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Running title: **Narrow-leafed lupin β-conglutins modulate the insulin signalling pathway**

Narrow-leafed lupin (*Lupinus angustifolius* L.) β-conglutin proteins modulate the insulin signalling pathway as potential type 2 diabetes treatment and inflammatory-related disease amelioration

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ABSTRACT

Scope: We have investigated the potential use of β-conglutin protein isoforms from narrow-leafed lupin (*Lupinus angustifolius* L.) as a diabetes treatment.

Methods and results: We produced purified recombinant β1-, β2-, β3-, β4-, and β6-conglutin proteins and showed that β1, β3 and β6 could bind to insulin. To assess β-conglutin proteins modulatory effect on insulin-activation meditated kinases, whole blood and peripheral blood mononuclear cell (PBMC) cultures from Type 2 diabetes (T2D) and healthy control subjects (C) were incubated with conglutin proteins. Treatment of PBMCs from T2D patients with β1, β3, and β6 proteins increased up to 3-folds mRNA and protein levels of genes important in insulin signalling pathways, namely IRS-1/p85 /AKT/GLUT-4. This was accompanied by a comparable fold-change decrease in the mRNA expression level of pro-inflammatory genes (iNOS and IL-1β) and proteins compared to healthy controls. The β2 and β4 isoforms had no effect on the insulin signalling pathway. However, these β-conglutin proteins elicited pro-inflammatory effects since levels of mRNA and proteins of iNOS and IL-1β were increased.

Conclusion: Our results raise the possibility of using these particular β-conglutin proteins in the prevention and treatment of diabetes, as well as their potential as anti-inflammatory molecules.

Keywords: Antioxidant; Anti-inflammatory; GLUT-4; IL-1β; Legumes; PI3-Kinase; Sweet lupins; Type 2 Diabetes; Vicilin.

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INTRODUCTION

Recently, the health beneficial effects of plant-derived compounds have been increasingly investigated [1], including proteins from legume seeds [2], as food supplements [3]. These studies showed the biological activities of some legume proteins and proteins from other plant species as modulaters of chronic diseases [4, 5]. However, most of these studies did not investigate the molecular mechanisms underlying these positive health effects. Furthermore, most studies used whole seeds, where thousands of compounds would be present, and thereby not defining the exact molecule(s) that promote particular health effects such as reduction of body weight, food intake [6], and a decrease in the LDL level in plasma [7].

Considerable interest has been focused towards legume seed proteins [8], especially those from lupins, a legume of the Fabaceae family. The seeds of the "Sweet Lupins" (*Lupinus angustifolius* or blue lupin; *Lupinus albus* or white lupin; and *Lupinus luteus* or yellow lupin) have low alkaloid content [9], and are attracting attention because of their nutritional attributes [10] and potential for disease improvement [11]. These properties are associated with the seeds' high protein, dietary fiber content, which help reduce blood pressure and the risk of cardiovascular disease [12], as well as impacting on the prevention and treatment of Type 2 diabetes (T2D) [13]. The question that needs to be answered is which compound(s) among the lupin seed content mediates these effects.

Narrow-leafed lupin (NLL), *Lupinus angustifolius* (L.) seeds are rich in proteins, which belong to four main families: α, β, γ, and δ conglutins [14]. Of these, the β conglutins, a vicilin or 7S globulin, is the major seed storage protein in NLL and in *Lupinus* species [15], which belong to the Cupin superfamily [16], and mainly associated (as storage protein funtion) with plant physiological proccesses through the supply of aminoacids during seedling germination [17]. NLL seed storage proteins are getting increased awareness and international recognition as a potential food for humans as its protein-rich seeds contain a

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large quantity of dietary fibre, are low in fat and starch, are gluten-free and have a very low Glycaemia Index [18, 19]. Recently, seven genes coding for individual β-conglutins namely conglutin β1 to conglutin β7 have been identified in NLL [14, 15; (http://www.lupinexpress.org)]. The β-conglutins are the most highly expressed conglutin family in NLL, and constitute 56% of the total conglutin transcript content in NLL seeds [15]. Despite of that, nothing is known so far about the potential and alternative functions that individual NLL β-conglutin proteins may exhibit in relationship to health benefits.

At present, T2D is one of the main global health concerns with its ensuing deleterious complications such as retinopathy, neuropathy, heart attack and atherosclerotic vascular disease [20]. The aim of our study was to test the effects of different purified recombinant β-conglutin proteins on whole blood cultures from T2D patients and healthy control subjects.

MATERIAL AND METHODS

Construction of expression plasmids

The expression system used for bacterial expression was a modified variant of a pET28a vector (Novagen) containing an N-terminal polyhistidine (6xHis) Tag. A pUC57 vector containing a synthetic gene encoding for each conglutin protein GenBank accession number HQ670409 (β1), HQ670410 (β2), HQ670411 (β3), HQ670412 (β4), and HQ670414 (β6), connected by restriction enzyme linkers consisting of ccatgg (NcoI) and ctcgag (XhoI) sequences were created synthetically by GenScript. The bacterial expression vectors pET28a(+)-β-conglutin -6xHis-Tag for β1, β2, β3, β4, and β6 were obtained via digestion of respective pUC57-β-conglutin construct using double NcoI and XhoI restriction enzymes digestion, followed by ligation of the β1 to β4 and β6 fragments into the pET28a(+) vector.

Beta-conglutin protein variants over-expression

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All β-conglutin proteins were expressed in Rosetta™ 2(DE3) pLysS Singles™ Competent Cells (Novagen). Proteins expression was performed by using the auto-induction method [21]. Briefly, a colony of *E. coli* containing the expression construct was isolated and grown for 20h in ZY-medium plus kanamycin at 50µg/ml at 37 ºC and continuous shaking (200 rpm). The culture was diluted 1:150 in Studier medium and grown for a further 5h until cell density reached 0.7 OD 600nm, to subsequently inducing overexpression of the proteins by adjusting the temperature to 19 °C for another 16 h. Cells were collected by centrifugation at 5000xg at 4 ºC. Bacterial cell pellet was rinsed two times with phosphate buffered saline (PBS), pH 7.5, removing the supernatant and the cell pellet was flash frozen using liquid nitrogen. The bacterial cell pellet was stored at -80 ºC until further use.

Purification of recombinant conglutin β1, β2, β3, β4, and β6 proteins

Overall, protein purification from bacterial pellets was performed following company protocol recommendations (Qiagen) for His-tagged proteins. Briefly, the steps consisted in breaking cells, following by nickel affinity chromatography using Ni-NTA spin columns, and 6xHis-Tag at the C-terminal part of each β-conglutin protein. After elution of proteins from the column with an increasing imidazole concentration gradient (10 to 300mM), 2.5 ml fractions were collected. Fractions containing protein were analyzed using SDS-PAGE and fractions showing a single band corresponding to the expected molecular weight were pooled, aliquoted and flash frozen in liquid nitrogen and kept at -80C until futher uses. Purity of the protein samples resulted in >95%. Typical yields after nickel affinity chromatography were ~5-15 mg/ml.

β-conglutin antibody production

A peptide, Nt – VDEGEGNYELVGIR - Ct, specific for the seven NLL β-conglutin proteins variants was synthesised (Agrisera, Sweden). This peptide was used to immunize rabbits and

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to produce polyclonal antiserum. The rabbit immune serum was affinity-purified against the same synthetic peptides (Agrisera, Sweden).

Immunoprecipitation assays

Immunoprecipitation (IP) was performed using the Thermo Scientific Pierce (Rockford, IL, USA) co-immunoprecipitation Kit 26149 following the manufacturer's protocol. Briefly, the monoclonal anti-6xHis-Tag antibody (Sigma-Aldrich) was first immobilized using AminoLink Plus coupling resin. The resin was then washed and incubated with individual purified conglutin β1, β2, β3, β4 and β6 recombinant protein and human recombinant insulin (Sigma) for 4h at 4°C. After incubation, the resin was washed and protein eluted using elution buffer. Negative controls were performed by immobilizing anti β-conglutin antibody (C1) or monoclonal anti-6xHis-Tag antibody (C2) to AminoLink Plus coupling resin, and using human recombinant insulin without adding any of the purified recombinant b-conglutins in the incubation, and further elution step.

