dionex). The gradient was as follows: 5% buffer B (10 min), 5-40% B (60 min), 40-50% B (10 min), 95% B (5 min) at a flow rate of 0.3 lL/min.

Mass spectrometry was performed using a LTQ-Orbitrap Velos (Thermo Fisher Scientific) equipped with a nanospray source (Proxeon Biosystems, now Thermo Fisher Scientific). Eluted peptides were directly electro-sprayed into the mass

spectrometer through a standard non-coated silica tip (New Objective, Woburn, MA, USA) using a spray voltage of 2.8 kV. The LTQ-Orbitrap was operated in positive mode in data-dependent acquisition mode to automatically alternate between a full scan (m/z 350–2000) in the Orbitrap and subsequent CID MS/MS in the linear ion trap of the 20 most intense peaks from full scan. Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific). Data Base searching was performed using the Sequest search engine contained in the Proteome Discoverer 1.1 software (Thermo Fisher Scientific). The following parameters were used: 10 ppm for MS and 0.5 Da for MS/MS tolerance, carbamidomethylation of Cys as fixed modification, Met oxidation and Ser/Thr/Tyr phosphorylation as variable modifications, trypsin (2 misses) as protease.

3.17 Transmission electron microscopy (TEM) and immune-gold

Labelling HepG2 cells, plated and grown on Transwell®, were incubated as above with/without 10^{-5} M γ-conglutin for 30 min, 3 and 24 h. Transwell membranes were washed with cold PBS and incubated for 1 h in PBS with 10 g/L heparin. Then, Transwell® membranes were fixed with 1.2% glutaraldehyde and 3.3% paraformaldehyde in 0.1 M PBS pH 7.4 at 4 °C for 3 h, dehydrated in an ethanol series and embedded in London Resin (Duranti, 1994). Immune-labelling was carried out on ultrathin sections at 4 °C overnight with an anti-γ-conglutin polyclonal serum (1:100) and goat anti-rabbit antibody (1:20) conjugated with 20

nm gold particles. Ultrathin sections were stained with 2% uranyl acetate and lead citrate and examined with a JEOL 100SX TEM (Jeol Ltd., Tokyo, Japan).

3.18 Homology 3D modelling

γ-Conglutin 3D model was prepared by homology modelling using the programme ESyPred3D (http://www.unamur.be/sciences/biologie/urbm/bioinfo/esypred/) availed on line. With the amino acid sequence sequence Q9FSH9_LUPAL and the soybean homologous protein, Bg7S, 3D structure as the template (PDB accession number: 3AUP).

3.19 Western blotting

3.19.1 Isoelectric focusing and γ-conglutin internalization and phosphorylation

For IEF/SDS–PAGE analyses, cells were incubated with/ without 10^{-5} M γ-conglutin for 6 h. At the end of incubation, cell medium was removed and the monolayers were incubated in PBS containing 10 g/L heparin for 1 h. After incubation HepG2 cells were lysed by a solution of 8 M urea, 2% CHAPS and 65 mM 1,4- dithiothreitol (DTT) (Amersham Biosciences, Milan, Italy). The protein extracts were centrifuged at 10.000 xg for 30 min and immediately analysed or kept frozen at -80 °C until use. Isoelectric focusing (IEF) was performed on 7 cm pH 3-10 linear IPG strips (Amersham Biosciences, UK) following the procedure described by Capraro et al. (Capraro et al., 2008). The separation was performed on 9 cm X 7 cm 12% polyacrylamide SDS–PAGE gels using a mini-PROTEAN

III cell (Bio-Rad, Milan, Italy). The 2-D separations were repeated three times for each analytical set. The proteins in gels were transferred to 0.45 μm-pore nitrocellulose membranes (Protran, Whatman, Dassel, Germany) by using the TE 77 PWR Semidry Transfer Unit (Amersham Biosciences, UK), according to Towbin et al. γ-conglutin was immune-detected as already described (Magni et al., 2005; Towbin et al., 1979).

After blocking, membranes were then soaked in PBS buffer containing rabbit anti-γ-conglutin (1:1500) and then with HRP-conjugate (1:2000). The labelled bands were detected with 0.6 g/l peroxidase (Bio-Rad) and hydrogen peroxide with 4-chloro-1-naphthol (Sigma-Aldrich) as substrate.

The antiserum to γ-conglutin was raised in rabbit using a non-glycosylated form of γ-conglutin in order to suppress potential carbohydrate unspecific binding, as described by Restani et al. (2005). The polyclonal antibodies were then immuno-affinity purified as described by Casey (1979). This antibody preparation was used for all experiments carried out in this work.

3.19.2 Insulin receptor pathway PI3K-AKT

HepG2 cells were incubated with or without γ-conglutin 10^{-5}M, Insulin 100 nM and Metfornin 10 mM in DMEM containing 11.1 or 30 mM Glucose for 6 or 24 h. After incubation for indicated times cells were lysated and the cell pellet was treated with Nonidet P-40 (NP-40) lysis buffer [10 mmol/L Tris · Cl, pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl2 and 0.5% (v/v) NP40, containing a protease inhibitor cocktail]. Cells were separated into cytosolic and nuclear fractions by

centrifugation at 12,000 xg for 10 min. In the supernatants containing cytosolic protein concentrations were measured using BCA protein assay kit (Sigma-Aldrich). Protein samples (20–50 μg prepared with the Laemmli method) were separated by 10 % SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA). The membranes were blocked with 5% BSA in TBST (Tris-Buffered Saline and Tween 20) and probed overnight at 4 °C with anti Akt (Cell Signalling) 1:2000, anti Phospho-Akt Ser 473 (Cell Signalling) 1:2000, anti AMPK (Cell Signalling) 1:2000, anti Phospho-AMPK (Cell Signalling) 1:2000 and anti β-actin (Sigma-Aldrich) 1:10000. After undergoing TBST washes, the membranes were incubated with peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized by enhanced chemiluminescence (BioRad ChemiDoc XRS).

7S α' experiment in cells cultures

3.20 Cells cultures

3.20.1 HepG2 cells

HepG2 cells were grown in monolayers in 90 mm-diameter Petri dishes, and maintained at 37 °C in a humidified atmosphere of 95% air, 5% CO_2 in minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acid solution (1%, v/v), penicillin (10^5 U/L), streptomycin (0.1 g/L), tricine buffer (20 mM, pH 7.4), $NaHCO_3$ (24 mM) and sodium pyruvate (0.11 g/L). For experiments, cells were seeded in 35-mm plastic dishes (3-5 x 10^5 cells) and used just before reaching confluence.

3.20.2 The 3T3-L1 preadipocytes

The 3T3-L1 preadipocytes were seeded at $3x10^4$ cell/well in 24-well plates and cultured in DMEM containing 1% sodium piruvate, 1% penicillin/streptomycin and 10% calf bovine serum (days 1 and 2). After reaching 100% confluence, the cells were differentiated in DMEM containing 1% sodium pyruvate, 1% penicillin/streptomicyn, 10% FBS, 0.5 mM isobuthylmethylxantine, 1 mM dexametasone and 1.7 mM insulin for 8 days, at which time >90% of cells were mature adipocytes with fat droplets (Martinez-Villaluenga et al., 2009). Cells were treated on day 8 of the differentiation process with 50 mM synthetic peptides dissolved in Dulbecco's phosphate buffer saline. The incubation lasted 48 h at 37°C in a 5% CO_2 atmosphere.

3.20.3 Macrophages cell line RAW 264.7

Macrophages cell line RAW 264.7 induced by LPS (1 mg/mL), were incubated for 24 h in medium containing DMEM, 1% penicillin/streptomicyn, 1% sodium piruvate, and 10% FBS at 37°C in 5% CO_2/95% air, in the presence of each peptide at 25 mM concentration.

3.21 Cell viability

Culture media from HepG2 cell lines exposed to synthetic peptides at different concentrations were tested by measuring the lactate dehydrogenase (LDH) activity, using a kinetic (LDH/LD) diagnostic kit (Sigma Diagnostics), essentially as previously described (Young, 1990)

Adipocytes and macrophages viability was tested by using the CellTiter 96 Aqueous One Solution Proliferation assay kit based on the novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium, inner salt (MTS), and an electron coupling reagent, phenazine ethosulfate (PES) (Promega Corporation, Madison, WI, USA) (Marshall et al., 1995).

3.22 RT-PCR

3.22.1 Lipid homeostasis evaluation

For experiments designed to evaluate LDL-R, SREBP-2, PCSK9 and HMGCoA reductase mRNA expression, HepG2 cells were incubated for 6-8-16 h in MEM +

5% LPDS in the presence of 7S α'peptides (1 μM and 10 μM) or simvastatin (1 μM). Total RNA from HepG2 cells was isolated using RNeasy Mini Kit (Qiagen, Italy) according to the manufacturer's instructions. All RNA samples were examined as to their concentration, purity and integrity based on the absorbance ratio at 260-280 nm and at 230-260 nm. Overall sample integrity was confirmed by agarose gel electrophoresis, showing sharp and intense 18S and 28S ribosomal RNA bands with the total absence of smears. In real time-PCR experiments five micrograms of total RNA from HepG2 was retrotranscripted with "iScript cDNA Synthesis Kit" (Bio-Rad Laboratories) according to the manufacturer´s instructions. RT-PCR assays were performed by the TaqMan technology on an ABI PRISM 7000 SDS (Applied Biosystems, Weiterstadt, Germany). Each PCR reaction contained 5 μL cDNA template, corresponding to approximately 50 ng of the total extracted RNA. We assessed *LDL-R* and *18 S (endogenous control)* mRNA expression levels using the human *LDL-R (Hs00181192_m1)*, and *18S (4319413E); HMGCoA red (hs00168352_m1), SREBP2 (hs00190237_m1)* and *PCSK9 (hs00845399_m1)* Pre-Developed TaqMan Assay Reagents (Applied Biosystems). Results were compared to the values detected in untreated cells (arbitrary unit = 1).

3.23 Oil Red O assay

3.23.1 Lipid quantification in 3T3-L1 adipocytes

Treated adipocytes were washed with cold Dulbecco's phosphate buffer saline and fixed with 10% formaldehyde for 1 h. Then, cells were washed with 60%

isopropanol and let air-dried. Oil Red O stock solution (0.2 g in 60% isopropanol) was filtered through a 0.22 μm membrane and added to lipid droplets for 10 min. After Oil Red O lipid staining, cells were washed with water four times and were air-dried. Oil Red O dye was eluted by adding 100% isopropanol after 10 min incubation at room temperature. Absorbance (A) at 510 nm of eluted isopropanol was measured using a microplate reader (Biotek Instruments, Winooksi, VA, USA). Inhibition of lipid accumulation in adipocytes was calculated using the following equation (Martinez-Villaluenga et al., 2009):

% inhibition of lipid accumulation= $(A_{control,\ 510\ nm} - A_{treatment,\ 510\ nm})/A_{control,\ 510\ nm}$ x 100

3.24 Fatty acid synthase (FAS)

FAS activity was assayed by spectrophotometric method using a Sinergy 2 Microplate Reader System equipped with a temperature controller (Biotek Instruments, Winooksi, VA, USA) as described (Martinez-Villaluenga et al., 2010). NADPH oxidation was followed at 37°C by measuring the decrease in absorbance at 340 nm in a 96-well clear-bottomed polystirene plate (Corning, NY, USA). Reactions were performed in a final volume of 150 μL containing 3 μM acetyl-CoA, 10 μM malonyl-CoA and 35 μM NADPH and 0.3 μM FAS in 0.1 M potassium phosphate buffer. Initial rates were calculated for the slope of the progress curves during the first 5 min. Inhibition studies were performed by measuring the residual FAS activity after enzyme preincubation with peptides at different concentrations for 20 min at 37 ºC.

3.25 Oxygen radical absorbance (ORAC) assay.

The ORAC assay was performed following Prior *et al.* (Prior et al., 2003) and Davalos *et al.* (Davalos et al., 2004) methods. Fluorescein reacted with free radicals generated by 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) yielding a non-fluorescent product. Loss of fluorescence was measured over time in fluorescent microplate reader, (Biotek Instruments, Winooksi, VA, USA) at 37°C and sensitivity 60. Readings were made every minute at λ_{exc} 485 nm and λ_{emi} 520 nm. Then, the area under curve (AUC) was calculated as previously reported (Darmawan et al., 2010) and compared to a standard antioxidant, Trolox (vitamin E analogue). Results were expressed as µmol Trolox equivalents (TE)/ µmol peptide.

3.26 Statistical analyses

Data were expressed as means ± standard deviation or ± SEM of at least three replicates. Results were compared using one-way analysis of variance (ANOVA) followed by Dunnett's test. *P* values $\leq$ 0.05 were considered as statistically significant.

4. RESULTS

Results of γ–Conglutin experiments

4.1 γ–Conglutin administration reduces plasma glucose in the "*in vivo*" model of type 2 diabetes

The dosage of 28 mg/kg b.w. γ-conglutin was selected according to lowest dosage of the previous trials consisting of acute glucose overloads in normal rats (Magni et al., 2004). Body Weight were not modified either by glucose feeding or by γ-conglutin treatment in all groups: controls, (244.6 ± 3.8) g; glucose-treated, (243.7 ± 5.5) g; glucose-treated + γ-conglutin, (241.00 ± 8.0) g. As depicted in **Fig.1a**, chronic glucose administration resulted in a statistically significant ($p \leq 0.01$) increase in fasting blood glucose (2.5 fold); conversely, simultaneous treatment with γ-conglutin attenuated the rise in glucose (1.9 fold) so that the glucose levels in these animals were reduced by 22% in comparison with those recorded in the glucose-treated rats.

In glucose-drinking rats, insulin levels increased by 170% (2.7 fold) **Fig. 1b**; $p \leq 0.05$); the treatment with γ-conglutin reduced this increase to 79% ($p \leq 0.05$), although the levels remained higher than in control animals. Chronic glucose feeding increased the IR index, as expressed by HOMA-IR, by 582% (**Fig.1c**; $p \leq 0.05$). γ-Conglutin treatment attenuated this increase by 252% in glucose-fed rats, and the comparison between the HOMA-IR indexes in the two groups of rats (glucose-treated and untreated) showed an improvement in IR by 48% ($p \leq 0.05$), following daily administration of γ-conglutin.

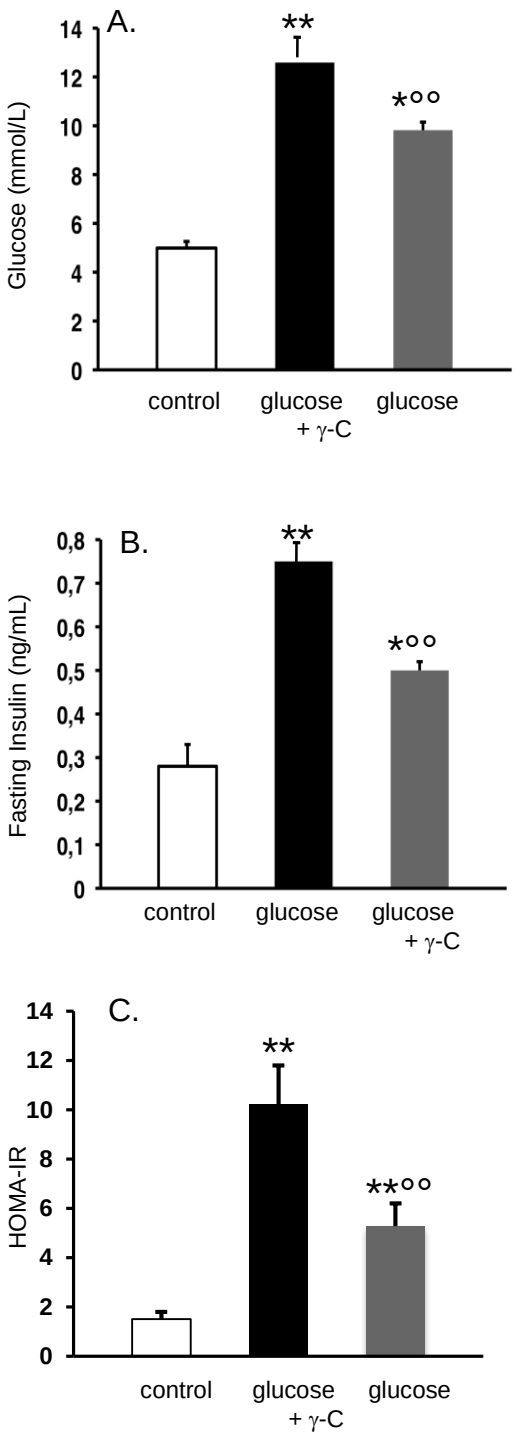

A.
Glucose (mmol/L)
14
12
10
8
6
4
2
0
**
*°°
control
glucose
glucose
+ γ-C
B.
Fasting Insulin (ng/mL)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
**
*°°
control
glucose
glucose
+ γ-C
C.
HOMA-IR
14
12
10
8
6
4
2
0
**
**°°
control
glucose
glucose
+ γ-C

Figure 1

Figure 1: Effects of chronic glucose drinking, combined with or without γ-conglutin (γ-C) supplementation (a) on plasma glucose levels expressed in mmol/l, (b) on plasma insulin levels expressed in ng/ml and (c) on index of insulin resistance (IR; plasma glucose x insulin/22.5= homeostasis model assessment (HOMA)). Values are means, with their standard errors represented by vertical bars, *n* 12. Mean values were significantly different from those of controls: * p≤0.05, ** p≤0.01. Mean values were significantly different from those of glucose group: †† p≤0.01.

The oral glucose tolerance test, carried out at the end of experimental period on all animals enrolled in the study, showed (**Fig**. **2a**) a statistically significant reduction upon 3-week treatment with γ-conglutin, both fasting blood glucose and postprandial blood glucose (2 h) were reduced (221 and 212 %, respectively; p≤0.05), suggesting improved insulin sensitivity in the treated animals (**Fig**. **2b**).