Elution samples were analyzed by SDS-PAGE and stained with coomassie blue, as well as by Western blotting using rabbit monoclonal anti-insulin (Abcam, Cambridge UK), and a horseradish peroxidase-conjugated secondary goat-IgG anti rabbit antibody. Chemiluminiscent detection was carried out as described below (quantitative immunoblotting section). Results visualization and documentation were performed with a C-DiGit blot scanner (LI-COR, USA).

Participant' study

A total number of twenty seven healthy control subjects, and thirtyone diabetic patients were recruited for this study, and respective informed full consents were obtained from each participant. The subjects were unrelated and diagnosed at the coverage area of basic area "Pedro Martínez" (A.G.S. NorthEast Granada, Spain). Samples consisted of venous blood, which was collected from the cubital vein in 4-ml lithium-heparin tubes by well-trained

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authorized personal. All study procedures were performed in accordance with a protocol previously approved by the Ethics Committee of Basic Area “Pedro Martínez” (A.G.S. NorthEast Granada, Spain). All participant provided full written, and informed consents for the procedures.

All experiments were performed in agreement to “The Principles of Good Laboratory Practice”, developed in accordance with the OECD and adopted by the EU (Directive 2004/9/EC, and Directive 2004/10/EC).

All methods and experimental protocols included in the “Methods section” corresponding to the current study were approved by The Estación Experimental del Zaidín, Granada (Spain), the institution belonging to the Spanish National Research Council (CSIC), where all experimental procedures were performed. In addition, all methods and experimental protocols were also approved by the Research Ethics Committee of the Granada province, Andalusian Health System (Spain).

Whole blood Culture

Venous blood was drawn into lithium–heparin tubes (BD Vacutainer System, Heidelberg, Germany) in the morning. Participants were fasted for 12 hours before blood collection (Fasting consisted of no food or drink intake but water). Within 3 h, whole unseparated blood was diluted 1:3 with Dulbecco’s modified Eagle’s medium (DMEM) and HEPES 4%; (Invitrogen, Karlsruhe, Germany), and agitated gently in 50-ml tubes (Greiner Bio-one, Solingen, Germany); 1ml aliquots were seeded per well of 24-well plates (Nunc, VWR International GmbH, Langenfeld, Germany) and cultured for 24 h at 37°C and a atmosphere of 5% CO_2. The whole blood assay procedure was established from pilot studies with blood from four healthy subjects. All parameters for each subject including blood withdrawal were measured in one assay on the same plates in order to ensure intra-individual cross-comparison of results. From each blood drawing, we performed triplicate incubations with

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negative controls, and the separate purified β-conglutin (20 μg/mL), or *E. coli* lipopolysaccharide (LPS, 5 pg/ml) and each β-conglutin protein in combination with LPS. Samples were incubated in triplicate with negative controls (PBS 25%; Sigma-Aldrich). The same lots of purified β-conglutin (β1, β2, β3, β4, β6 respectivelly), LPS and PBS were used for all experiments. Blood cultures were removed from each well to be centrifugate at 700xg for 5 min at 20°C, and supernatants were aliquoted and stored at -20°C until further analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density-gradient centrifugation on Histopaque 1077 (Sigma, St. Louis, MO), washed three times in Hanks' balanced salt solution (Life Technologies, Grand Island, NY), and resuspended in complete medium including RPMI 1640 (Sigma) supplemented with penicillin, streptomycin, and L-glutamine (100 U/ml, 100 μg/ml, and 2 mM, respectively) (Sigma).

Relative mRNAs quantification of IRS, GLUT-4, iNOS and IL-1β

Real time quantitative PCR technology was used to assay Insulin Receptor Substrate 1 (IRS-1), Glucose Transporter Type 4 (GLUT-4), Phosphoinositide 3-Kinase (PI 3-kinase), Inducible Nitric Oxide Synthase (iNOS) and Interleukin 1 beta (IL-1β) mRNAs expression on 25 samples from each experimental group.

Total messenge ribonucleotic acid (mRNA) was isolated from culture supernatants by using the mRNeasy min kit (Qiagen, Netherlands) and Total mRNA was extracted from the cultured PBMCs with RNeasy mini kit 50 (Qiagen, Netherlands) according to the instructions of the manufacturer. First strand complementary DNA (cDNA) was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems, Weiterstadt, Germany). For gene expression assays, cDNA was amplified using 7500 Fast Real-Time polymerase chain reaction (PCR) System (Applied Biosystems). TaqMan primers and probes were derived from the commercially available TaqMan Gene Expression Assays (GenBank accession no:

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NM_005544.2, Assay ID: Hs00178563_m1; GenBank accession n˚: NM_001042.2, Assay ID: Hs00168966_m1; GenBank accession n˚: AF049656.1, Assay ID: Hs01075529_m1 and GenBank® accession no: NM_000576.2, Assay ID: Hs01555410_m1, Applied Biosystems respectivelly). Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of β-Actin (GenBank accession n˚: NM_001101.3, Assay ID: Hs99999903_m1) detection, referred to as ΔCT. PCR efficiency was determined by TaqMan analysis on a standard curve for targets and endogenous control amplifications, which were highly similar.

Quantitative immunoblotting

The analyses were performed on culture supernatants obtained from whole blood culture. Total protein concentration was determined for each sample with the Bradford assay (Bio-Rad) using bovine serum albumin as a standard. Aliquots corresponding to 20 μg of total proteins were heated to 95°C for 5 min with an equivalent volume of sample buffer, containing 2% SDS and 5% mercaptoethanol, bromophenol blue and 20% glycerol, and loaded onto 10% and 12% polyacrylamide gels for protein separation. Proteins separated by SDS-PAGE were electrotransferred to PVDF membranes and probed with appropriate antibodies. Membranes were blocked for 1 h at 37ºC in a blocking solution containing 3% BSA, 0.05% Tween-20, and PBS (pH 7.4) and incubated overnight at 4ºC with primary antibodies in the blocking solution. These primary antibodies consisted of anti- [IRS-1 (107-185 kDa), PI3-Kinase (p85) (85kDa), GLUT-4 (50-60kDa), Protein kinase B (AKT) (60kDa), respectively], which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA.), and the antibody against iNOS (130 kDa) and IL-1β (17kDa) were purchased from Abcam (Cambridge, UK). Membranes were rinsed three times with washing solution (0.05% Tween-20 in PBS) for 10 min each, followed by incubation for 1 h at room temperature in a 1:5000

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dilution of goat anti-rabbit IgG-HRP, purchased from Santa Cruz Biotechnology (Santa Cruz, CA), with 3% BSA in PBS. The membranes were then washed three times for 10 min each in the same washing solution. Chemiluminiscent detection was carried out with the Immun-StarTM WesternCTM Chemiluminescence Kit (Bio-Rad, cat# 170-5070) according to the manufacturer´s instructions. Visualization and documentation of the results was performed with a C-DiGit blot scanner (LI-COR, USA). Quantitative (densitometry) analysis of protein bands was performed using ImageJ v1.47 (Bio-Rad).

Statistical analysis

Data are expressed as means ± SEM of 6 patients for group. Two-tailed Student's t-test was used to compare between two groups. One-way ANOVA followed by Dunnett's test was used to compare among three or more study groups. $P < 0.05$ is regarded as statistically significant.

RESULTS

Purification of β-conglutin proteins and immune precipitation assay

The SDS–PAGE analyses indicated a single protein band of approximately 65 kDa (**Figure 1**). Recombinant β-conglutins-6xHis-Tagged were obtained at high purity level (>95%), and a concentration between 5-15 mg/mL (**Figure 1**). Analysis by immunoblotting using anti-β-conglutin protein antibody produced in the current study confirmed the identity of the recombinant protein (asterisks, **Figure 1**).