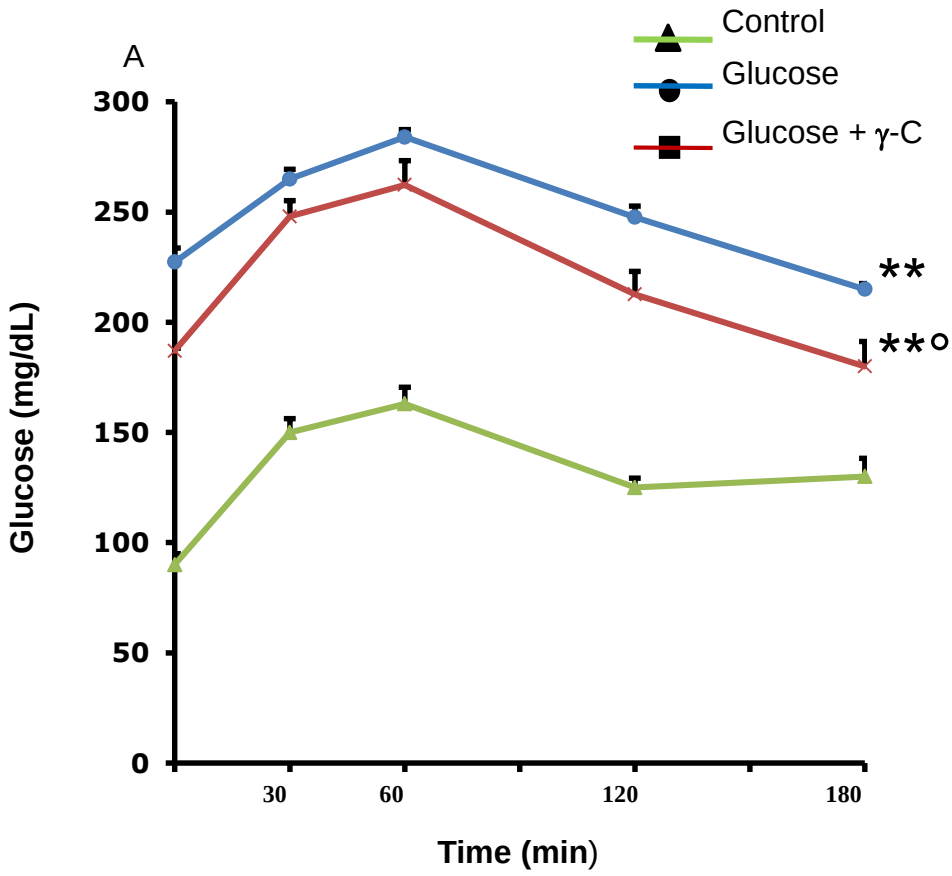


Figure 2

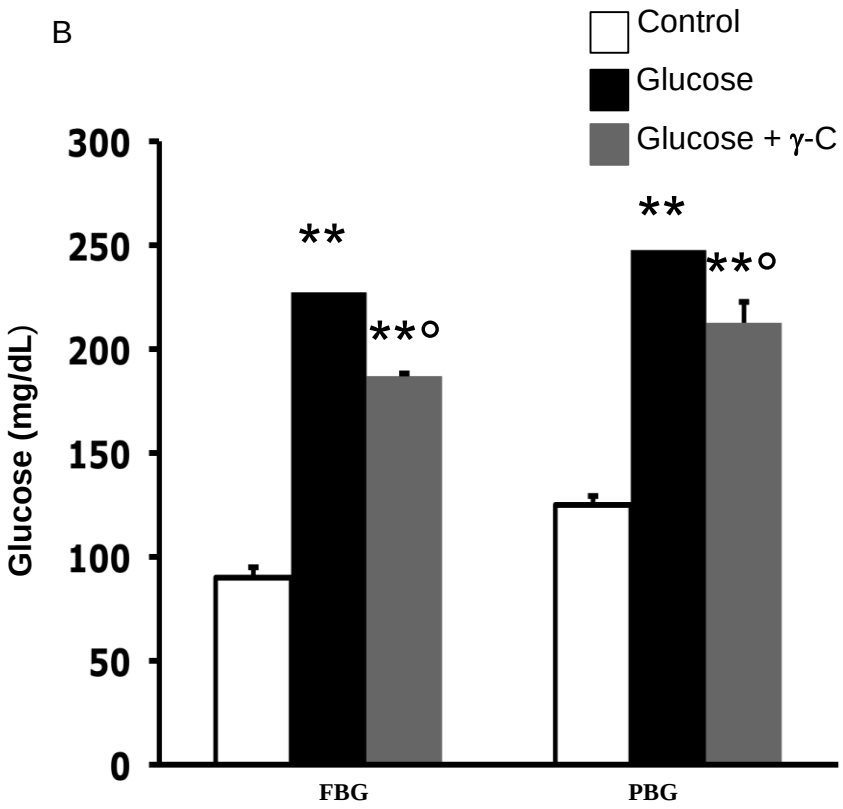


Figure 2 b

Figure 2: (a) Influence of γ-conglutin (γ-C) supplementation on plasma glucose concentrations of rats during oral glucose overloading trials (2 g/kg b.w.). Plasma glucose was assayed in rats at various times from glucose overload as reported in the 'Materials and methods' section. (b) Fasting blood glucose (FBG) and 2 h postprandial blood glucose (PBG) determined during glucose overload experiment in the rats. Values are means, with their standard errors represented by vertical bars, *n* 12. ** Mean values were significantly different from those of controls ($p \leq 0.01$). † Mean values were significantly different from those of glucose group ($p \leq 0.05$).

4.2 γ-Conglutin improved glucose metabolism *"in vitro"*

Glucose consumption was examined in HepG2 cells following incubation with purified γ-conglutin. The dose of 10^{-5} M γ-conglutin was selected from previous experiments, where an up-regulation of LDL receptors was detected in HepG2

cells after pre-incubation with γ-conglutin (Sirtori et al., 2004). Cells were grown in DMEM containing different glucose concentrations in order to simulate normal (5.5 mM =99 mg/dl) or moderate high glucose (11.1 mM = 200 mg/dl) and severe hyperglycaemia (16.5 mM~300 mg/dl) in human subjects. Moreover, insulin (100 nM and metformin (10 mM) were used alone or in combination with γ-conglutin to assess the potential synergism/antagonism in glucose consumption following 24 or 48 h incubation. As depicted in **Fig**. **3**, the addition of insulin or metformin to HepG2 cells grown in DMEM containing different amounts of glucose induced a statistically significant increase ($p \leq 0.05$) in the glucose consumption after 24 and 48 h incubation. When the glucose in the culture medium was normal (5.5 mM), no statistically significant effect on glucose consumption was detected on the addition of γ-conglutin in all experimental conditions (control, insulin and metformin). On the other hand, the effect of γ-conglutin in glucose consumption ($p \leq 0.05$) was evident in cells grown in DMEM with moderate (11.1 mM) or elevated (16.5 mM) glucose content; moreover, the concomitant addition of γ-conglutin to HepG2 cells exposed to insulin or metformin increased further the glucose consumption, normally stimulated by both compounds. In particular, when the glucose concentration in the culture medium increased from 5.5 to 11.1 mM, the glucose expenditure induced by γ-conglutin, after 24 h of incubation, was elevated to 100, 60 and 18 %, respectively in controls, insulin- and metformin-treated cells (**Fig**. **3b**). When the glucose concentration increased from 11.1 to 16.5 mM, the amount of glucose consumed in 24 h was enhanced by 109, 33 and 43 %, respectively in controls, insulin- and metformin- treated cells by the addition of

γ-conglutin addition (**Fig**. **3c**). This trend was shared by HepG2 cells incubated for 48 h in the same experimental conditions: the addition of γ-conglutin induced a statistically significant increase in the amount of glucose consumed as depicted in the **Fig. 3** (e and f). The glucose-lowering effect of γ-conglutin observed in HepG2 cells was not linked to an increment in cell number, due to the glucose level, since we did not observe any change in methyltetrazolium salts optical density (data not shown) In addition, the results obtained following the exposure of HepG2 cells to γ-conglutin were compared for each time and glucose concentration with the respective controls. Moreover, a previous experiment (data not shown), in which mannitol was added to 5.5 mM glucose DMEM, pointed out that the present results were due to the activity of tested compounds (γ-conglutin, insulin and metformin) and not to the effect of hyperosmolarity.

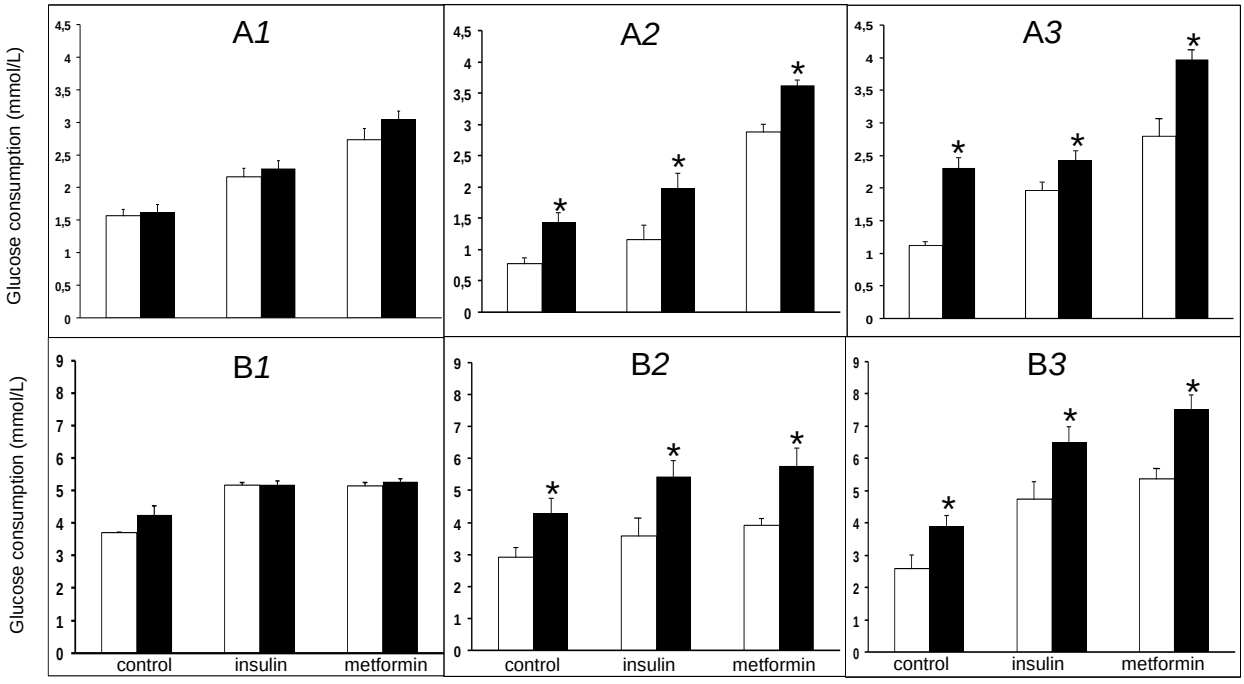


Figure 3

Figure 3: Glucose consumption by HepG2 cells after 24 (panels A) and 48 h (panels B) growth at different glucose concentrations (1: 5.5 mM; 2: 11.1 mM and 3: 16.5 mM). The trials were: cells alone (control), treated with insulin (100 nM) or metformin (10 mM) in the absence (white bars) or presence (black bars) of 10^{-5} M γ-conglutin. The tests were performed in DMEM supplemented with 0.2% BSA as detailed under Methods. Data are means ± S.E.M. of 3 independent experiments, each performed in quadruplicate. * $p \leq 0.05$ *vs* the γ-conglutin untreated trials.

4.3 Flow cytometry glucose uptake assay

The 2-NDBG uptake was examined in HepG2 cells following incubation with 10^{-5} M purified γ-conglutin. Insulin (100 nM) was used alone or in combination with γ-conglutin to assess the potential synergism/antagonism in glucose analogue uptake following 6 h incubation, as already observed in previous works (Terruzzi et al., 2011).

As shown in **Figure 4**, γ-conglutin alone or in combination with insulin stimulated, in a statistically significant way, the HepG2 cell glucose uptake by 64 % and 184 %, respectively *vs* the untreated cells. Insulin alone increased glucose uptake by 119%. γ-Conglutin did not show any binding capacity toward glucose, as assessed by affinity chromatography and changes in refractometric indexes (Capraro 2011, unpublished results), therefore any direct involvement of the lupin protein in glucose transport into the cell could be ruled out.

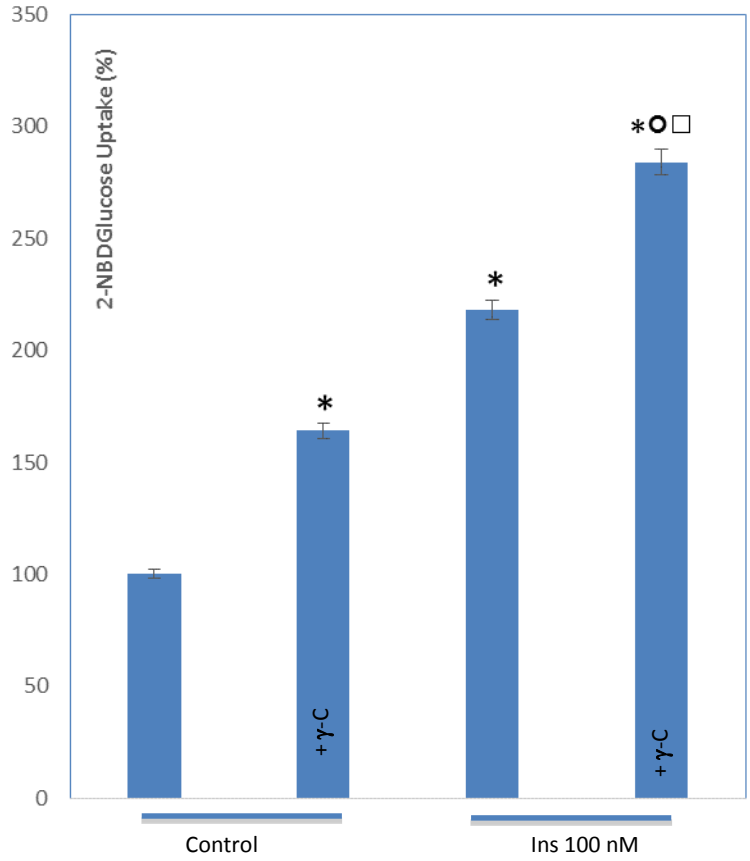


Figure 4: Effect of γ-conglutin on basal and insulin-stimulated 2-NBDG uptake in HepG2 cells. Cells were incubated in DMEM containing 11.1 mM glucose in the absence/presence of 2-NBDG (50 μM) with and without 10^{-5} M γ-conglutin and 100 nM insulin for 6h. At the end of incubation cells were collected for the flow cytometry analysis. The relative fluorescence intensities minus the background levels were used for subsequent statistical analyses. Vertical bars show the mean ±SEM of three independent experiments, each performed in quadruplicate. Significant differences are marked by * $p \leq 0.05$ *vs* control cells, o $p \leq 0.05$ *vs* γ-conglutin treated cells and □ $p \leq 0.05$ *vs* insulin and γ-conglutin treated cells

4.4 Time course of γ-conglutin uptake by HepG2 cells as assessed by confocal microscopy using γ-conglutin antibodies

The uptake of γ-conglutin by HepG2 cells, as monitored with fluorescently-labelled γ-conglutin antibodies in confocal microscopy, is shown in **Figure 5**, thirty min after the treatment, it was already possible to observe some fluorescent spots around the cells (**Fig. 5A**), suggesting the presence of protein aggregates leaning against cell membranes. Whether these aggregates were only stacked outside the membrane or, at least in part, already inside the cells it was not clear at this time (**Fig. 5A**).

Conversely, the intracellular uptake of some of these aggregates was more evident 3 h later (**Fig. 5B).** A very different distribution pattern of fluorescence was observed at 6 h after the treatment, when the whole cytoplasm showed a diffuse and intense fluorescence, suggesting that γ-conglutin had spread in it, without a specific localization or aggregation in dense bodies. Some fluorescence spots were still visible at the cell border, possibly outside membranes. This scenario completely changed at 24 h, when the diffused cytoplasm fluorescence was much less intense and large brilliant spots were again visible at the cell periphery. At any considered time, nuclei were not labelled.

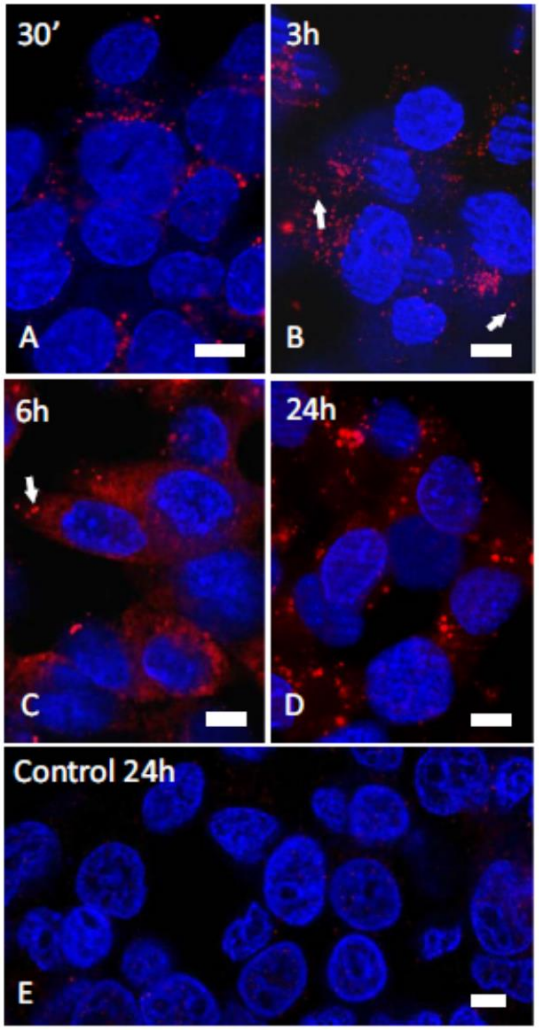


Figure 5: Immune-detection by confocal microscopy of HepG2 cells at different incubation times with γ-conglutin (red); nuclei are counterstained in blue by DAPI. Nuclei were not labelled at any considered time, as well as control cells without γ-conglutin treatment (panel E). All bars = 20 μm.