We further investigated whether human recombinant insulin was able to interact with the β-conglutin proteins (β1, β2, β3, β4, or β6) by immunoprecipitation experiments. Precipitated species were detected by coomassie staining (**Figure 2A**), and confirmation of the presence of insulin in the eluated samples was performed by immunoblotting using an anti-insulin antibody (Abcam, Cambridge UK) (**Figure 2B**). Insulin was found at relatively high quantity

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in the eluate samples when incubated with purified conglutin β1, β3, and β6 (**Figure 2**), when compared to controls.

Participant information

The clinical parameters of the population studied are summarized in **Table 1**. Healthy control subjects and patients with T2D showed significant differences for age and body mass index (BMI), as expected. There were significant differences between study groups in the levels of Fasting Glycaemia ($P<0.001$), Blood Pressure ($P<0.03$), Heart Rate ($P<0.001$) and HbA1c ($P<0.001$).

Challenging assayes using PHA, LPS, PMA+IO exhibited comparable values

We investigated whether incubation with PHA, LPS, and PMA+IO, respectively, had potential effects on the release of cytokines from whole blood samples. Challenging with PHA, LPS, or PMA+IO induce a potent stimulis of spontaneous cytokine IL-1β and iNOS production in whole blood cultures. The mean concentration values of IL-1β in culture supernatants of healthy control subjects after 24 h after incubation with PHA, LPS, or PMA+IO are shown in Figure 3. We evaluated *ex vivo* IL-1β production with these three stimulis. Due to that the patterns of producction were similar in all subjects tested leading to similar concentations of IL-1β, a whole blood culture system with LPS appeared to be most suitable to assess in our study (see exemplary for IL-1β, **Supporting information Figure S1**).

Differential expression of genes related to the insulin molecular signalling pathway

We assessed whether the effect of each purified β-conglutin protein (β1 to β4, and β6, respectivelly) on blood culture of T2D and controls (C) was releted to the activation of the insulin signalling pathway. Expression levels of the IRS-1 (Insulin receptor substrate 1) gene were significantly lower in T2D patients [-75%, -75%, -81%, -91%, and -74% (**Figure 3A**)] when compared to C in all experimental groups. Interestingly, 24h after challenging using β1, β3 and β6, mRNA levels of the IRS-1 gene in T2D patients experienced a significant increase

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[+244%, +347%, and +280% *versus* T2D patients (**Figure 3A**)]. No differences were observed using β2 or β4 conglutin for challenge assays when compared to T2D (**Figure 3A**). After LPS challenge, we found significantly stimulated IRS-1 gene expression in T2D patients in all the experimental groups [+548%, +678%, +412%, +720%, and +710% *versus* T2D (**Figure 3A**)]. Challenge experiments using LPS+β2 and LPS+β4 enhanced mRNA expression of IRS-1 [+531%, and +620% *versus* T2D (**Figure 3A**)]. On the other hand, a significant reduction was found for challenges using LPS+β1, LPS+β3 and LPS+β6 [+85%, +128%, and +146% *versus* T2D (**Figure 3A**)].

Interestingly, challenge assays using β1, β3, β6, significantly stimulated the expression of the IRS-1 gene in C subjects [+111%, +238%, and +116% (**Figures 3B**)], and no significant differences were found in the expression levels of mRNA of the IRS-1 gene with β2 and β4 challenges *versus* C (**Figures 3B**). The mRNA expression levels of the IRS-1 gene were upregulated in LPS challenging assays [+497%, +333%, +601%, +636%, and +411%] in all experimental groups (**Figure 3B**). In addition, mRNA expression levels of the IRS-1 gene for challenges using LPS+β2 and LPS+β4 caused a significant induction [+326%, and +534% *versus* C (**Figure 3B**)] when compared to LPS+β1, LPS+β3 and LPS+β6 [+63%, +20%, and +45%], in which mRNA levels also were partially increased when compared to control (**Figure 3B**).

Protein level of IRS-1 and P85 (Mitogen-activated protein kinase) in culture supernatants were significantly reduced in T2D (IRS-1: -55%, -74%, -67%, and -87%; and p85:-72%, -69%, -58%, -46%, and -70%) in comparison to C in all experimental groups (**Supporting Information Figures S2A and S3A**). Noteworthy, challenges using β1, β3 and β6 showed that IRS-1 and p85 protein levels were significantly higher in plasma of diabetic patients [IRS-1: +187%, +299%, and +229%, respectively (**Supporting Information Figure S2A**); p85: +337%, +356%, and +417% (**Supporting Information Figure S3A**)] when compared

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to T2D. No significant changes were found with β2 and β4 when compared to T2D (**Supporting Information Figures S2A S3A**).

Furthermore, a significant increase in IRS-1 and p85 protein levels were found for all different experimental groups using LPS in T2D subjects [IRS-1: +498%, +427%, +366%, 364%, and +385% (**Supporting Information Figure S2A**) *versus* T2D; p85: +495%, +392%, +166%, +392%, and +334% (Figure S2A) *versus* T2D]. In addition, IRS-1 and p85 protein expression levels exhibited an increase in challenges using LPS+β2 and LPS+β4 [IRS-1: +517%, and +412% (Figure S1A) *versus* T2D; p85: +412%, and +357% (**Supporting Information Figure S3A**) *versus* T2D] compared to LPS+β1, LPS+β3 and LPS+β6 [IRS-1: +116%, +174%, and +187%; p85: +170%,+145%, and +105% *versus* T2D], in blood cultures of T2D subjects (**Supporting Information Figures S2A S3A**).

A statistically significant increase of plasma IRS-1 and p85 proteins was observed in challenge assays using LPS in controls for all experimental groups [IRS-1: +254%, +332%, +432%, +332%, and +443% *versus* C (**Supporting Information Figure S2B**); p85: +154%, +298%, +121%, +215%, and +254% *versus* C (**Supporting Information Figure S3B**)].

We also found an increase of IRS-1 and p85 proteins levels using β1, β3 and β6 [IRS-1: +345%, +467%, and +413% (**Supporting Information Figure S2B**); and p85: +275%, +276%, and +312% (**Supporting Information Figure S3B**)] when compared to controls. No differences were found in the protein expression levels of IRS-1 and p85 when challenges were performed using β2 and β4 in control subjects (**Supporting Information Figures S2B and S3B**).

Beta conglutin challenges result in a differential expression of genes related to glucose transport pathway

We also examined whether challenges with particular β conglutins proteins would trigger the activation of the glucose transport pathway, by quantification of the expression levels of

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GLUT-4 (Glucose transporter type 4) gene. T2D exhibited a significant reduction in the mRNA levels of GLUT-4 [-69%, -65%, -79%, -33%, and -80% (**Figure 3C**)] versus samples from healthy control subjects. The reduced expression of GLUT-4 in the T2D group was clearly restored under β1, β3 and β6 challenges as illustrated in **Figure 3C** [+428%, +430%, and +230% *versus* C]. β2 or β4 caused no changes in the levels of mRNA of GLUT-4 when compared to T2D (**Figure 3C**). Stimulation with LPS exherted a significant increase in the expression of GLUT-4 in all the experimental groups [+428%, +182%, +428%, +430%, and +230% (**Figures 3C**)].

Furthermore, GLUT-4 mRNA expression levels were increased after using LPS+β2 and LPS+β4 in challenge assays [+243%, and +648% (**Figure 3C**)]. No significative changes were noticed using LPS+β1, LPS+β3 or LPS+β6 [+135%, +143%, and +101% *versus* T2D (**Figure 3C**)]. However, LPS+β1, LPS+β3 and LPS+β6 showed recovery values of GLUT-4 mRNA expression levels when compared to LPS+β2 and LPS+β4 in challenge assays.