4.5 Two-D electrophoresis and mass spectrometry evidence of γ-conglutin internalisation and phosphorylation by HepG2 cells

In order to confirm the intracellular uptake of lupin γ-conglutin by treated HepG2 cells and monitor the status of the internalized protein, 2D IEF/SDS–PAGE of the cell lysates at 6 h incubation, when the cells showed a 64% increase of glucose uptake, was performed. The resulting 2D electrophoretic maps of the untreated control cells and cells after 6 h treatment with γ-conglutin are shown in **Figure 6,** panels A and A', respectively. The two maps showed a very similar pattern. However, some spots, which were not present in the control sample, appeared in the γ-conglutin treated sample. These two positions in the electrophoretic map corresponded to that of γ-conglutin subunits (Magni et al., 2007), which consists of a main small subunit around 17 kDa and pI 6.2 and large ones of 30 kDa and pI 8.7. For further evidence, the 2D maps of untreated and treated cells were blotted and revealed with anti- γ-conglutin antibodies (**Fig. 6 B and B'**). With the untreated cells, no labelling was visible throughout the map, suggesting that no protein component of the cells cross-reacted with γ-conglutin antibodies. Conversely, in panel B', two main spots around 30 kDa and other less distinct ones at 17 kDa were detected by the antibodies, thus confirming the presence of intact γ-conglutin subunits. Other unforeseen spots reacting with γ-conglutin antibodies were found in the blotted map (**Fig. 6, panel B'**) at the large subunit position, but with unusually low pIs. The acidic spots in the blotted map, indicated as spot 1 (S1) and spot 2 (S2), as well as a main spot in the canonical position of γ-conglutin large subunit (S3), were excised, trypsin treated and submitted to MS/MS

spectrometry, as described under Methods. The results of mass analyses are detailed in **Table 1**. The analysis allowed to unequivocally identify the 3 spots as γ-conglutin, in agreement with the Western blot data. Moreover, the presence of phosphorylated amino acids was detected in various tryptic peptides of spots 1 and 2, whilst none was seen in the unmodified γ-conglutin. The position of the phosphorylated amino acids in γ-conglutin sequence is shown in **Table 1**. The analysis of the predicted 3D structure of γ-conglutin confirmed the expected location of the modified amino acids at the surface of the molecule (not shown). However, due to the incompleteness of the sequence coverage, the presence of other phosphorylated amino acids could not be excluded

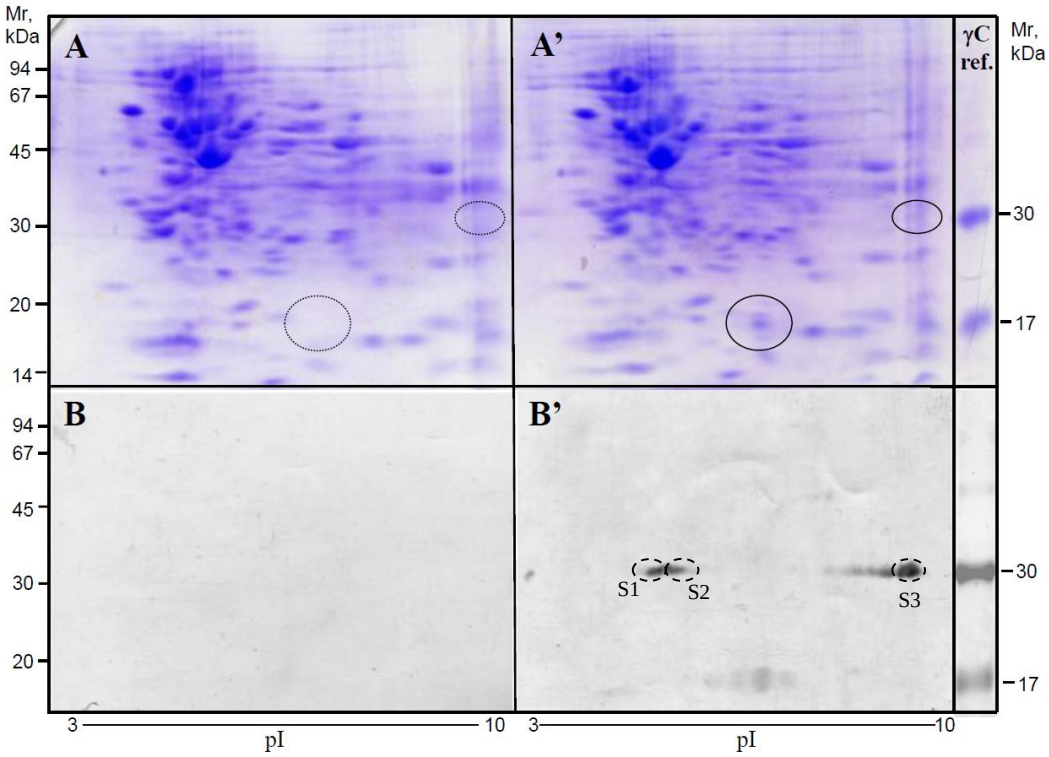


Figure 6: Two-D electrophoretic maps of proteins from HepG2 cell lysates incubated for 6 h in DMEM without (A) and in presence (A') of the lupin seed protein γ-conglutin (10^{-5} M). Figure B and B', show antibody revelation of γ-conglutin in the blotted maps of

cells incubated with (B') or without (B) γ-conglutin. Reference γ-conglutin is also showed. In panel A', the spots corresponding to the 30 kDa and 17 kDa γ-conglutin subunits were circled after identification by comparison to the reference map (see the white lupin 2D reference map on http://www.lupinproteinteam.unimi.it). These spots are not present in the untreated sample (panel A, dotted circles to locate the empty areas). Antibody revelation evidences the large (marked as Spot 3) and small subunits of γ-conglutin in the treated sample (panel B'). Two acidic spots, revealed by the same antibodies, are marked Spot 1 and 2, respectively.

Sequence	Identified phosphorylated residue	Position [a]	Xcorr	Charge	*m/z*(Da)	MH^+ (Da)
Spot S1						
HSIFEVFTQVFANNVPK	T	36	1.58	3	686.33496	2056.99033
RTPLMQVPVLLDLNGK	T	298	1.74	3	625.34088	1874.00809
KISGGVPSVDLIMDK	S	327	1.87	4	410.46106	1638.82241
AVGPFGLCYDTKK	Y	321	1.43	2	768.34937	1535.69145
SCSNLFDLNNP	S	409	1.60	2	680.76617	1360.52507
Spot S2						
RTPLMQVPVLLDLNGK	T	36	2.07	3	625.33844	1874.00077
KISGGVPSVDLIMDKSDVVWR	S	327	1.42	3	799.72986	2397.17502
IPQFLFSCAPTFLTQK	T	149	1.11	3	659.99481	1977.96988
Spot S3						
Various peptides belonging to γ-conglutin sequence	No phosphorylated residues	-	-	-	-	-

[a] The residue position is referred to sequence Q9FSH9.

Table 1: List of phosphorylated peptides in 2D-gel spots S1, S2 and S3.

4.6 Uptake and fate of γ-conglutin in HepG2 cells by TEM immune-labelling

To shed light on the intracellular uptake pathway and the presence of γ-conglutin aggregates, TEM immune-localization of the protein at 30 min, 3 h and 24 h was performed. γ-Conglutin aggregation occurred around the numerous microvilli present on HepG2 cell membranes (**Figure 7A and B**), consistently with the above mentioned fluorescent spots leaning against cell membranes. These microvilli

seemed to trap the protein, sometimes in large aggregates (**Fig.7B**). At 3 h treatment, some small aggregates were visible both stacked to the cell membrane (**Fig.7C**) or inside the cytoplasm (**Fig.7D),** with no clear signs of endocytosis, possibly because the lack of osmication during the fixation process prevented membrane visualization. Moreover, intense labelling was localized both in the protein aggregates stacked to microvilli and in some dense bodies in the cytoplasm (**Fig. 7E**). These bodies, being only labelled in some parts, were possibly formed by other substances, besides γ-conglutin, and corresponded to the brilliant fluorescent spots observed in the cytoplasm by confocal microscope at the same time course. Intriguingly, dense bodies were not present at 24 h, but the labelling was scattered in the cytoplasm, with rare aggregation (**Fig. 7F)**. Serial sectioning showed that some of these protein aggregates were invaginated into the cell membrane, however still outside the cell (**Fig. 7G**).

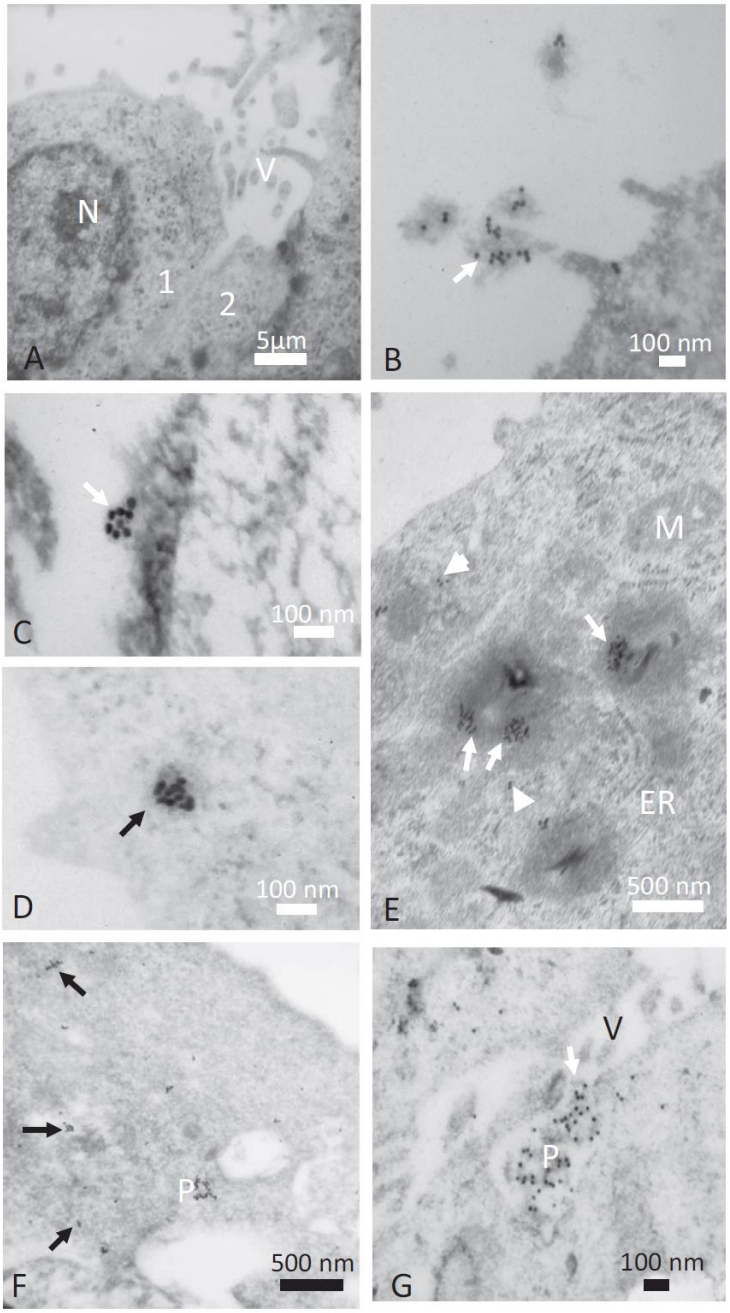


Figure 7: TEM ultrathin sections of HepG2 cells at 0 min (A), 30 min (B), 3 h (C-E) and 24 h (F, G) incubation with γ-conglutin. γ-Conglutin is sometime localized in electron-dense bodies (panel E, arrows) or scattered throughout the cytoplasm (panel E, arrowheads) and rare protein aggregates (P) are present. (G) Serial sectioning of an aggregate showing that its invagination into the cell membrane (arrow) (**V**, microvilli). **ER**, endoplasmic reticulum; **M**, mitochondrion.

4.7 Effect of endocytosis inhibitor on the cellular uptake of FITC-γ-conglutin

To clarify the mechanism of internalization of γ-conglutin into the HepG2 cells, inhibitory experiments of γ-conglutin cellular uptake were carried out using specific endocytosis inhibitor. The effect of the following endocytosis inhibitors on internalization of the protein were examined: filipin (5μg/ml) and genistein (200 μM) for caveolae/lipid raft mediated endocytosis, chlorpromazine (25 μM), methyl-β-cyclodextrin (5 mM) for clathrin-mediated pathway and amiloride (5 mM) for macropinocytosis. To study whether incubation with inhibitors of endocytosis would affect internalization of γ-conglutin, cells were preincubated for 30 minutes at 37 °C with inhibitors and then, exposed to FITC-γ-conglutin (50 μg/ml) for 4 h at 37°C in the presence of inhibitors (**Figure 8**). Treatment of the cells with amiloride significantly affect the FITC-γ-conglutin internalization, with an approximately 69% decrease in the amount of cellular uptake. Treatment with filipin had no significant effect on γ-conglutin internalization. Methyl-β-cyclodextrin, chlorpromazine and genistein showed a lower significant reduction in the internalization of protein (40%, 33% and 14% respectively). These results indicate that in HepG2 cells, the γ-conglutin was internalized preferentially through macropinocytosis pathway.

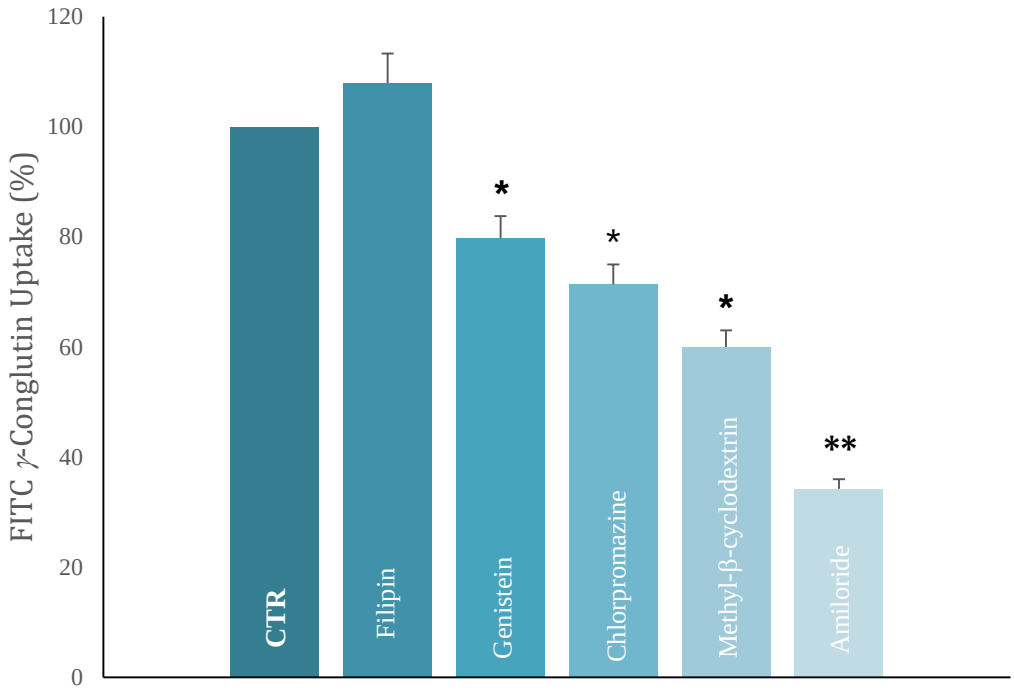


Figure 8: Effect of endocytosis inhibitors on FICT-γ-conglutin uptake: HepG2 cells were pretreated for 30 minutes with or without inhibitors of caveolae/lipid raft-mediated endocytosis [filipin (5 μg/ml) and genistein (200 μM)], of clathrin-mediated pathway [chlorpromazine (25 μM), methyl-b-cyclodextrin (5 mM) and of macropinocytosis [amiloride (5 mM)]. Then, cells were incubated with FITC-γ-conglutin (50 μg/ml) for 4 h at 37°C in the presence of inhibitors. Subsequently, the cells were analyzed by FACS. *$p \leq 0,05$ *vs* controls, ** $p \leq 0,01$ *vs* controls.

4.8 Effect of macropinocytosis inhibitor on FITC-γ-conglutin internalization

To confirm the potential role of macropinocytosis in the uptake of FICT-γ-conglutin, confocal microscopy study was carried out on HepG2 cells using amiloride (5 mM). As showed in **Figure 9** after 4 h of incubation with γ-conglutin and in the absence of inhibitor, cytoplasm showed a diffuse and intense fluorescence due to the presence of FITC-protein. When cell were treated with

amiloride a very different distribution pattern of fluorescence was observed in panel C. FITC-γ-conglutin was in fact visible at the cell border, outside membranes. This result confirmed the previous data, suggesting the macropinocytosis as main via of γ-conglutin internalization.