Protein expression levels of GLUT-4 and the insulin-dependent kinase AKT protein were decreased in T2D *versus* C group [GLUT-4: -77%, -57%, -88%, and -87% (**Supporting Information Figure S4A**); AKT: -82%, -75%, -73%, -63%, and -67% (**Supporting Information Figure S5A**)]. Interestingly, this effect was reversed using β1, β3 and β6 in the challenge assays [GLUT-4: +342%, +386%, and +318% (**Supporting Information Figure S4A**) *versus* T2D; AKT: +396%, +385%, and +103% (**Supporting Information Figure S5A**) *versus* T2D]. No significant effect was found with β2 or β4 conglutins when compared to C.

In addition, **Supporting Information Figures S4A** and **S5A** also showed a significant enhancement of GLUT-4 and AKT in LPS challenges for all the experimental groups [GLUT-4: +500%, +380%, +364%, +298%, and +341% *versus* T2D; AKT: +420%, +320%, +440%, +450%, and +445% *versus* T2D]. However, T2D group showed higher levels of

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GLUT-4 and AKT protein expression in challenges with LPS+β2 [+400%, and +394% versus T2D (**Supporting Information Figure S4A**)], and LPS+β4 [+264%, and +320% *versus* T2D (**Supporting Information Figure S5A**)] compared to LPS+β1, LPS+β3, and LPS+β6 [GLUT-4: +175%, +175%, and +173% (**Supporting Information Figure S4A**)]; IRS-1:+103%, +100%, and +83% (**Supporting Information Figure S5A**)]. In addition, challenge using LPS promoted significant increases in protein levels for GLUT-4 and AKT in control subjects [GLUT-4: +332%,+323%,+312%, +225%, and +276% (**Supporting Information Figure S4B**); AKT: +165%,+154%,+184%, +112%, and +121% (**Supporting Information Figure S5B**)], as well as in LPS+β2 and LPS+ β4 challenges [+174%, and +176% (**Supporting Information Figure S4B**) versus C; +134%, and +144% (**Supporting Information Figure S5B**) versus C]. A more moderate increase was found for challegenges using LPS+β1, LPS+β3 and LPS+β6 [+54%, +54%, and +38% (**Supporting Information Figure S4B**); +65%, +75%, and +46% (**Supporting Information Figure S5B**)]. Blood culture of control subjects challenged with β1, β3 and β6 significantively increased protein levels of GLUT-4 and AKT [GLUT: +365%, +326%, and +323% (**Supporting Information Figure S4B**) versus C; AKT: +294%, +299%, and +298% (**Supporting Information Figure S5B**) versus C]. No changes were found when using β2 and β4 in challenge assays.

Variation in genes expression related to the insulin pathway in PBMCs isolated from blood of T2D subjects

IRS-1 and GLUT-4 were significantly reduced in T2D subjects when compared to control [IRS-1: -85%, -90% -77%, -69%, and -69% (**Figure 5A**); GLUT-4: -75%, -80% -67%, -99%, and -89% (**Figure 5C**)].

Therefore, challenge assays using β1, β3 and β6 raised the levels of IRS-1 and GLUT-4 mRNA in PBMC of T2D subjects [IRS-1: +230%, +210%, and +238% (**Figure 5A**); GLUT-4: +301%, +298%, and +259% (**Figure 5C**)].

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Beta conglutins affect the expression levels of inflammation related genes important in T2D subjects

T2D is characterized by impaired insulin secretion and/or insulin sensitivity [22], and sustained by chronic subclinical inflammation. In order to investigate the possible potential antinflamatory effects of purified β-conglutin proteins, we assessed the expression levels of iNOS (Inducible nitric oxide synthase) and the pro-inflammatory cytokine IL-1β (Interleukin 1 beta).

iNOS mRNA and protein levels were substantially increased in T2D in all experimental groups when compared to control [+642%, +603%, +735% +779%, and +855% (**Figure 4A**)]. β1, β3 and β6 significantly suppressed iNOS mRNA expression and protein levels in T2D [-610%, -735%, -713% (**Figure 4A**) *versus* T2D]. In contrast, iNOS mRNA and protein levels remained significantly high [+284%, and +130% (**Supporting Information Figure S6A**) *versus* T2D] when β2 and β4 were used in challenge assays. We also found iNOS mRNA and protein levels significantly increased in LPS+β2 and LPS+β4 challenges [+230%, and +260 (Figure 4A) versus T2D; +87%, and +15% (**Supporting Information Figure S6A**) versus T2D], while LPS+β1, LPS+β3 and LPS+β6 challenges resulted in a substantial reduction in the mRNA and proteins levels [-512%, -625%, and -641% (**Figure 4A**) versus C; -510%, -548%, and -409% (**Supporting Information Figure S6B**) versus C] when compared with LPS+ β2 and LPS+β4 challenges.

β1, β3, β6 did not alter mRNA and protein expression levels of iNOS in control subjects (**Figure 4C**). However, mRNA and protein levels of iNOS were significantly increased using β2 [+734%, and +265% (**Figure 4C**) *versus* C], and β4 [+938%, and +206% (**Figure 4C**) *versus* C].

In this regard, gene expression and protein levels of IL-1β showed a similar pattern to iNOS (**Figure 4C, 4D**; and **Supporting Information Figures S7A and S7B**).

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Figure 4B shows a significant increase of mRNA expression and protein levels of IL-1β in T2D subjects in all the experimental groups [+642%,+603%, +735% +776%, and +755% (**Figure 4B**); and +338%,+245%, +237% +227%, and +320% (**Supporting Information Figure S7A**)]. Interestingly, this effect was reduced after 24h of incubation with β1, β3 and β6 [-610%, -735%, and -713% (**Figures 4B**) *versus* T2D; -310%, -202%, and -67% (**Supporting Information Figure S7A**) versus T2D]. In addition, the mRNA expression level and protein quantity of IL-1β were significantly induced in LPS challenge assays in all experimental groups [+1252%, +1174%, +1134%, +1222%, and +1134% (**Figure 4D**) *versus* C], and [+238%, +245%, +367%, +387%, and +390% (**Supporting Information Figure S7B**) versus C]. LPS+β2 and LPS+β4 challenges significantly increased the IL-1β levels of both, mRNA and protein [+817% and +554%, respectively *versus* C (**Figure 4D**); +265% and +376 (**Supporting Information Figure S7B**) versus C]. No differences in IL-1β cytokine levels were found when challenges were performed with LPS+β2 and LPS+β4 in comparison to the LPS group or the T2D group. Nevertheless, a moderate induction was found after LPS+β1, LPS+β3 and LPS+β6 challenge assays when compared to LPS+β2 and LPS+β4 challenges in the T2D subject groups.

Inflammation related genes response in PBMCs of T2D patients

Levels of iNOS and proinflammatory cytokine IL-1β mRNA were significantly higher in PBMC isolated from T2D [iNOS: +389%, +567%, +428%, +711%, and +698% (**Figure 5B**); IL-1β: +438%, +693%, +740% +850%, and +439% (**Figure 5D**)] when compared to C. This induction was notably suppresed after 24h of challenge with β1, β3 and β6 [-391%, -390%, and -377% (Figures 5B); and -475%, -480%, and -467% (**Figures 5D**)], whereas levels of iNOS and proinflammatory mediator IL-1β remained significantly high after β2 and β4 challenges in PBMC from T2D subjects (**Figure 5B and 5D**).

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Beta conglutin pool affects the expression levels of insulin pathway and inflammation related genes in PBMCs of T2D patients

In order to prove the join effect of the purified β-conglutins proteins in our study, challenging assays were preformed using all individual β-conglutin isoform proteins together (β1 + β3 + β6 + β2 + β4) in T2D patients and measured variations of IRS-1, GLUT-4, iNOS and IL-1β levels through qRT-PCR. IRS-1 and GLUT-4 were significantly reduced in T2D patients when compared to C [-71% (**Supporting Information Figure 8A**; and -82% (**Supporting Information Figure 8B**), respectively]. Furthermore, challenging assayes using β-conglutin pool caused the activation of mRNA synthesis levels of IRS-1 and GLUT-4 in stimulated PBMC of T2D [+398%, (**Supporting Information Figure 8A**); and +438 (**Supporting Information Figure 8B**), respectively].

iNOS and IL-1β mRNA levels were significantly high in PBMC from T2D [+689% (**Supporting Information Figure 8C**); and +711% (**Supporting Information Figure 8D**), respectively] when compared to C. Interestingly, challenge using β-conglutin pool suppressed the induction levels of iNOS and proinflammatory mediator IL-1β in T2D patients [-748% (**Supporting Information Figure 8C**); and -739% (**Supporting Information Figure 8D**), respectively].