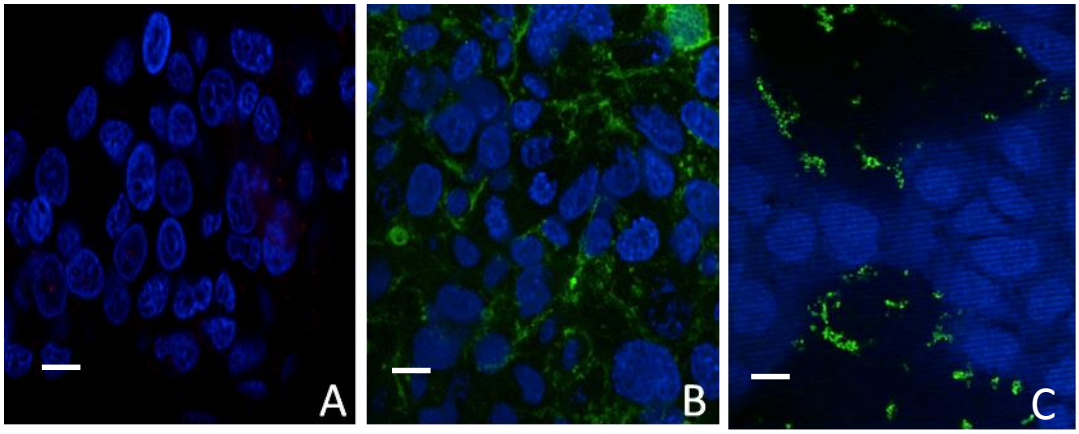


Figure 9: Immune-detection by confocal microscopy of HepG2 cells at 4h incubation with FITC-γ-conglutin (green); and with amiloride (5 mM) (panel C). Nuclei are counterstained in blue by DAPI, as well as control cells without γ-conglutin treatment (panel A). All bars = 20 μm.

4.9 γ -Conglutin: Involvement in PI3K-AKT insulin receptor pathway

4.9.1 Effects of γ-conglutin on AMPK phosphorylation

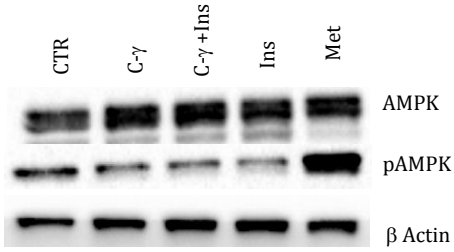
We monitored the ability of both insulin and γ-conglutin to stimulate the same signaling pathway in HepG2 cells. Insulin and Metformin, the first-line oral anti-diabetic drug, was used as a positive control.

To start the study of the potential effect of γ-conglutin on key proteins of the insulin signaling, AMPK, a sensor of energy status for maintaining cellular energy

homeostasis (Hardie, 2011), was evaluated by analyzing the levels of total and phosphorylated proteins in cell lysates by Western blot analysis.

Figure 10 showed that the 24 h treatment with γ-conglutin, alone or in concomitant presence of insulin did not induce an increase in the levels of AMPK phosphorylated proteins. Metformin, as aspect, showed the high levels of the p-AMPK/AMPK ratio.

Figure 10 A



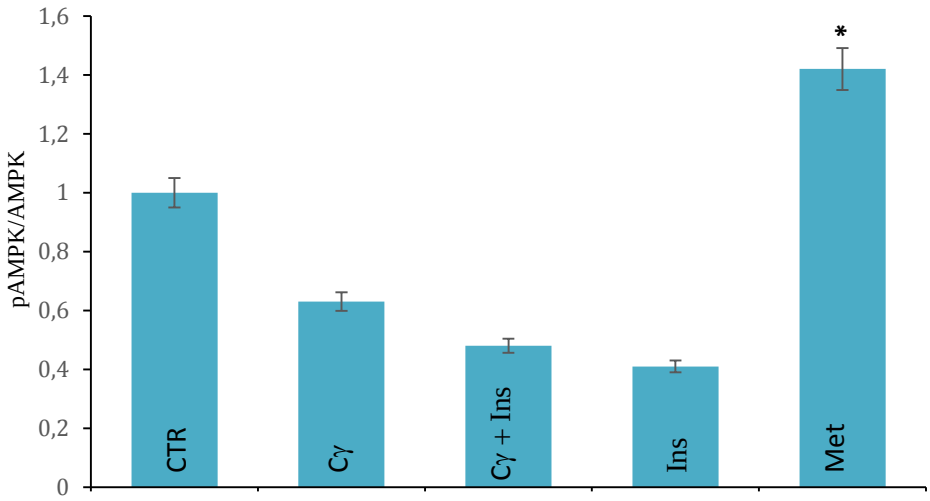


Figure 10 B

Figure 10: Effect of γ-conglutin on phosphorylated 5'-AMP-activated protein kinase (AMPK) levels in HepG2 cells after 24 hour treatment. (A) Western blot analysis of

representative experiments. Equal loading of Western blots was ensured by β-actin. (B) Values of p-AMPK/AMPK ratio relative to the control condition. (*) Indicate statistically significant differences ($p \leq 0.05$).

4.9.2 Effects of γ-conglutin on AKT phosphorylation

Akt, serine/threonine-specific protein kinase, is the molecular key in mediating the metabolic effects of insulin signaling. It lays downstream of PI3K and facilitates glucose uptake and glycogen synthesis in the liver (Whiteman et al., 2002). To test the modulation of Akt by γ-conglutin, phosphorylated and total Akt were evaluated in cell lysates by Western blot analysis after 6 and 24 hour incubation in DMEM containing 11.1 or 30 mM Glucose. **Figure 11 A** and **B**, show the results of cells treatment with γ-conglutin, insulin and metformin in the different experimental condition.

After 6 h incubation in 11.1 mM Glucose DMEM , in cells treated with γ-conglutin we have a 5 fold increase in the phosphorylation of Akt which goes up to 25 fold when cell were treated in concomitant presence of insulin. A similar rise in p- Akt value is present in all experimental conditions

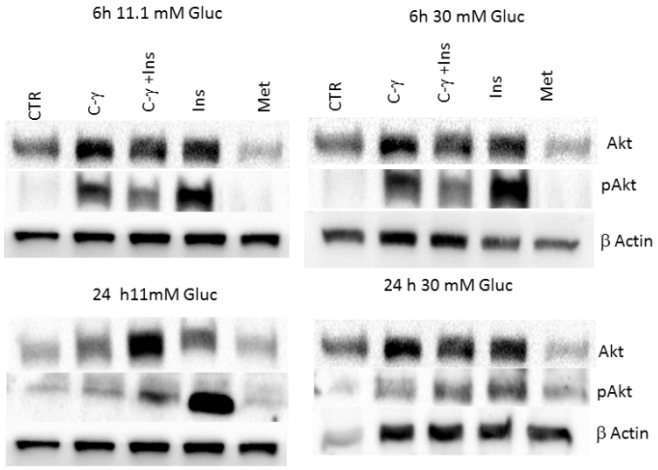


Figure 11A

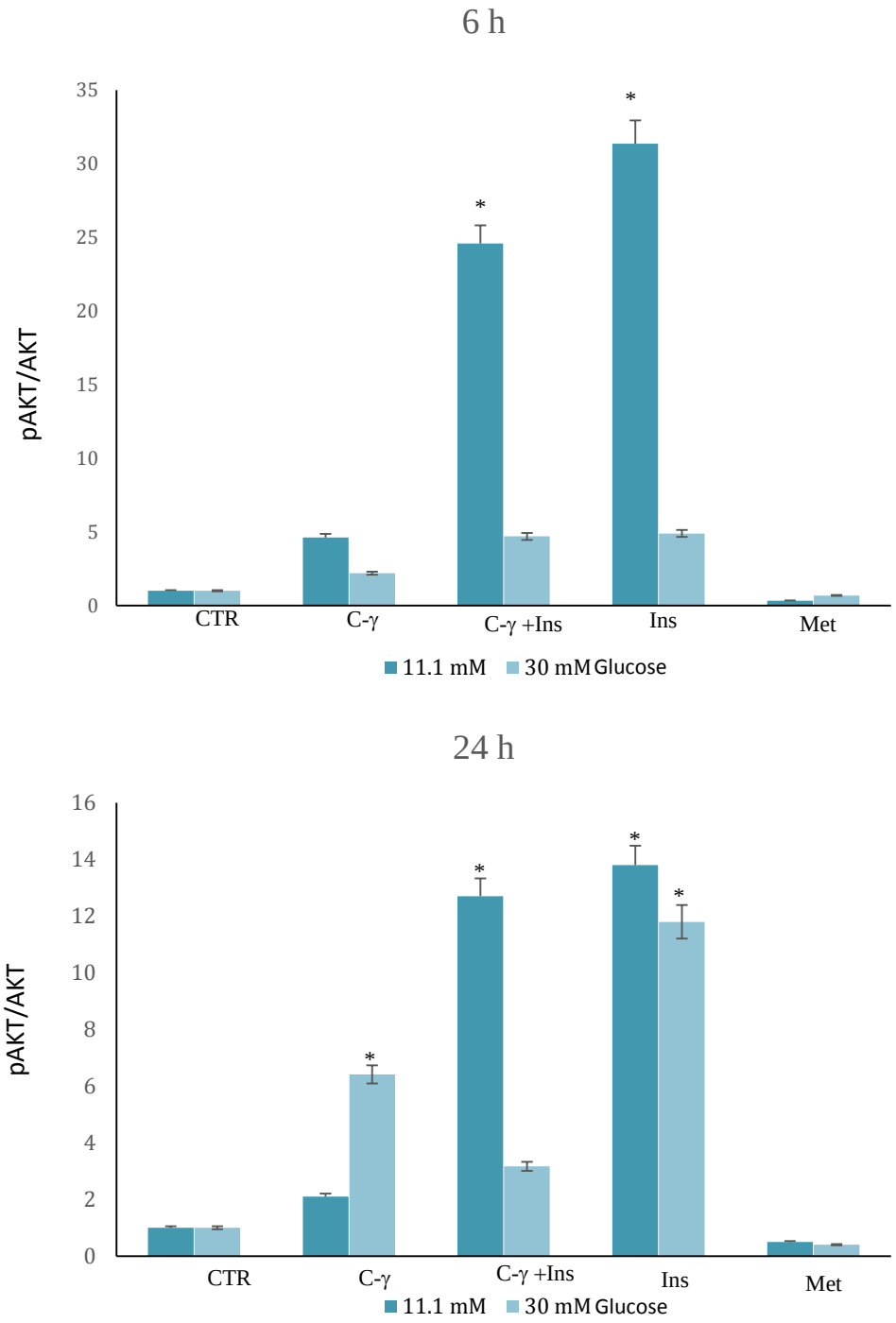


Figure 11B

Figure 11: Effect of γ-conglutin on levels of phosphorylated and total Akt protein, in HepG2 cells, following 6 and 24 h incubation in DMEM with 11.1 or 30 mM glucose. (A) Bands of representative experiments. Equal loading of Western blots was ensured by

β-actin. (B) Data of p-Akt/Akt ratio; values are expressed as relative to the control condition and are means ± SD* ($p \leq 0.05$).

A different pattern was observed in HepG2 cells after 24 h of incubation in 11.1 mM DMEM where in presence of γ-conglutin was observed a decrease in the phosphorylated form of protein. Because is now that PI3K is involved in Akt activation, in order to confirm the role of PI3K/Akt pathway in the effect of γ-conglutin the cells were expose to a selective inhibitor of PI3K/Akt (LY294002) and the levels of Akt and its phosphorylated form were assayed and compared with the values recorded in cells incubated in the absence of inhibitor. In **figure 12** (A/B) data show that LY294002 treatment decreased p-Akt levels in all experimental condition (6 or 24 h incubation) whereas the increased ratio of p-Akt/Akt were recovered in HepG2 cells in the presence of γ-conglutin alone or in the concomitant presence of insulin when cells are not exposed to LY294002 *vs* values detected in control groups. However, neither LY294002 than γ-conglutin, significantly affected the levels of total Akt indicating that the inhibition in phosphorylation at Ser-473 Akt was not due to a nonspecific effect.

This data, suggest that PI3K/AKT pathways may be involved in the glucose homeostasis effect induced by γ-conglutin-induced effects**.**

Figure 12 A

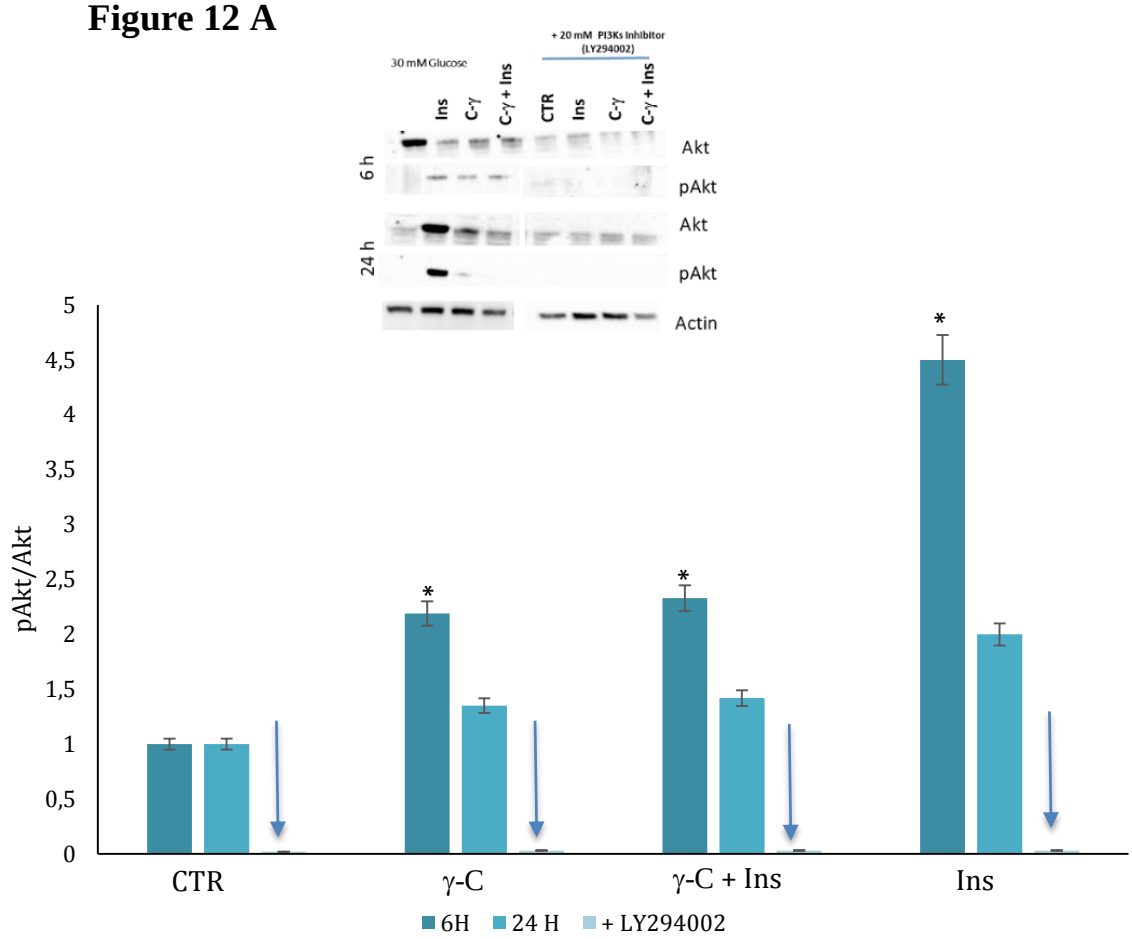


Figure 12 B

Figure 12: Effect of γ-conglutin and selective inhibitor LY294002 (LY) on total Akt and p-Akt levels in HepG2 cells. Cells were incubated in the presence or absence of 20 μM LY for 1 h and later with 10^{-5}M γ-conglutin or 100 nM Insulin for 6 or 24 h. (B) Data of p-Akt/Akt and (A) Bands of representative experiments. * indicate statistically significant differences ($p \leq 0.05$).

Results of α' 7S soy protein experiments

4.10 Body weight and food consumption in animal treated with α' 7S subunit

At the end of the experimental period (four week) the oral administration of IDN 6442 **(**7S−α'enriched), IDN 6443 (α'E) (Supplied by INDENA, Scientific department Milano, Italy), and clofibrate, in rats maintained on a hypercholesterolemic diet, or feed on standard diet, body weight and food intake was comparable with that of the untreated rats. **Table 2** shows that in fact the increase (Δ) of body weight recorded at the end of treatment was similar in the different groups, both treated and non-treated rats with the test compounds, indicating that the amounts of recombinant polypeptide (α'E) used in this experiment did not affect either the normal process of growth of the animals neither on the food consumption that was equal to 14.6 gr/day for rats fed in hypercholesterolemic diet. This value, due to the higher caloric content of the diet itself, is lower than that of the rats fed with on standard diet (20 gr/day).

4.11 Plasma levels of total cholesterol, triglycerides and blood glucose of hypercholesterolemic rats

It can be observed that the hypercholesterolemic diet is able to induce a statistically significant increase, ($p \leq 0.001$), in the levels of total cholesterol, plasma triglycerides, and glucose compared to the values found in animals fed the standard diet (**Table 3**). In particular, plasma cholesterol and triglycerides levels increased

by 399 and 70% respectively in rats fed HC diet respect to the rats fed standard diet at the end of the treatment. The oral treatment with proteins under investigation caused a statistically significant reduction in the cholesterol and triglyceride levels compared to the values found in untreated animals (* $p \leq 0.05$, ** $p \leq 0.001$).

Group	Body weight gr		Δ	Food intake
	Pre	Post	gr	gr/day
Standard diet	160±2	341±12	181±11	20±0.8
HC diet	151±3	326±10	175±9	14±0.9
+IDN 6442 (20 mg/Kg)	153±2	331±7	178±7	14±1.0
+IDN 6443 (25 mg/Kg)	148±2	350±7	201±7	15±0.8
+IDN 6443 (50 mg/Kg)	154±2	334±7	180±6	15±1.2
+ Clofibrate (200 mg/Kg)	152±2	321±6	169±7	15±0.9

Table 2: Effect of administration of IDN 6443, IDN 6442 and clofibrate on the performance of body weight in rats fed on hypercholesterolemic diet. $p \leq 0.05$ *vs* standard diet.

As show in **Table 3** as show that, IDN 6442 (7S-α' rich), administered at a dose of 20 mg / kg b.w (around to 15.42 mg of 7S protein enriched in α' subunit which correspond to 5.53 mg/kg b.w. of protein of α' subunit) induced a reduction in the levels of cholesterol and triglycerides (-27% and -34%, respectively) comparable to that previously published (Duranti et al., 2004). IDN 6443 (α 'E), was used at concentrations of 25 mg/kg b.w. (5.65 mg protein /Kg b.w.) and 50 mg /Kg b.w.

(11.3 mg protein /kg b.w.), and although the concentration of active principle is minimal compared to the amount of product administered, is characterized by a remarkable reduction activity of both total cholesterol (-29 and 26%, respectively with 25 and 50 mg/kg b.w.) and triglycerides (-13 and - 23%, respectively with 25 and 50 mg/kg b.w.). The lipid-lowering effect is comparable to that observed in rats treated with 200 mg/kg of clofibrate, as a reference drug. No statistically significant change in plasma glucose levels were induced by the treatment with the test compounds.

	Cholesterol mmol/L	Triglycerides mmol/L	Glucose mmol/L
Standard diet	1.19±0.09	0.71±0.05	4.95±0.1
HC diet	5.94±0.16	1.21±0.06	6.43±0.67
+ IDN 6442 (20 mg/Kg)	4.34±0.16**	0.80±0.07*	5.21±0.56
+ IDN 6443 (25 mg/Kg)	4.24±0.15**	1.05±0.04**	6.63±0.42
+ IDN 6443 (50 mg/Kg)	4.39±0.27**	0.93±0.06**	6.07±0.14
+ Clofibrate (200 mg/Kg)	4.06±0.26**	0.79±0.11*	6.36±0.44

Table 3: Concentrations of total cholesterol and triglyceride in plasma rats fed HC diet for 28 day treated or untreated IDN 6442, IDN 6443 and clofibrate respectively. Doses (20; 25, 50; 200) are mg of product administered for (kg body weight for day). Values are means ± SEM, *n = 9* rats. *p≤0.05, **p≤0.01 versus HC rats.

4.12 Serum lipid and lipoprotein concentrations

In rats fed with standard diet, cholesterol is mainly carried (about 63%) by the high-density lipoprotein (HDL). The switch to hypercholesterolemic diet, is characterized by an evident increase in the concentration of cholesterol and triglycerides parallel by a marked increase (values ranging between 61 and 77% in relation to the treatment) in the concentration of lipoproteins of density less than <1.006 gr/ml (VLDL) that become the main transporter of cholesterol with a decrease in HDL fraction.

		Hypercholesterolemic diet				
	Standard Diet	Control	IDN 6442 20 mg/Kg	IDN 6443 25 mg/Kg	IDN 6443 50 mg/Kg	Clofibrate 200 mg/Kg
cholesterol mmol/L						
Total	1.19±0.09	5.94±0.16	4.34±0.16**	4.24±0.15**	4.39±0.27**	4.06±0.26**
VLDL	0.33±0.01	4.10±0.31	2.63±0.15**	2.80±0.13**	2.73±0.08**	3.10±0.09**
LDL	0.12±0.01	0.39±0.05	0.29±0.03*	0.24±0.02*	0.25±0.04*	0.08±0.01**
HDL	0.75±0.02	1.45±0.08	1.42±0.08	1.22±0.09*	1.43±0.05	0.85±0.04**
AI	0.6±0.01	3.01±014	2.05±0.08	2.49±0.08	2.08±0.05	3.74±0.04
triglycerides mmol/L						
Total	0.71±0.05	1.21±0.06	0.80±0.07**	1.05±0.04*	0.93±0.06**	0.79±0.11**
VLDL	0.67±0.02	1.15±0.06	0.72±0.07**	0.99±0.04*	0.88±0.04**	0.75±0.04**
LDL	0.04±0.00	0.04±0.00	0.03±0.00	0.04±0.00	0.03±0.00*	0.02±0.00**
HDL	0.01±0.00	0.03±0.01	0.04±0.01	0.02±0.01	0.02±0.00**	0.01±0.00**

Table 4: Plasma total cholesterol and triglycerides and their distribution in the separated lipoproteins of rats fed on a standard or the hypercholesterolemic diet with or without treatment with IDN 6442, IDN 6443 and Clofibrate. AI (atherogenic risk index, estimated in term of VLDL-chol + LDL-chol to HDL-chol ratio). Values are means

± SEM of 9 rats per group for plasma total cholesterol and triglycerides. The cholesterol and triglycerides in the different lipoproteins are from 3 pools of 3 animals per group. Value are means of 3 replicate.*$p \leq 0.05$, **$p \leq 0.001$ *vs* control.

The treatment with the different products (IDN 6442, IDN 6443 and clofibrate), at the concentrations tested in this study, leads to a statistically significant reduction ($p \leq 0.001$, $p \leq 0.05$) in the amount of cholesterol carried by VLDL with little variation in that transported by LDL and HDL, when compared with values detected in hypercholesterolemic untreated rats (**Table 4**). Moreover, it is worth to note that the treatment with the different products based on soy proteins are able to reduce markedly the atherogenic risk index, estimated in term of VLDL-chol + LDL-chol to HDL-chol ratio, compared to that of rats fed on HC diet. In rats treated with 7S α'-rich or the α' E polypeptides at different doses (25 and 50 mg/Kg b.w.), the index was, in fact, reduced by 54, 43 and 52 %, respectively (**Table 4**). It should however be noted that the 7S soybean polypeptides administration did not induce a return to the lipid distribution in the various lipoprotein classes as showed in **Table 5**, in which the data are expressed not as an absolute value (mg/dl) but as a percentage. The same trend is observable as regards the transport of triglycerides by the different lipoprotein fractions in hypercholesterolemic rats.

		Hypercholesterolemic Diet				
	Standard diet	Veicolo	+ IDN 6442 20 mg/Kg	+ IDN 6443 25 mg/Kg	+ IDN 6443 50 mg/Kg	+ Clofibrate 200 mg/Kg
COL (mmol/L)						
Total	1.19±0.09	5.94±0.16	4.34±0.16**	4.24±0.15**	4.39±0.27**	4.06±0.26**
%VLDL	27.8	68.4	60.6	65.7	62.0	76.7
%LDL	10.1	6.7	6.7	5.5	9.5	2.0
%HDL	60.5	24.8	32.7	28.7	32.4	21.2
TG (mmol/L)						
Total	63±4.6	107±5.5	71±6.2**	93±3.3*	82±5.2**	70±9.8**
%VLDL	92.4	95.0	88.4	93.7	94.7	95.8
%LDL	6.5	2.9	4.5	3.9	3.1	2.3
%HDL	1.9	2.3	6.1	2.4	2.2	1.8

Table 5: Effect of administration of IDN 6442 and IDN 6443 on the distribution of lipoproteins cholesterol and triglycerides (percentage) in rats on a hypercholesterolemic diet. Data are expressed as mean ± S.E.M. of 9 rats per group. Treatment: 28 days. Significance between the different groups of animals in hypercholesterolemic diet: ** $p \leq 0.001$ *vs* vehicle.

4.13 Liver lipids

As expected, the administration of the Nath's hypercholesterolemic diet induced a significant increase (approximately 2-fold) of the liver weight, together with a parallel increase in levels of free and esterified cholesterol, compared to animals feed with the standard diet. The free/esterified ratio was 9-fold lower in HC animals (**Table 6**). However, no treatment with the test substance (IDN 6442: 7S α' enriched; IDN 6443 α' extension and clofibrate) was able to induce a substantial change in terms of statistically significant differences compared to the value detected in untreated hypercholesterolemic rats either as lipid content (total cholesterol and triglycerides) or as a distribution (free and esterified cholesterol).

The only statistically significant difference observed was related to the lower content of esterified cholesterol in the groups treated with IDN 6442 and clofibrate. This trend is confirmed if it is taken into account the overall content of esterified cholesterol in the liver. It can be observed, in fact, that the treatment with IDN 6422 (E.C. 178 total mmoles $p \leq 0.05$) and with clofibrate (E.C. 171 total mmoles, $p \leq 0.05$) have been able to induce a statistically significant reduction of the esterified cholesterol to the hypercholesterolemic control group (E.C. 227 total mmoles). Regarding the total content of triglycerides in the liver it can be observed that the treatment with clofibrate was able to induce a clear reduction of liver triglycerides contenent (from 470 total mmoles in hypercholesterolemic *vs* 323 total mmoles in clofibrate treated rats); this reduction is also found when the data are expressed as mmoles of triglyceride per gram of liver tissue. The treatment with IDN 6422 and IDN 6443 did not lead to a statistically significant variation of the content in hepatic triglycerides compared to the values found in hypercholesterolemic controls.

	Standard diet	HC diet				
		Control	IDN 6442	IDN 6443		clofibrate
			20 mg/kg	*25 mg/kg*	*50 mg/kg*	*200 mg/kg*
Body weight, g	341±12	326±10	331±7	350±7	334±7	321±6
Liver weight, g	9.53±0.68	18.90±1.38	17.41±0.67	21.62±2.16	18.41±0.94	19.26±1.23
FC, mmol/g	3.98±0.18	5.92±0.31	6.36±0.44	4.50±0.36	5.61±0.31	9.41±0.08
EC, mmol/g	0.87±0.09	11.99±0.28	10.20±0.12*	12.99±1.41	16.03±0.18	8.86±0.14*
FC/EC mmol/ mmol	4.57	0.49	0.62	0.35	0.35	1.06
TG, mmol/g	7.25±0.77	24.88±1.90	29.74±1.37	35.54±1.29	28.29±1.92	16.82±1.63**

Table 6: Data are expressed as mean ± SEM of three pool for 9 rats in the group. Each analysis was carried out in triplicate. * $p \leq 0.05$, ** $p \leq 0.001$ *vs* vehicle.

4.14 Evaluation of the LDL receptor expression by RT-PCR

The treatment with α'E-polypeptides has induced an increased expression of the mRNA of LDL receptor (LDL-R) compared to that recorded in animals fed on hypercholesterolemic diet and treated with vehicle alone, as show in **Figure 13**. In particular, the administration of IDN 6442 and IDN 6443 at the highest concentration (50 mg/kg b.w.), induced an increased expression of the mRNA of LDL-R (+1.7 fold) compared to values recorded in control group. The compound IDN 6443, at a concentration of 25 mg/kg body weight, resulted in a 1.8-fold increase, whereas the treatment with clofibrate induced a 2 fold increase in the

expression of mRNA of LDL-R compared to those observed in the hypercholesterolemic control group.

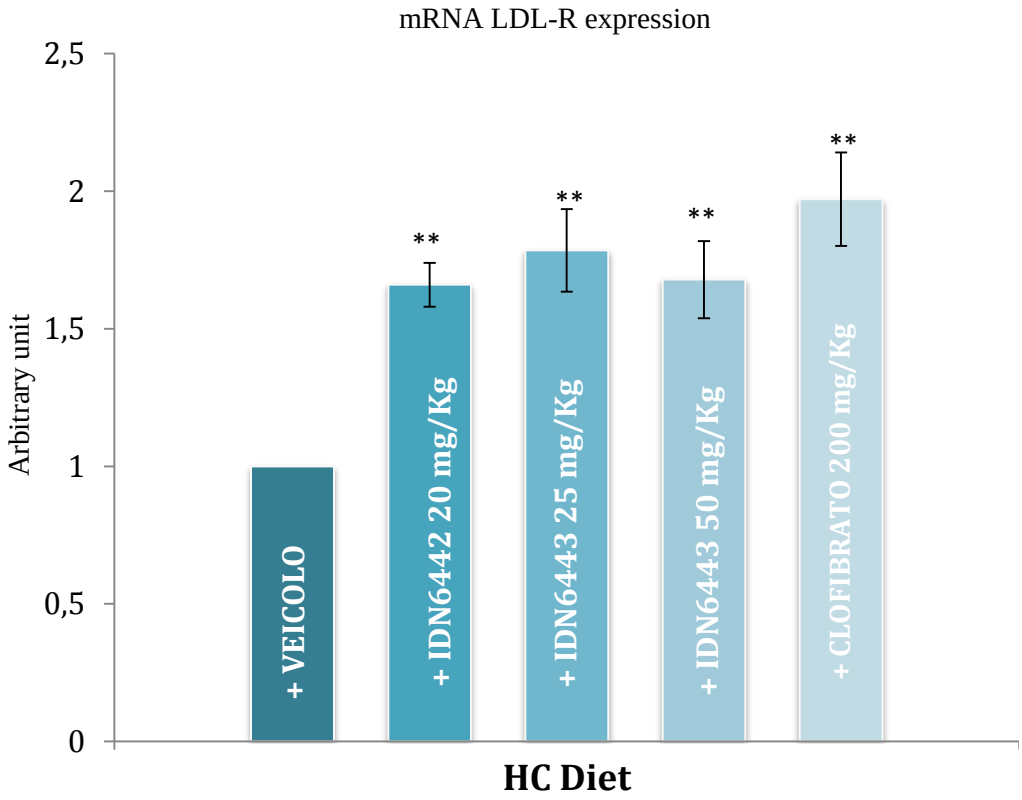


Figure 13: Effect of the administration of the peptides IDN6442 (20 mg/Kg), IDN6443 (25 and 50 mg/kg) and clofibrate (200 mg/kg), in hypercholesterolemic rats, the mRNA expression of the LDL receptor compared to the HC control treated with CMC. Data are expressed as mean ± S.E.M. (pool n. 3) relative to 9 rats per group. Each analysis was conducted in triplicate. Significance between the different groups of animals fed on hypercholesterolemic diet: ** $p \leq 0.001$ *vs* vehicle.

4.15 Effect of synthetic peptides on lipid homeostasis in HepG2 cells

Sterol response element-binding protein SREBP-2 preferentially regulates genes involved in cholesterol homeostasis by activating the transcription of 5-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoA Red) and low density lipoprotein receptor (LDL-R) (Brown and Goldstein, 1997)**.** The results obtained in the present study pointed out that some of peptides, belonging to α', α and β subunits of 7S soy globulin, when exposed to HepG2 cells displayed different effect on the expression of LDL-R, SREBP-2 and HMGCoA red mRNAs, as recorded by real time PCR. As expected (**Figure 14**) simvastatin induced a marked increase of LDL-R (+1.5 fold), SREBP-2 (+1 fold) and a marked decrease of HMGCoA red (-60%) *vs* untreated cells. Peptide C (RKQEEDEDEEQQREE) and E (KNPQLR) tested at 1 μM concentration displayed increased expression of mRNA LDL-R (+2.3, and +1.9 fold), SREBP-2 (+1.8, and +3.3 fold) and a decreased expression of mRNA HMGCoA red (-30, and -70 %, respectively) *vs* values detected in untreated cells (arbitrary unit = 1). This trend was similar to that observed in HepG2 cells treated with 1 μM simvastatin. Peptide D showed similar behavior at higher concentration (10 μM); increased expression of mRNA LDL-R (+0.5 fold), SREBP-2 (+2 fold) *vs* untreated HepG2 cells have been detected. Although the amino acid sequences of peptides A and B are present in α' extension (α'E) polypeptide they were ineffective, at both tested concentrations, on LDL-R and SREBP-2 mRNA expression; although an inhibition of HMGCoA red

expression was observed. This result could suggest that these amino acid sequences are not completely involved in the lipid lowering mechanism.