DISCUSSION

Finding an effective and natural alternative anti-diabetic agent would be of enormous interest and importance for the prevention, and treatment of type 2 diabetes world-wide [23].

Various bioactive (nutraceutical) properties of *L. albus* or white lupin seed compounds have been reported such lowering plasma cholesterol, and triacylglycerol concentrations in hypercholesterolemic animal models [24].

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Post-prandial experiments have shown that healthy subjects that have consumed bread added with lupin flour [25; 26] or kernel fibre [27], had decreased levels of glucose, insulin, and satiety responses compared with those control subjects comsuming regular white bread. Therefore, when lupin was compared to soy, post-prandial glycaemic lowering response for bread consumption was more efficient for lupin than soy in diabetic subject [28]. In addition, a basic globulin protein (gamma conglutin family) isolated from *L. albus* seeds have shown that it reduces the plasma glucose levels in rats in a dose dependent manner [29], as well as glucose and insulin blood in rats treated with a hyperglycaemic diet [30].

In the present study, we have investigated the role of the *lupinus angustifolius* major seed protein, β-conglutin/vicilin proteins family, as a diabetes treatment. We have evaluated through challenge assays whether purified β-conglutin proteins of NLL are able to activate the intrinsic tyrosine kinases, triggering cell-signalling responses, commonly shared among the insulin signalling molecular cascade, i.e. IRS-1 pathway. Tyrosine phosphorylation of the IRS-1 protein allows its functional interaction with the regulatory subunit of PI3-K protein. This in turn activates PI3-K to a functional protein, necessary for insulin signalling for glucose transport [31].

Outcomes showed that whole blood culture challenges using purified β1, β3 and β6 promote the activation of the intracellular IRS-1/PI3-kinase pathway eventually involved in glucose homeostasis and protein synthesis stimulation in T2D patients in comparison to control T2D subjects. Moreover, purified β1, β3 and β6 proteins were able to stimulate IRS-1 (**Figures 3 and Supporting Information Figure S2**) and P85 (**Supporting Information Figure S3**) increasing their protein levels in control (healthy) subjects.

The mechanism by which activation of PI3-K through tyr-residue phosporylation [31, 32] induces GLUT- 4 translocation and increases glucose uptake by the cells has not been defined. A downstream effector of PI3-K protein (serine-threonine kinase - AKT) may also

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play a fundamental role in insulin's effects and glucose transport [33]; however the role of this kinase in glucose transport is controversial [34]. In this regard, 24h of challenge assays using β1, β3 and β6 resulted in the up-regulation of mRNA and an increase in protein levels of GLUT-4 and AKT in plasma of T2D subjects. In contrast, GLUT-4 and AKT remained unaltered when β2 or β4 were used in the challenge assays. Since recombinant human insulin has been shown in the current study to have a preference for binding to β1, β3, and β6, these proteins may be involved somehow in the variations of mRNA and protein levels of some of the above markers of the insulin pathway.

Taking all these results into consideration, and to the best of our knowledge, we propose for the first time that β1, β3 and β6 proteins have the ability to regulate the level of mRNA and protein synthesis of crucial genes involved in the insulin molecular signalling pathway, thereby modulating the activation and response of these regulatory genes leading to variation in insulin-mediated, plasma glucose levels.

The administration of LPS has been previously applied as an experimental model to mimic some of the clinical findings of human T2D [35]. The kind of vicious stimulus, at the same time, leads to severe metabolic disorder [36]. Thus, endotoxemia would induce acute insulin resistance and adipose inflammation, characteristic of that observed chronically in metabolic syndrome and diabetes.

LPS triggers inflammation, which is associated with marked changes in glucose metabolism; thus, LPS-induced glucose metabolism disorder is recently getting more attention as a prominent pathological problem [37]. Indeed, LPS challenge assays performed in the current study resulted in a significant activation of protein levels and gene transcriptional expression of kinases, IRS-1 and GLUT-4 in all different experimental groups. Mediators of the inflammatory response have been shown to impair insulin signalling to muscles and blood flow [38]. However, little work has been done in order to examine functional interaction

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among these particular factors. Fan et al. [36] found that LPS leads to whole body insulin resistance in rats and impaired insulin signalling. A recent *in vivo* study using a hyperinsulinemic-euglycemic clamp in mice was unable to detect impairment in insulin action following an LPS injection [39]. Therefore, it is unclear how much of the impairments are signalling mediated.

While a number of factors can contribute to inhibition of insulin-stimulated glucose uptake by cells in the presence of an LPS challenge, there is scarce information about the relationship between the LPS induced inflammatory response, insulin signalling, and decreased muscle glucose delivery [40].

Importantly, when LPS and purified β-conglutin proteins were jointly added to the challenge assays, we found that LPS+β1, LPS+β3 and LPS+β6 significantly reduced the level of intracellular mRNA for these genes belonging to the intracellular insulin signalling molecular pathway, in comparison to LPS challenge in T2D and in control (healthy) subjects; conversely no effects were found in challenge assays using LPS+β2 and LPS+β4, results that might be associated with the antioxidant properties and anti-inflammatory effects of β1, β3 and β6. To the best of our knowledge, this is the first time on the ability of specific NLL β-conglutin proteins to modulate the chronic subclinical inflammation caused by T2D.

At present, T2D is associated with oxidative stress and chronic inflammation by increasing the formation of reactive oxygen species and causing reduction in the antioxidant levels [41]. Therefore, inhibitors of the pro-inflammatory cytokines have been considered as candidates for anti-inflammatory drugs [42]. This type of activation is associated with an elevated production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), IL-6, and IL-1, ROS and nitrogen intermediates such as the inducible isoform of nitric oxide iNOS [43]. The increase in the incidence of inflammation related disorders has led to the search for active biopeptides derived from plant proteins with anti-inflammatory properties [1]. In the

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present study we investigated the potential anti-inflammatory activity of five purified NLL β-conglutin proteins. We observed a markedly pronounced activation of iNOS, an IL-1β, in culture supernatants of T2D patients. Notably, IL-1β and iNOS showed a significant inhibition after 24 h of incubation with either β1, β3 or β6 conglutin proteins in T2D patients. Gene expression of IL-1β and iNOS showed a similar pattern in PBMC in accordance to the results observed in blood culture supernatants of T2D patients.

Therefore, we analysed the effects of each purified β-conglutin protein in plasma, with the presence of stimuli of the innate immune system, (endotoxin LPS). In our study, LPS represents a strong immunostimulatory signal that induces a systemic inflammatory response with increases in proinflammatory cytokines such as IL-1β and iNOS in all experimental groups (T2D and control) [44]. In parallel experiments, we also found that LPS+β1, LPS+β3 and LPS+β6 challenge assays strongly reduced the mRNA expresion of IL-1β when compared to LPS alone in T2D subjects. On the other hand, no significant differences were observed between LPS+β2 and LPS+β4 challenges when compared to LPS. Previous studies using dietary proteins from animal and plant sources have demonstrated specific effects going beyond merely nutrient supply [1]. However, our results indicate for the first time that β1, β3 and β6 trigger the release of pro-inflammatory capacity of cells by diminishing IL-1β and iNOS mRNA expression and protein levels, leading to amelioration of the inflammatory process associated with T2D in whole blood cultures.