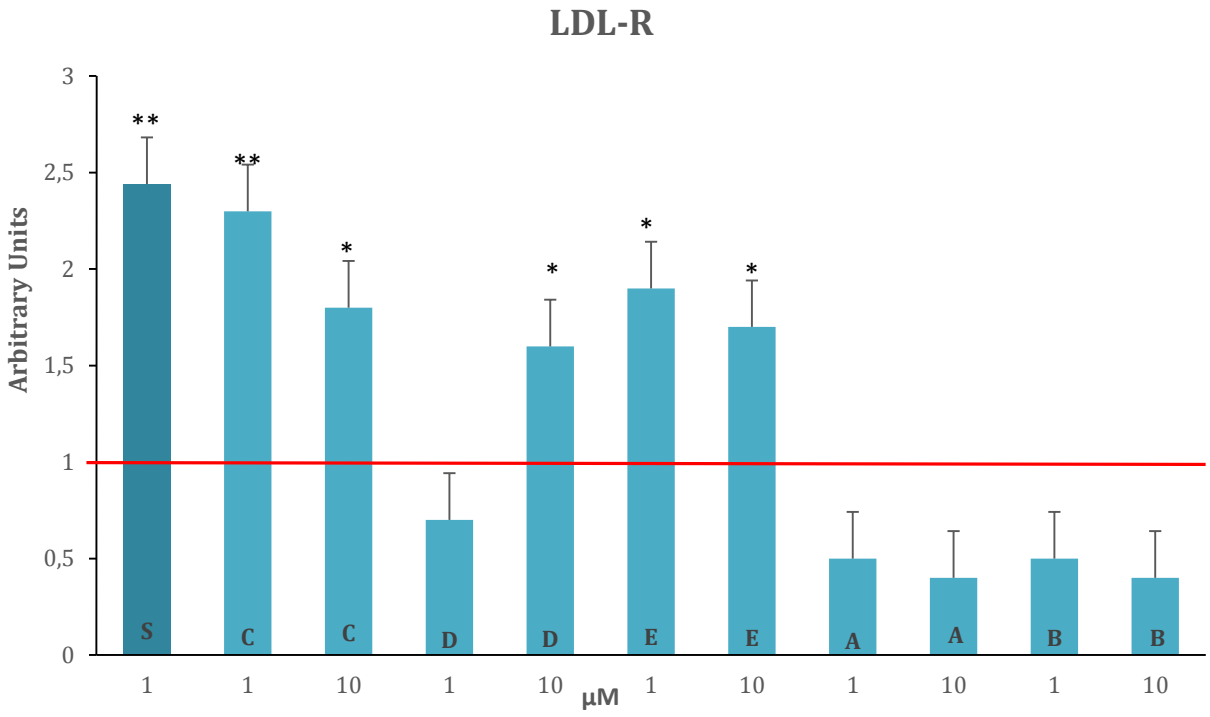


Figure 14

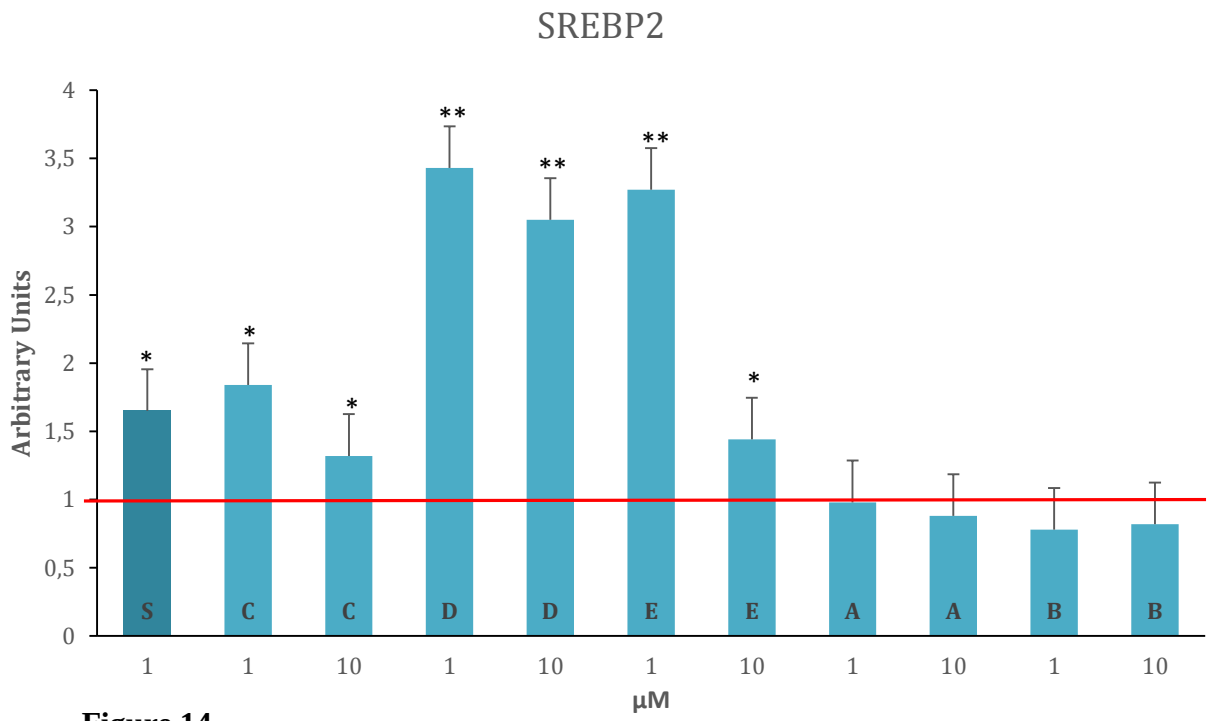


Figure 14

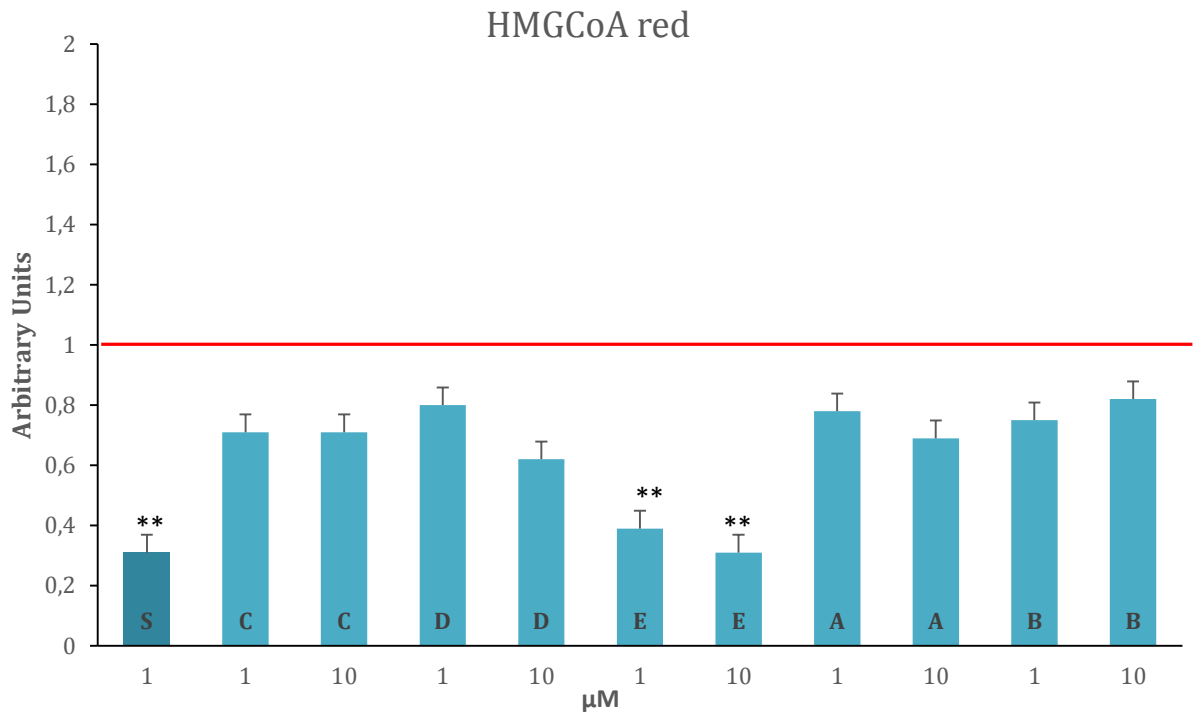


Figure 14: Effect of synthetic peptides on the expression of LDL-R, SREBP-2, and HMGCoA red mRNAs by RT-PCR. HepG2 cells were incubated for 16 h in MEM + 5% LPDS in the presence of each peptide (1 and 10 μM) or simvastatin (1 μM). Results were

compared to the values recorded in untreated cells (arbitrary unit=1, red line). Values are the mean ±S.D. of three independent triplicates. Abbreviation: S=simvastatin. ** $p \leq 0.005$; *$p \leq 0.05$ *vs* negative control (untreated cells)

4.16 Effect of synthetic peptides from β-conglycinin on lipid accumulation, FAS activity and anti-oxidant activity in 3T3-L1 adipocytes

Table 7 presents the percentage inhibition of lipid accumulation in 3T3-L1 adipocytes after 48 h of treatment with 50 μM peptides compared to their controls. Treatment with synthetic peptides from β-conglycinin decreased lipid accumulation in the adipocytes from 12 to 27 % compared to negative controls (untreated cells). All synthetic peptides were potent inhibitors of FAS activity. In particular, the large peptide C (RKQEEDEDEEQQRE) resulted the most active compound to reduce lipid content. It was shown, in fact, a higher inhibitory effect (-27% vs untreated cells) on lipid accumulation and a significantly higher ($p \leq 0.05$) potency (IC_{50}= 16 mM) on FAS inhibitory response compared to the other peptides under study. Moreover, peptides B (QKEEEKHEWQ) and D (EITPEKNPQLR) showed decreased lipid accumulation (18 and 19%, respectively) and the same potency (26 and 25 μM, respectively). Peptides A (SEEEEEDQ) and E (KNPQLR) were characterized by higher IC_{50} and lower inhibition of lipid accumulation in comparison with the other peptides tested. These results confirm previous data where the FAS inhibitory potency of soy peptides also correlated

with their molecular mass; in addition the large inhibitory peptide (RKQEEDEDEEQQRE) bond to thioesterase domain of human FAS blocking the active site of such domain, as predicted by molecular docking (Martinez-Villaluenga et al., 2010). The ORAC assay is widely used to determine antioxidant capacity (AC) *"in vitro"* by measuring the scavenging activity of peroxyl or hydroxyl radicals (Davalos et al., 2004) . The amino acid sequence of peptides tested belong to α', α and β subunit of β-conglycinin none of them showed a significant anti-oxidant capacity, since the results obtained varied between 11 and 15 μmol Trolox equivalent/mmol peptide (**Table 7**).

Peptide	*% inhibition of lipid accumulation*	*FAS activity IC_{50} (μM)*	*AC*
A	12 ± 1.3*	56*	11
B	18 ± 2.0*	26*	15
C	27 ± 3.2**	16**	11
D	19 ± 3.1*	25*	13
E	14 ± 1.8*	73*	13

Table 7: Antioxidant activity and inhibitory effect of synthetic peptides (50 mM) on lipid accumulation in 3T3-L1 adipocytes after 48 h and on fatty acid synthase (FAS) activity. AC= anti-oxidant capacity (μmol Trolox equivalents/mmol peptide). Values are the mean ± S.D. of three independent triplicates. ** $p \leq 0.005$; *$p \leq 0.05$ *vs* negative control (untreated cells).

4.17 Effect of synthetic peptides on inflammation markers in RAW 264.7 cells

It is known that inflammation is part of the defense mechanism against infectious agents and injury. We studied the effect of each peptide (25 μM concentration) on the protein expression of iNOS in macrophages using Western blot. **Table 8** shows that the expression of iNOS was differently affected by synthetic peptides. Western blot analyses (data not shown) pointed out the protein expression of iNOS was barely detected in the non-stimulated cells. However, the level increased markedly after 24 h of LPS treatment. Peptide A (SEEEEEDQ), B (QKEEEKHEWQ), and E (KNPQLR) reduced the expression of iNOS by -71, -75 and -44%, respectively. COX-2 appears to be the dominant source of prostaglandin formation in inflammation, although its role in the reduction of inflammation was indicated in COX-2 Knock Out mice. To evaluate the role of synthetic peptides in inflammation, expression of COX-2 in LPS-stimulated RAW 264.7 cells was measured. As indicated in **Table 8**, COX-2 expression was markedly reduced by peptide A, B, and E by 54, 34, and 79 %, respectively. Peptide C (RKQEEDEDEEQQRE), and peptide D (EITPEKNPQLR) showed lower inhibitory activity on both inflammation markers. These data support previous results indicating that peptides released by *"in vitro"* or "*in vivo"* enzymatic digestion from different protein components present in the soybean seed are characterized by different physiological functions.

Peptide	*% inhibition of iNOS*	*% inhibition of COX-2*
A	71 ± 3.8**	54 ± 1.6 **
B	75 ± 2.1 **	34 ± 2.5 **
C	43 ± 2.9 *	20 ± 1.7 *
D	25 ± 1.7 *	25 ± 2.3 *
E	44 ± 2.8 **	79 ± 3.6 **

Table 8: Effect of synthetic peptides (25 mM) on pro-inflammatory responses in LPS-induced RAW 264.7 macrophages after 24 h. Values are the mean ± S.D. of three independent triplicates. ** $p \leq 0.005$; *$p \leq 0.05$ *vs* negative control (untreated cells).

4.18 Effect on plasma lipids and LDL receptor expression of the "*in vivo*" treatment with synthetic peptide (NPDNDE)

Total cholesterol and triglyceride were analysed at the end of 21 days of treatment with NPDNDE (5 mg/Kg b.w. /day), results are shows in **Figure 15**. The data were compared with that obtained in rats treated either with whole α' (20 mg/Kg b.w.) or clofibrate (200 mg/Kg b.w.), as reference drug. The oral administration of NPDNDE peptide resulted in lower plasma lipid level, cholesterol (-37%, triglycerides, -38%) *vs* value recorded in rats fed casein-cholesterol diet alone. Four-fold amount of α' chain reduce cholesterol and triglyceride by 37 and 25% respectively. Similar results were obtained in clofibrate-treated rats. Moreover, synthetic peptide increased the expression of mRNA LDL-R (+53 %) (**Fig 15 B)**, thus restoring receptor activity suppressed by HC diet.

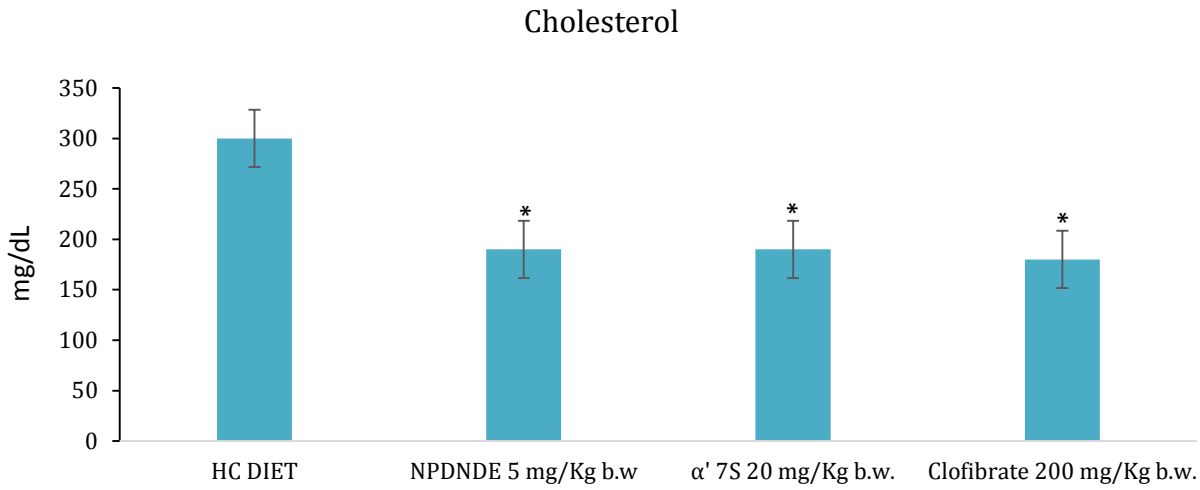


Figure 15 A

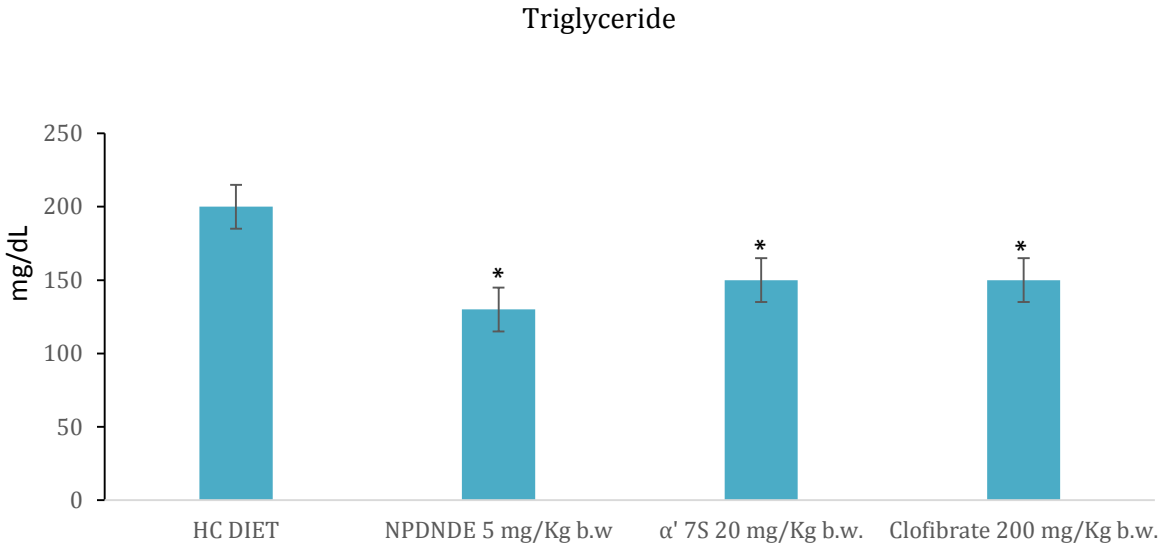


Figure 15 B

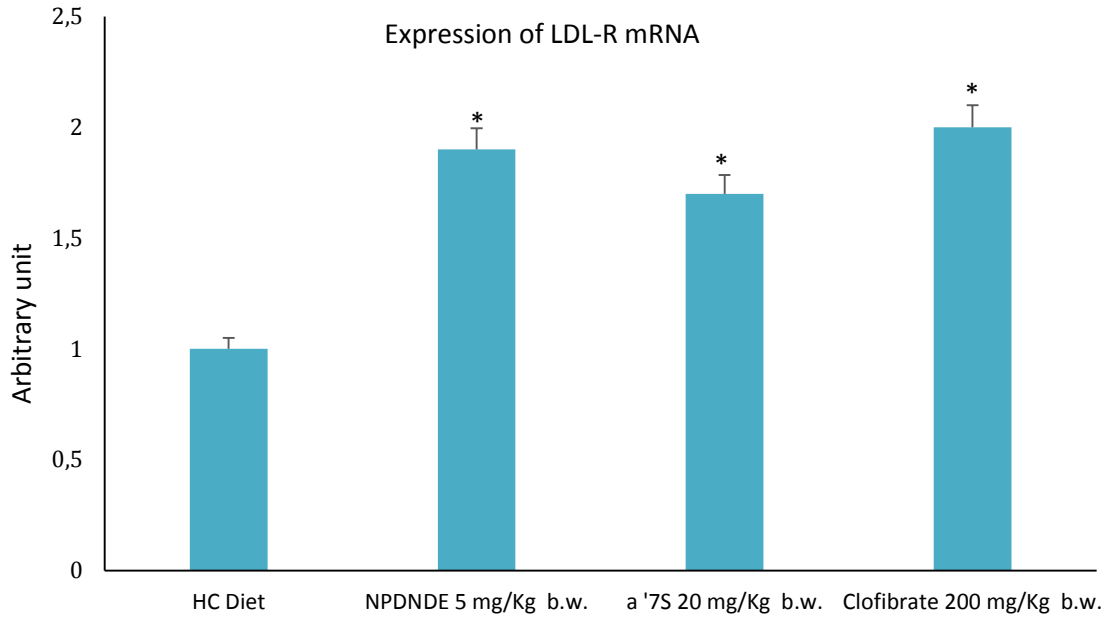


Figure 15 C

Fig 15: (A) Plasma lipid levels, cholesterol and triglycerides in HC fed rats after 21 days oral administration of NPDNDE peptide. **(B)** Expression of LDL-R mRNA in liver of HC rats treated with NPDNDE peptide. Results were compared to the values detected in untreated rats (arbitrary unit = 1) *p≤0.05 *vs* control.