Most importantly, our study provides novel and interesting information, particularly on how β1, β3 and β6 proteins from NLL have a completely different phenotype at the gene expression level of T2D patients in comparison to β2 and β4, through their ability to modulate the intracellular IRS-1/PI3-Kinase/AKT7-GLUT-4 signalling pathway, that are eventually involved in protein synthesis stimulation, as well as their antioxidant and anti-inflamatory nature.

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An initial study has showed the structural differences of the seven β-conglutin proteins modelled structures [45], where the sequences identity ranged from 73-99% (**Supporting information Figure S9**). This relatively high level of protein sequence polymorphism might be reflected in functional differences at the physiological level in plant developmental processes [46, 46], but also differential roles as potential bioactive peptides [48]. Thus, we can not rule out that β-conglutin proteins sequence variability may be, at least partially, responsible of differential activities concerning genes transcriptional and proteins translational estimulatory effects in the insulin signalling pathway.

In conclusion, and to the best of our knowledge, this is the first report using this experimental *ex-vivo* system to challenge human blood samples of T2D and C patients with purified NLL conglutin proteins, and in combination with LPS. β1, β3, and β6 triggered increasing expression levels of IRS-1, p85, AKT and GLUT-4 genes in T2D and C subjects, and attenuated expression levels of IL-1β and iNOS, with potential amelioration of the inflammatory state caused by T2D. Furthermore, this study showed for the first time the interaction of recombinant human insulin with various NLL β-conglutin proteins, particularly with β1, β3 and β6, which might be a possible mechanism to alter the expression levels of genes in the insulin pathway.

These results hightlight the potential use of β1–, β3–, β6-conglutin proteins as food ingredients and as alternative approaches for T2D prevention, management and/or treatment. Chronic inflammation is a hallmark of several pathologies, such as Type 2 Diabetes rheumatoid arthritis, inflammatory bowel disease, atherosclerosis and cancer. Our work and its future applications can help to develop novel as well as culturally relevant plant-based therapeutic approaches against inflammation attenuation, and at the same time can serve as quality control tools to foster reliable and effective plant-based treatments. Moreover, this

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methodological approach offers an interesting opportunity to identify potential new bioactive plant peptides and metabolites with anti-diabetic activity, and opens the gate for possible plant-based therapeutic approaches to prevent inflammatory disorders, since the potential role of NLL β1, β3 and β6 having antioxidant and potential anti-inflamatory effects.

Finally, implementation of breeding programs using this experimental knowledge as molecular tools would be helpful in terms of NLL seeds uses in food-derived products with positive human health benefits by producing lupin plants with increased expression and storage of particular β-conglutin isoforms with known effects in T2D and related-inflammatory disseases.

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AUTHOR CONTRIBUTIONS

JCJ-L and EL-C conceived and designed the study. JCJ-L, EL-C and VA performed the study. JCJ-L, EL-C, VA, GM, KBS, RCF, SA, and JDA, analyzed, discussed, and assessed the resulting data. JCJ-L, JDA, KBS, and VA contributed reagents/materials/analysis tools. JCJ-L, EL-C, GM, KBS, RCF, SA and JDA wrote the paper. All authors have read the final manuscript.

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COMPETING FINANCIAL INTERESTS

The authors have declared that no competing interests exist.

FIGURE LEGENDS

Figure 1. Purification of conglutin β1, β2, β3, β4, and β6.

The Coomassie-stained shows the 5 purified β-conglutin proteins. Immunoblotting shows the 5 purified β-conglutin proteins identified by the anti-β-conglutin antibody (asterisks). **MW** = molecular weight standard, kDa.

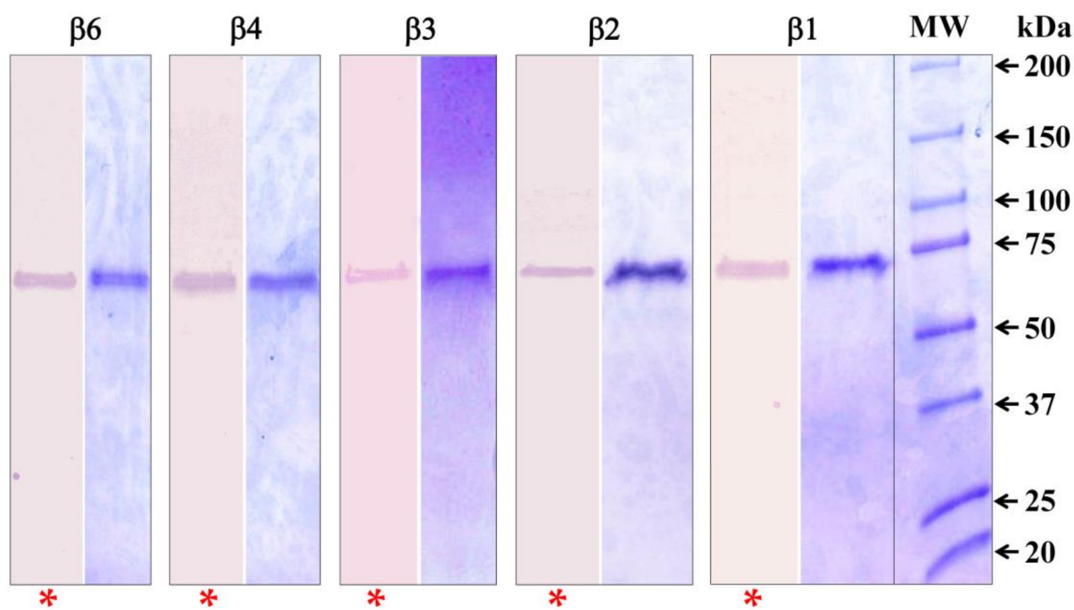

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Figure 2. Insulin preferentially interacts with conglutin β1, β3, and β6.

The Coomassie-stained SDS-PAGE (**a**) shows the eluate fractions. Insulin was mainly found in samples corresponding to β1, β3, and β6 incubations. Negative controls (lane 2: anti-β-conglutin + insulin, and absence of any purified β-conglutin; and lane 3: anti His-Tag + insulin and absence of any purified β-conglutin) showed no presence of insulin in these assayes. Immunoblotting showed the presence of insulin mayoritaritly in β1, β3, and β6 lanes (**b**).

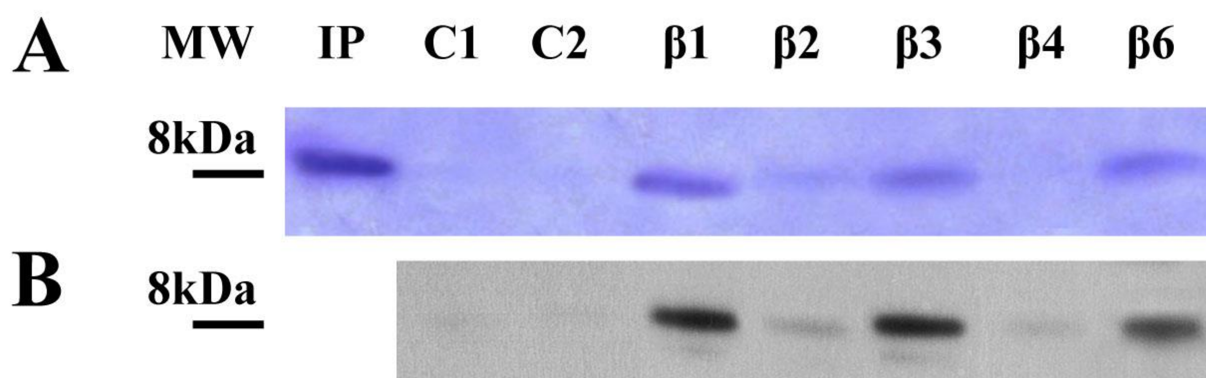

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Figure 3. mRNA levels of IRS-1 and GLUT-4 gene in culture supernatants of Type 2 Diabetic and healthy control groups.

Whole blood cells were incubated for 24h in the presence of LPS, LPS + β1, β3, β6, β2, β4, individually, or β1, β3 β6, β2, β4 alone. Bar graph shows IRS-1 and GLUT-4 culture supernadants from T2D (**a**, **c**), and C (**b**, **d**).

p* <0.05 T2D *versus* C; p**<0.05 LPS + β-conglutin *versus* T2D; p*** <0.05 β-conglutin *versus* T2D; p # <0.05 LPS versus T2D.

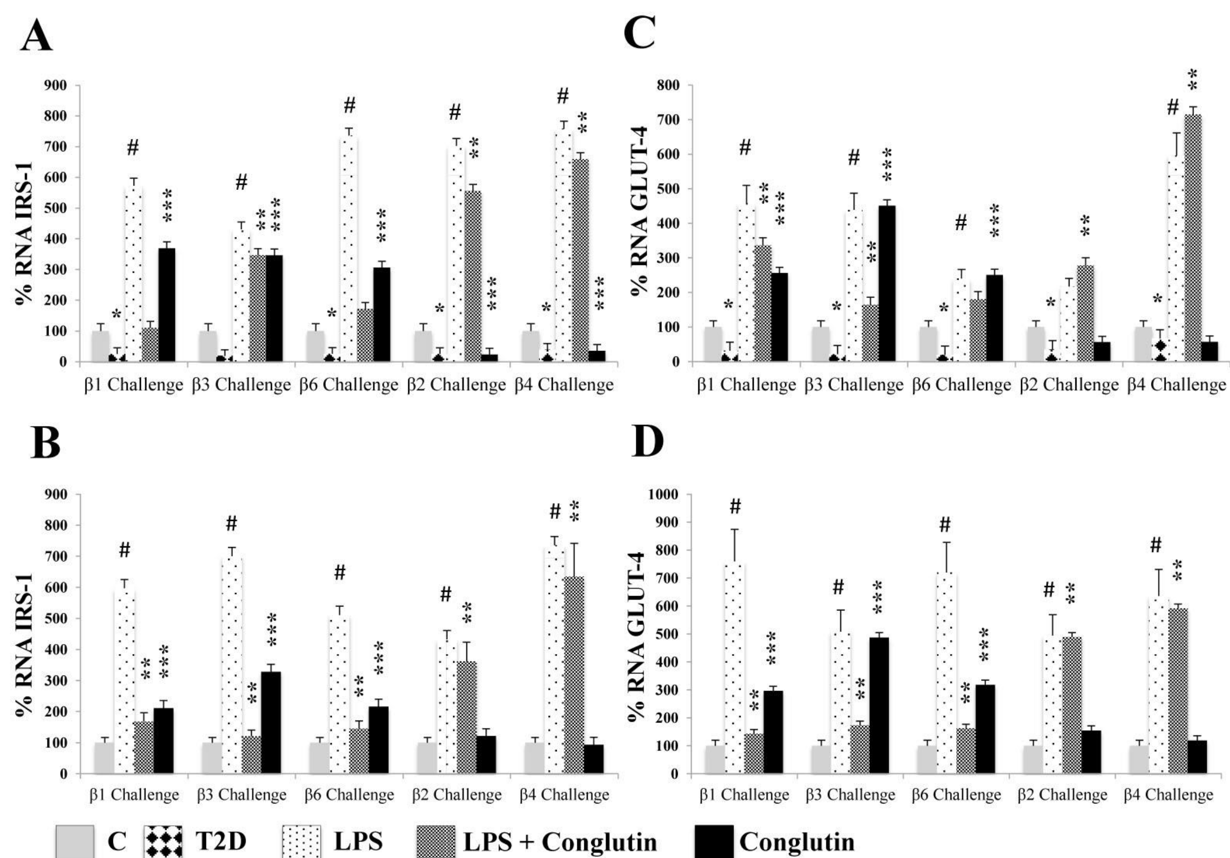

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Figure 4. mRNA levels of the iNOS and IL-1β genes in culture supernatants of Type 2 Diabetic and healthy control groups.

Whole blood cells were incubated for 24h in the presence of LPS, LPS + β1, β3 β6, β2, β4, individually, or β1, β3, β6, β2, β4 alone. Bar graph shows iNOS and IL-1β culture supernatants from T2D (**a**, **c**) and C (**b**, **d**) groups.

p*<0.05 T2D *versus*C; p**<0.05 LPS +β-conglutin *versus* T2D; p***<0.05 β-conglutin *versus* T2D; p # <0.05 LPS *versus* T2D.

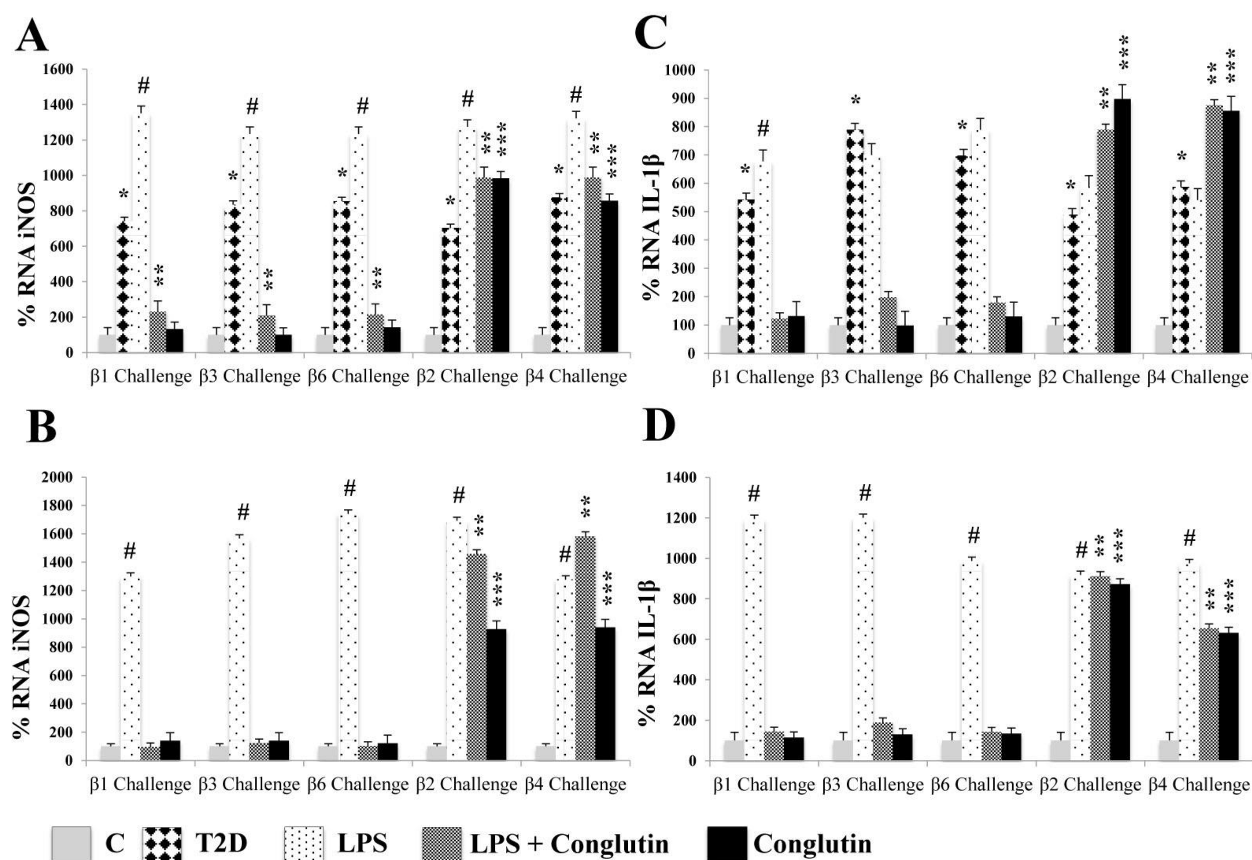

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Figure 5. mRNA levels of IRS-1, GLUT-4, iNOS, and IL-1β in peripheral mononuclear cell culture of Type 2 Diabetic and healthy control groups.