4.19 Modulation of SREBP-2, LDL-receptor, and PCSK9 mRNA expression in HEPG2 by NPDNDE peptide

To evaluate the potential effect of NPDNDE peptide on the modulation of LDL-R, SREBP-2 and PCSCK9, HepG2 cells were incubated for 6, 8 and 16 h in MEM +5% LPDS w/wo whole α' subunit (3.5 μM), or synthetic peptide (10^{-5} M), or simvastatin (1 μM), as a positive control. At the end of incubation, cells were processed for LDL-R, SREBP2 and PCSK9 mRNAs by RT-PCR. Increased mRNA expression of LDL-R at 6h by + 2.5 fold (for both substances) (**Figure 16 A**), for SREBP2 (8h: +1.5, +1.2 fold), and PCSK9 (8h: +3,5; +1.5 fold) were

detected in cells exposed to NPDNDE, respectively *vs* α'and simvastatin exposed cells (**Fig. 16 B-C**).

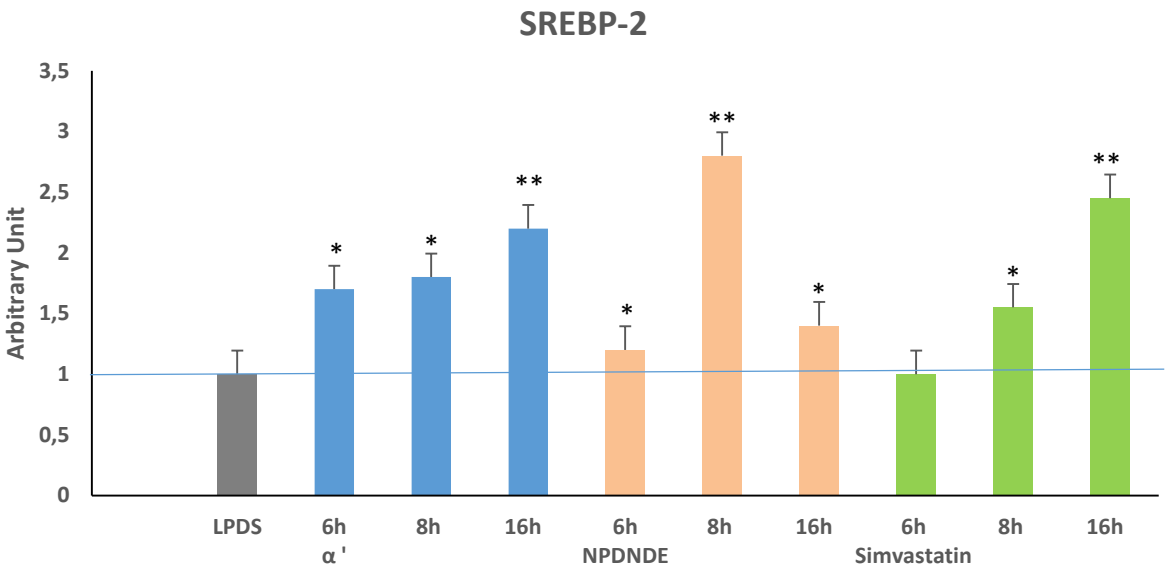


Figure 16 A

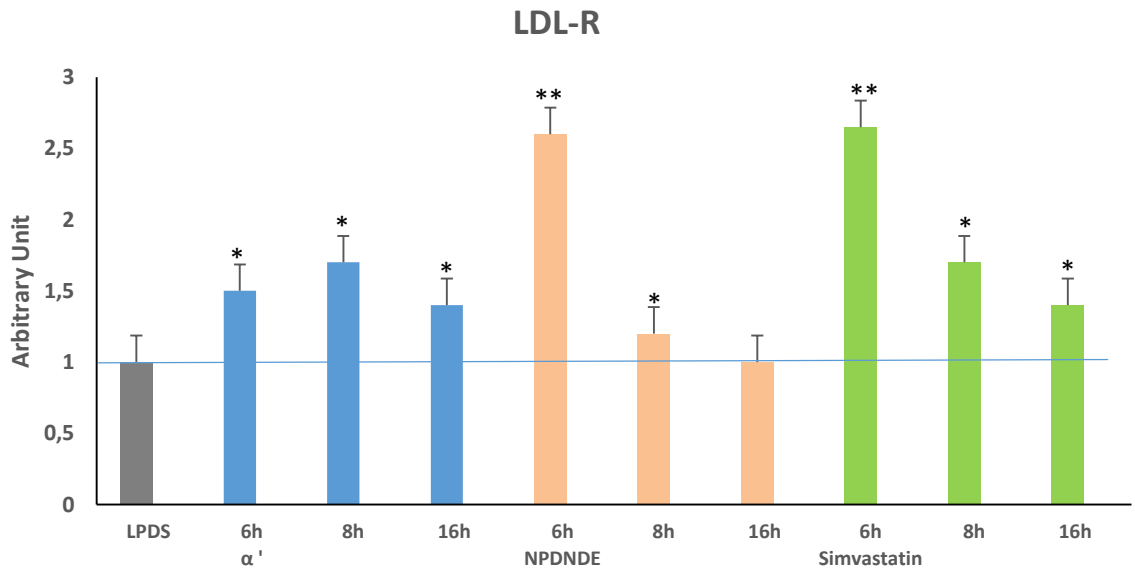


Figure 16 B

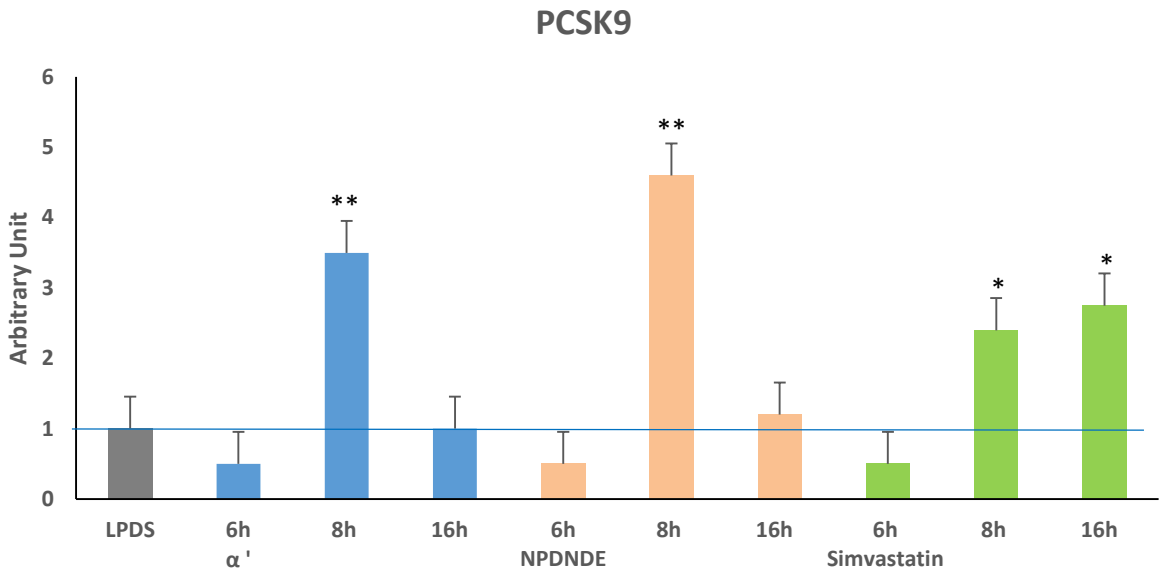


Figure 16 C

Fig.16: Effect of NPDNDE peptide on the expression of LDL-R, SREBP-2 and PCSK9 mRNA by RT-PCR. HepG2 were incubated with 3.5 µM α'7S subunit or synthetic peptide (10^{-5} M). Results were compared to the values recorded in untreated cells (arbitrary unit =1). Values are the mean ± S.D. of three independent triplicates. * p≤0,05; **p≤0,005 *vs* negative control.

5. DISCUSSION AND CONLUSION

Type 2 diabetes is a chronic metabolic disorder, often characterized by Insulin resistance, which leads to several secondary complications, including hypertension, atherosclerosis, coronary artery disease and hyperlipidemia (King et al., 1998). Approximately 150 million people worldwide are affected by the disease at present, with a projection of 300 million people being affected by 2025. Diabetes has become a serious public health problem, particularly in developed countries (Parikh et al., 2007). Research in an effective anti-diabetic agent, in addition to those already available, would be of great interest for the treatment of type 2 diabetes. Legume seeds, due to the nutraceutical potentialities of some of its proteins, may provide an alternative to the usual treatment of glucose metabolism disorders. Specifically, lupin flours, such as other pulses, are characterized by a low glycaemic index, so they can be useful in the prevention of IR in human subjects (Duranti, 2006).

Lupin seeds are characterized by a high content of protein, about 35%, and by low levels of isoflavones and anti-nutritional factors (Champ, 2001). The anti-diabetic activity of toasted lupin seeds was initially described, in the middle of the last century, by Ferranini & Pirolli (Ferranini, 1937) and by Orestano (Orestano, 1940), who proposed lupin as a substitute for the insulin therapy in mild-to-medium diabetes mellitus, but no further studies have been carried out to identify the molecule responsible of this biological effect. A few years ago the isolated lupin protein was found to bind insulin in vitro, by using both affinity chromatography on an insulin-bound matrix and Surface Plasmon Resonance (SPR). In parallel, suitable amounts of isolated protein were tested in glucose overloading trials on

rats. The results, reported in a significant decrease of the under-curve area of time course of glycaemic concentration with an effect similar to that of metformin at about half the dose of the lupin protein (Magni et al., 2004). Studies on both the biological activity of lupin γ-conglutin and its metabolic effects are currently ongoing.

In the attempt of identifying the active principle responsible of the glucose controlling capacity attributed to a lupin seed component, a study on a specific lupin protein, named conglutin γ, was initiated. γ-Conglutin was considered a good candidate on the basis of previous finding that an homologous soybean seed protein, named Bg7S, was found to display a binding capacity to some small regulatory proteins, including insulin (Hanada and Hirano, 2004). However, these precursor studies primarily focused on the modalities and possible physiological role of the interaction between the soybean protein and an endogenous regulatory peptide, thus neglecting the potential effect(s) of the soybean protein on the human body carbohydrate metabolism. Due to the amino acid sequence similarity (63%) between soybean Bg7S (SwissProt Database accession number: P13917) and lupin γ-conglutin (SwissProt Database accession number: Q9FSH9), a molecular and metabolic study on the latter protein was recently undertaken.

In this work we have studied the potential effect of γ-conglutin treatment glucose lowering effect in "*in vivo*" and "*in vitro*" models, monitor the protein internalization in cells and studied the interference whit the insulin receptor pathway.

In the first study, the addition of 10% glucose in drinking water induced hyperglycaemia and hyperinsulinaemia in rats similar to that observed in human subjects. No side effects have been detected during the experimental period, such as those recorded in animals undergoing streptozotocin (Dabros et al., 2004) or alloxan (Pari and Saravanan, 2002) treatment to mimic diabetes. γ-Conglutin administration has been demonstrated to counteract the plasma glucose increase as well as to improve the insulin sensitivity, normally reduced by the glucose rich drinking-water. In the γ-conglutin treated rats, the insulin sensitization was increased significantly, as indicated by the 48% reduction in the homeostasis model of insulin resistance. It is worth noting that the hypoglycaemic effect in vivo was obtained by the use of a preparation, which contained a γ-conglutin amount corresponding to the lowest dose previously used in acute trials of glucose overload (Magni et al., 2004). Moreover, lower glucose levels were detected in γ-conglutin treated rats following oral glucose overload; these results were confirmed by lower glycaemia in fasting and two hours postprandial conditions. Recently, Terruzzi et al. (Terruzzi et al., 2011) have demonstrated that γ-conglutin may regulate muscle energy metabolism, protein synthesis and major histocompatibility complex gene transcription through the modulation of insulin signaling pathway. Moreover, γ-conglutin resistance to proteases at neutral pH values (Capraro et al., 2009) could explain the maintenance of its activity after 48 h of incubation, as we have observed in HepG2 cells.

In the present report the role of γ-conglutin in controlling glucose concentrations has been assessed using the purified protein in cell assays, however, the synergic effect of other protein/peptide components present in the type F sample used in "*in vivo*" experiment, cannot be excluded. In addition, the reduced increase in plasma glucose (-24%) and insulin (-33%) levels, recorded in rats following γ-conglutin treatment *vs* the values found in the pair fed animals, could be of pharmacological relevance. It is noteworthy, in fact that these decreases have been obtained by the use of a single daily administration of a purified food protein. The prerequisite for any biological activity is the interaction of the component under study with components present on plasma membrane or its direct entry into the target cells. This, in turn, implies that the protein can reach the district of its action in an intact or, at least, still active form. A number of plant and seed proteins have been found to be fully or partially resistant to proteolytic enzymes into the gastro-intestinal tract in force of their peculiar amino acid sequences and or structures (Clemente et al., 2000). In this respect, γ-conglutin was shown to undergo an 'all or none' mechanism of proteolytic degradation "*in vitro*". In fact, when γ-conglutin native conformation was lost, the protein became susceptible to proteolytic enzymes; conversely, if the native structure was preserved, as it occurs at any pH value greater than 3.0, no degradation was observed (Capraro et al., 2009). In this work we used a different approach to get complementary information on the internalization and possible covalent modifications of γ-conglutin in HepG2 cells, as the basis for further studies aimed at unveiling the mechanism of action of this protein. Remarkably, microscopy showed that the γ-conglutin did get in contact

with the cell in the form of molecular aggregates. The spontaneous formation of these aggregates at neutral pH values (Capraro et al., 2010) may facilitate the interaction with the cell structures devoted to captation of foreign materials, such in the cases of albumin in astrocytes (Bento-Abreu et al., 2009) and human 1-acid glycoprotein (AGP) (Komori et al., 2012). Intracellular accumulation of γ-conglutin is particularly evident in confocal microscopy at 6 h incubation, when also glucose uptake is significantly increased (64%). The 2D electrophoresis was carried out on HepG2 cell lysates at the same time of treatment and the lupin protein was detected. The position of its two subunits in the map suggested that the covalent integrity of the protein was preserved. However, a shift in the map of γ-conglutin related spots to more acidic pH values denoted a significant pI change of some polypeptides. Mass spectrometry unequivocally showed that this pI drift was due to multiple phosphorylation of the protein. No phosphorylation events have been previously described for γ-conglutin.

To elucidate the cell internalization routes of γ-conglutin, experiments using specific inhibitors of endocytosis, were carried out on HepG2 cells and the uptake of the protein was detect by fluorescence-activated cell sorting (FACS). We have chosen Chlorpromazine (CPZ) and methyl-β-cyclodextrin (MβCD) to inhibit the clathrin-mediated endocytosis pathway (Wang et al., 1993) (Rejman et al., 2005) (Manunta et al., 2004; Subtil et al., 1999), filipin and genistein (Aoki et al., 1999; Orlandi and Fishman, 1998; Rejman et al., 2005) to inhibit the raft/caveolae-mediated endocytosis pathway and amiloride to inhibit macropinocytosis pathway (Hewlett et al., 1994). The treatment with amiloride significantly affected the

FITC-γ-conglutin internalization, with an approximately 69% decrease in the amount of cellular uptake. The treatment with other inhibitor such as MβCD, CPZ and genistein showed a lower reduction in the uptake of protein (40 %, 33% and 14% respectively). Pretreatment of the cells with filipin, had no significant effect on γ-conglutin uptake. These results suggest that in HepG2 cells γ-conglutin was internalized preferentially trough macropynocytosis pathway.

On the other hand, the macropinocytotic pathway is one of the major physiological mechanism for the endocytic uptake of certain peptide sequences (Kaplan et al., 2005; Nakase et al., 2004; Wadia et al., 2004) compared with other routes of entry inside the cells.

HepG2 cells are largely used for biochemical and nutritional studies as a cell culture model of human hepatocytes because maintain their morphology and most of their function in culture (Brandon et al., 2006; Nakajima et al., 2000). Moreover, HepG2 cells has been used to study the hepatic glucose production and the modulation of the insulin pathway "*in vitro"*system (Lin et al., 2007; Lin and Lin, 2008).

To assess whether the γ-conglutin was involved in the activation of intracellular kinases like to the insulin signaling cascade on IRS-1/PI-3-kinase pathway on the glucose homeostasis (Saltiel and Kahn, 2001), we have processed cell protein from HepG2 cells. Firstly observed that incubation of cells with γ-conglutin alone or in the presence of insulin, did not induce an increase in the levels of AMPK phosphorylated proteins; metformin, as expect (Hawley et al., 2002) showed the highest levels of the pAMPK/AMPK ratio.

However when cell was treated with γ-conglutin, for 6 hours we observed a five fold increased in the phosphorylation of AKT and a similar rise in p-AKT value is present in all experimental conditions. To confirm this data, HepG2 cells were exposed to a selective inhibitor of AKT (LY294002); data showed that after 6 or 24 hours of treatment, LY294002 decreased the p-AKT levels in all cells incubated with this inhibitor with or without presence of γ-conglutin or insulin alone or in combination. These data suggest that, this protein triggers in the PI3K/AKT metabolic pathways involved in the glucose homeostasis. The linkages between diet and health are no longer a matter of discussion. Moreover, the specific and/or limited effects of current drug treatments for diabetes, combined with dangerous effects that most of them induce, have fueled the search for alternative medicine. Furthermore, the specific role of many food components, their synergies and antagonism are still a largely unexplored area. The case of dietary proteins/peptides is particularly intriguing due to the dramatic changes they may undergo from food production to food digestion. Our findings, by showing that γ-conglutin can be taken up by HepG2 cells in an intact form and is modified by multiple phosphorylation, open the way to more focused studies aimed to understand the mechanisms of action of this bioactive lupin seed protein able to lower glycaemia in animals and humans.

Further studies, aimed at understanding the protein moiety of γ-conglutin responsible for the glucose-lowering effect and the molecular mechanism thereby, are currently being undertaken. Moreover, the hypothesis that γ-conglutin could act as an insulin-like agent should not be excluded. In conclusion, the present study

provides the "*in vivo*" and "*in vitro"* evidence of the involvement of γ-conglutin on cell glucose homeostasis, thus suggesting the potential use of this food protein in the control of glycaemia in patients with manifest or pre-clinical diabetes as well as for applications as functional foods and dietary supplements.

Soybean (Glycine max) seed proteins have proved to be active in plasma cholesterol and triglyceride lowering activity (as demonstrated in the recent meta-analysis involving different randomised controlled intervention studies (Anderson and Bush, 2011; Harland and Haffner, 2008). Controversial discussions on the key role played, respectively, by proteins, isoflavones, fibers and other minor components are still ongoing, as witnessed by another review on the topic (Dewell et al., 2006). But, well as the fact that each compound is probably involved in different pathways and mechanisms on the role of soybean proteins, in October 1999, the U.S. Food and Drug Administration (FDA) approved a health claim that allowed food label claims for reduced risk of heart disease on foods that contain more than 6.25 g of soybean protein per serving ((FDA), 1999). In particular, a daily soybean protein intake of 25 g was considered beneficial, based on a number of previous clinical observations.

Although the substitution of animal with plant proteins in the diet of hypercholesterolemic individuals is known to be associated with a significant cholesterol reduction since long (Hodges et al., 1967) and the identification of the responsible molecules, including phytoestrogens, dietary fibers, protein components, etc., is still matter of debate, this claim is one of the rare examples of an official acknowledgement of this beneficial effect of a dietary protein on health.

However, the amounts of soybean proteins needed to trigger the biological activity are extremely large from the dietetic point of view. Therefore in the last years the identification of the active molecule(s) has been strongly pursued as a strict requirement for the design of suitable intervention protocols. The first *"in vivo"* evidence of the involvement of the 7S globulin family of soybean storage proteins was obtained in 1992 by Lovati et al. (Lovati et al., 1992). In this study, the administration of 7S soybean globulin significantly reduced cholesterolemia (-35%) *vs* casein fed rats. These findings were confirmed by studies on the stimulation of low density lipoproteins (LDL) receptors and degradation of LDL in cultured hepatocytes (Lovati et al., 1992). Later on, an evidence of the involvement of one of the 7S globulin subunits, namely the α′ subunit, was indirectly shown by the lack or reduced activity of a soybean cultivar naturally devoid of this polypeptide chain (Manzoni, 1998). Further studies (Manzoni et al., 2003) confirmed these previous findings, by showing the up-regulation of LDL-R by the α′ subunit. The direct demonstration of the key role played by the α′ subunit in reducing plasma lipids in rats fed cholesterol-rich diet came later, thanks to the isolation of relatively large amounts of this subunit from the 7S globulin oligomer (Duranti et al., 2004). This achievement was based on the observation that the N-terminal region of the α′ subunit, the so-called extension region, differed from the corresponding region of α subunit being richer in histidine residues (SwissProt Database accession numbers of α′ and α subunits: P11827 and P13916, respectively). On this basis, a preparative metal affinity chromatography column, was used to isolate the α′ subunit in gram amounts, making it possible to test the

isolated subunit in hypercholesterolemic animal models as well as in cell assays (Duranti et al., 2004). These results, by showing that the α′ subunit oral administration to rats significantly reduced plasma cholesterol and triglyceride levels, confirmed "*in vivo"* the results obtained with soybean proteins, 7S globulin and α′ subunit, in isolated cell systems, ruled out any isoflavone effect (Fukui et al., 2002) and substantiated the role of a legume dietary protein in the management of dislipidaemia. In addition, the up-regulation of the β-VLDL receptors in liver cells from hypercholesterolemic rats in response to oral treatment with this polypeptide was demonstrated (Duranti et al., 2004). Nonetheless, since the purification procedure devise is not easy to scale up, by implying a denaturation step to dissociate the 7S globulin oligomer, the possibility of cloning and expressing in proper host organisms the active subunit or a fragment thereof, bearing the biological activity, is currently being investigated by own group. Although the mentioned results represent a relevant point in the utilization of a dietary protein for the control of cholesterol and triglycerides hematic levels, still the mechanism of action of this protein is not understood. From a recent study, it appeared that soybean protein in the diet can also increase the size of LDL, a known protective effect against arterial diseases (Desroches et al., 2004). Moreover, the interaction of the soybean 7S globulin with thioredoxin 1 and cyclophilin B, two cell protein components involved in the protection from oxidative stress, was demonstrated (Manzoni et al., 2003). Due to the complexity of the metabolic and regulatory pathways involved, further research activities with suitable models and experimental approaches are needed to identify underlying

mechanism(s). All these direct evidences support the epidemiological association between soybean protein intake and reduced cardiovascular risk (Anderson and Major, 2002). Indeed, a positive effect of soybean proteins on carotids lesions in high fat diet fed rabbits (Castiglioni et al., 2003) and a global anti-atheromatous effect in mice (Adams et al., 2004) suggest a link between soybean protein consumption and atherosclerosis reduction.

In this context, also the involvement of pulses other than soybean in the control of lipidaemic homeostasis has been considered (Dabai et al., 1996). In particular, a report on the reduction of plasma total and LDL-cholesterol induced by lupin proteins in rats on a high fat diet has appeared (Sirtori et al., 2004). In attempt to identifying the putative responsible molecule, γ-conglutin, a lupin protein which will be extensively mentioned in the following paragraph, has been put forward. Previously, another legume seeds, namely faba bean (Vicia faba) was proved to have a beneficial impact on lipid profiles. In human studies, Weck et al. (Weck et al., 1983) demonstrated that in hypercholesterolemic subjects, faba bean proteins had a cholesterol-reducing efficacy comparable to that of soybean protein. This effect was later confirmed on hypercholesterolemic rats by Macarulla et al. (Macarulla et al., 2001).

In this study we tested different peptides from 7S soy globulin in "*in vitro*" and "*in vivo*" models.

The results obtained in the present study, indicated that the compound IDN 6443 (α'E), administered to rats fed hypercholesterolemic diet, while not being able to reduce the levels of liver lipids, was effective in inducing a stimulation, not dose

dependent , in the expression of the LDL receptor. In particular, the administration of the reference compound (7Sα'-rich, IDN6442) (50 mg/kg b.w.), induced an increased expression of the LDL-R (+1.7 fold) compared to the values recorded in the control group. The compound IDN 6443 (α'E), at the concentration of 25 mg/kg b.w. resulted in a 1.8 fold increase, whereas the treatment with clofibrate induced a 2 fold increase in the expression of mRNA of LDL-R compared to those observed in the hypercholesterolemic control group. These results confirmed previously published data (Duranti et al., 2004) on the positive modulation of the LDL receptor in rats treated with the reference compound (IDN 6442).

More studies aimed to identify the cellular molecular target in view of a possible association of this product with other lipid-lowering drugs, are at present object of evaluation. In addition, the results obtained in this experiment indicate that IDN 6443 (α'E), administered to rats fed hypercholesterolemic diet, was able to reduce, in a statistically significant manner, the levels of plasma lipids, both total and associated with the different lipoprotein classes. IDN 6443 (α'E), was used at concentrations of 25 mg/kg b.w. (5.65 mg protein/Kg b.w.) and 50 mg/Kg b.w. (11.3 mg protein/kg b.w.); although the concentration of active principle is minimal compared to the amount of product administered, this product was characterized by a remarkable reduction activity of both total cholesterol (-29 and 26%, respectively with 25 and 50 mg/kg b.w.) and triglycerides (-13 and - 23%, respectively with 25 and 50 mg/kg b.w.). The lipid-lowering effect is comparable to that observed in rats treated with 200 mg/kg of clofibrate, as a reference drug.

Alteration of cell lipid homeostasis plays a key role in the development of chronic diseases, such as heart failure and metabolic syndrome, which are typical of the affluent countries. Previous studies indicated that β-conglycinin lowers plasma levels of cholesterol and triglycerides in humans (Kambara, 2002; Ma et al., 2013), as well as in animal models of human hyperlipidemia (Duranti et al., 2004; Ferreira Ede et al., 2011) and induces an up-regulation of LDL high affinity receptors as recorded in hypercholesterolemic patients (Lovati et al., 1987) and cell cultures (Lovati et al., 1992; Mochizuki et al., 2009). Although, "*in vitro"* and "*in vivo"* data suggest the α' subunit as responsible of the biological effect, it is not possible to exclude that other subunits of β-conglycinin can be involved in lipid modulation and/or in activities to them related. Moreover the results obtained in the present study pointed out that some of peptides, belonging to α', α and β subunits of 7S soy globulin, when exposed to HepG2 cells displayed different effect on the expression of LDL-R, SREBP-2 and HMGCoA red mRNAs, as recorded by real time PCR. As expected simvastatin induced a marked increase of LDL-R (+1.5 fold), SREBP-2 (+1 fold) and, a marked decrease of HMGCoA red (-60%) *vs* untreated cells. Peptide C (RKQEEDEDEEQQREE) and E (KNPQLR) tested at 1 μM concentration displayed increased expression of mRNA LDL-R (+2.3, and + 1.9 fold), SREBP-2 (+1.8, and +3.3 fold), and a decreased expression of mRNA HMGCoA red (-30, and -70 %, respectively) *vs* values detected in untreated cells. This trend was similar to that observed in HepG2 cells treated with 1 μM simvastatin. Peptide D showed similar behavior at higher concentration (10 μM);

increased expression of mRNA LDL-R (+0.5 fold), SREBP-2 (+2 fold) *vs* untreated HepG2 cells have been detected.

In a previous work, a marked reduction of apo B accumulation has been found in the media of HepG2 cells exposed both to whole 7S soy globulin and to a commercial isoflavone-poor soy concentrate (Croksoy^{R}70) (Lovati et al., 2000). In addition, an increased up-regulation of LDL-R was detected in the same cell line after incubation with a synthetic peptide (MW 2271 Da) corresponding to positions 301-324 of α' subunit of 7S soy globulin (Lovati et al., 2000). Moreover, Mochizuki et al. (Mochizuki et al., 2009) showed that short peptides (7S-peptides) derived from highly purified β-conglycinin suppressed the secretion of apolipoprotein B-100 in the medium and increased the cleaved nuclear form of SREBP-2 in HepG2 cells. Recently, a LDL-R transcription stimulating peptide (FVVNATSN), deriving from 7S globulin β chain, has been identified from a soybean hydrolysate prepared by a protease from *Bacillus amyloliquefaciens* and then by chemical synthesis (Cho et al., 2008). In HepG2 cells in fact an increased LDL-R transcription (+148%) was detected at a concentration of 100 μM of this peptide. Although the mechanism underlying the increased expression of LDL-R and SREBP-2 mRNAs and the reduced expression of HMGCoA reductase mRNA remains to be elucidated in details, it is worth to note that all peptides were potent inhibitors of FAS activity as reported below.

Recently, a number of studies have suggested that FAS is a potential target for drug discovery. In contrast to normal tissues, high levels of FAS expression have

been found in many human cancers, including carcinoma of the breast, prostate, colon, ovary and in adipose tissue (Berndt et al., 2007; Sheng et al., 2009).

The discovery and development of pharmacologic FAS inhibitors are the premise for the treatment of cancer, diabetes, hyperlipidemia, obesity and related diseases. Plant-derived compounds have been discovered as potential FAS inhibitors (Brusselmans et al., 2005; Na et al., 2006; Rivkin et al., 2006). Previous "*in vitro"* studies have shown that soybean β-conglycinin contains active peptides that inhibit FAS (Gonzalez de Mejia, 2009) and fatty acid biosynthesis in adipocytes (Martinez-Villaluenga et al., 2008). Treatment with synthetic peptides from β-conglycinin decreased lipid accumulation in the adipocytes from 12 to 27 % compared to negative controls (untreated cells). Previous data correlate the FAS inhibitory potency of soy peptides to their molecular mass. The large inhibitory peptide (RKQEEDEDEEQQRE) bond to thioesterase domain of human FAS blocking the active site, as predicted by molecular docking (Martinez-Villaluenga et al., 2010). The intake of 5 g of β-conglycinin per day decreased visceral fat in humans and increased the activities of hepatic β–oxidation enzymes in mice and rats (Moriyama et al., 2004). Newly, it was shown that soybean hydrolysates play a role in regulating body weight (Vaughn et al., 2008) and controlling lipid accumulation (Martinez-Villaluenga et al., 2008).

Recent research have suggested that oxidative stress is related to atherosclerosis as well as to diabetes and cancer, although the exact impact of this association has still to be determined (Song et al., 2007). None of the peptides tested in present work which belong to α', α and β subunit of β-conglycinin showed a significant

anti-oxidant capacity, since the results obtained varied between 11 and 15 μmol Trolox equivalent/μmol peptide (**Table 7** in results capitol). Chen *et al.* reported that soy peptides containing histidine are characterized by antioxidant properties. Since the peptides studied are lacking in histidine amino acid, this could justified their inefficacy as antioxidant compounds. Moreover, although the interaction of α'chain, or fragment thereof, with thioredoxin 1 and cyclophilin B has been previously indicated (Manzoni et al., 2003) we have not information about the potential interaction of peptides with cell membrane components as well the endocytotic pathway involved. Despite the results obtained in the present study, Elias *et al*. demonstrated the improvement of the antioxidant capacity of soybean when protein was hydrolyzed into peptides (Elias et al., 2008).

It is known that inflammation is part of the defense mechanism against infectious agents and injury. On the other hand, it is also associated to many chronic diseases such as cardiovascular disease, diabetes and cancer (Agarwal et al., 2009). From this, the reduction of expression or activity of the enzymes iNOS/COX-2 is the target to prevent chronic diseases related to inflammation. Nitric oxide synthases, (NOSs) are a family of enzymes catalyzing the production of nitric oxide (NO) from L-arginine. Nitric oxide, plays an important role in the regulation of immune function, anti-apoptosis, and neurotransmission (Chung et al., 2001). Although it was demostrated (Martinez-Villaluenga et al., 2009) that protein hydrolysates from soybean reduced prostaglandin 2 (PGE2) production in LPS-induced macrophages, there is no information about the effect of purified peptides from β–conglycinin on the iNOS and COX-2 following treatment of RAW 264.7.

We studied the effect of each peptide (25 μM concentration) on the protein expression of iNOS in macrophages using Western blot. Results shows that expression of iNOS was differently affected by synthetic peptides, peptide A (SEEEEEDQ), B (QKEEEKHEWQ), and E (KNPQLR) reduced the expression of iNOS by 71, 75 and 44%, respectively. To evaluate the role of synthetic peptides in inflammation, we also measured the expression of COX-2 in LPS-stimulated RAW 264.7 cells. As indicated in Results section, COX-2 expression was markedly reduced by peptide A, B, and E by 54, 34, and 79 %, respectively. Peptide C (RKQEEDEDEEQQRE), and peptide D (EITPEKNPQLR) showed lower inhibitory activity on both inflammation markers. These data support the hypothesis that peptides, released by "*in vitro"* or "*in vivo"* enzymatic digestion from different protein components present in the soybean seed, are characterized by different physiological functions.

The NPDNDE synthetic peptide corresponding to the position 314-319 of α' chain 7S soy globulin was tested "*in vitro"* and *"in vivo"* experiments. Results obtained after treatment with this peptide showed an increased mRNA expression of SREBP2 (+1.5; 1.2 fold), LDL-R (+2.5; 2.5 fold) and PCSK9 (+3.5; +1.5 fold) were detected in cells exposed to NPDNDE and simvastatin, respectively vs control cells. "*In vivo*" experiment rats fed hypercholesterolemic diet were daily treated by gavage with synthetic peptide NPDNDE (5 mg/Kg b.w.) for 21 days; the results were compared with that obtained in rats treated either with whole α' (20 mg/Kg b.w.) or clofibrate (200 mg/kg b.w.), as reference drug. The oral administration of peptide resulted in lower plasma lipid levels (cholesterol, -37%;

triglycerides, -38%) *vs* values recorded in rats fed casein-cholesterol diet alone. Four-fold amounts of α' chain reduced cholesterol and triglycerides by 37 and 25%, respectively. Similar results were obtained in clofibrate-treated rats. Moreover, synthetic peptide increased the expression of mRNA of LDL-R (+ 53%), thus restoring the receptor activity normally suppressed by the HC diet. This is the first "*in vivo"* evidence of potential activity of the NPDNDE synthetic peptide from α'chain 7S soy globulin on lipid homeostasis through SREBP-2 and PCSK9 pathways.

The results presented in this 7S soy protein study pointed out that peptides, designed considering the differences among the amino acid sequences of the three subunits of 7S soy globulin and chemically synthesized, as well as a biotechnological product such as α 'E were characterized by biological properties making them a suitable source of active peptides. Moreover, this study showed that the protein component and not any other potentially bioactive compounds, such as isoflavones, are responsible of the detected activities. These soy peptides played, in fact, a key role in the complex mechanism of cell physiology and this depends on the different cell line used. These molecules, following confirmation of their biological activity in "*in vivo*" models, will open to the sector of prevention, generating a strong impact on businesses as well as on the consumer health. Moreover, the biotechnological product (α'E) can effectively reduce cholesterolemia upregulating LDL receptor activity, a widely accepted mechanism of cholesterol reduction associated with the intake of vegetable proteins. The

growing interest in this product suggest its potential therapeutic use in hypercholesterolemia.

In conclusion the results of these studies are the basis for the development of functional foods with beneficial effects on various diseases, including hyperlipidemia, diabetes, cardiovascular disease and so on, to be used alone or in combination with drug therapies. Moreover, the knowledge of the amino acid sequence responsible of the biological effect could be of help for the selection of soy cultivars or the production of genetically modified soybean crops rich in bioactive compounds.

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