Whole blood cells were incubated for 24h with each individual purified β-conglutin protein (β1, β3, β6, β2, β4, respectively). Bar graph shows IRS-1 (**a**), GLUT-4 (**c**), iNOS (**b**), and IL-1β (**d**) mRNA levels of PBMCs from T2D and control groups.

p#<0.05 β-conglutin *versus* C; p*<0.05 T2D *versus* C; p** <0.05 β-conglutin *versus* T2D.

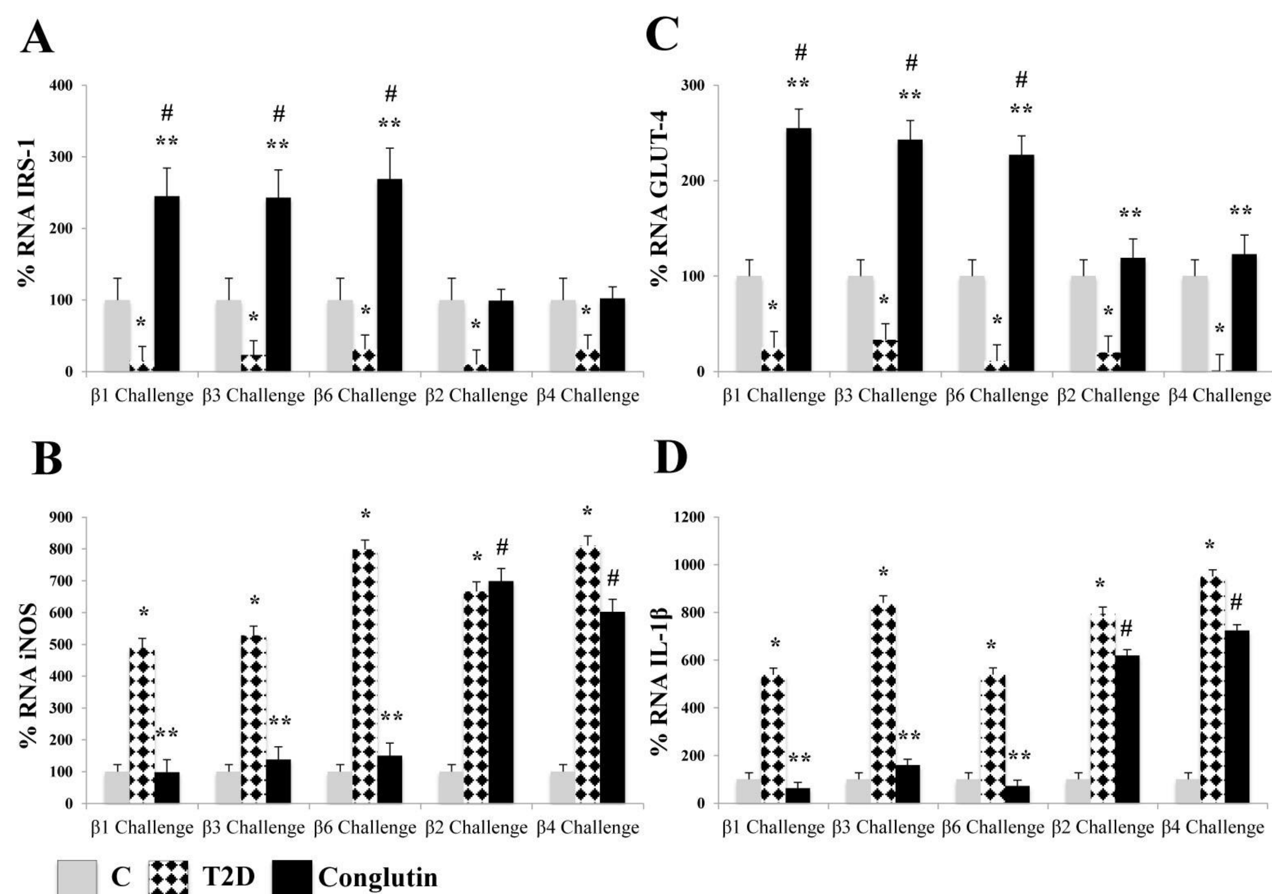

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Table 1. Characteristics of the study groups.

Values represent the median (25th percentile, 75th percentile); to detect differences between groups we used analysis of variance (ANOVA). Significant P-values (<0.01) are highlited with an asterisk. **BMI**: Body-mass Index; **HbA1c**: Glycosylated haemoglobin 1c; **BPM**: beats per minute; **T2D**: Type 2 Diabetes.

	Healthy Control Subjects	**Type 2 Diabetes**	***P*-value**
Male	27	31	
Age	50 (40,53)	48 (45,58)	0.007*
Fasting Glycaemia (mg/dL)	83 (80, 91)	165 (132, 198)	<0.001*
Blood Preasure (mmHg)	12/7 (11/7, 12/7)	15/8 (14/8, 157/)	<0.001*
BMI (kg/m^2)	24.8 (23, 26.6)	33.3 (27.5, 46.7)	<0.001*
Heart Rate (bpm)	64 (65, 76)	84 (96, 80)	<0.001*
HbA1c %	5.5 (5.4, 5.7)	6.7 (6.0, 7.4)	<0.001*

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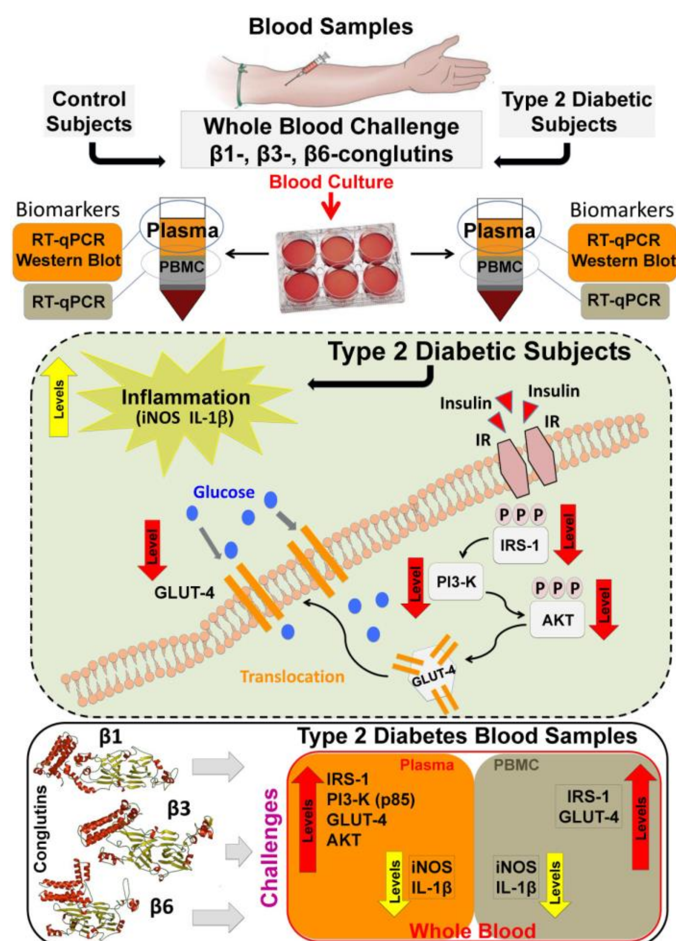
GRAPHICAL ABSTRACT CAPTION

This study provides new insights about the potential use of β-conglutin seed proteins from the legume *Lupinus angustifolius* L. in the Type 2 Diabetes prevention and treatment, and as anti-inflammatory molecules:

1) β1-, β3- and β6-conglutins have the ability to modulate the expression levels of crucial genes involved in the insulin molecular signalling pathway.

2) The same conglutins trigger the release of the pro-inflammatory capacity of cells by diminishing IL-1β and iNOS expression levels, leading to amelioration of the inflammatory process associated with Type 2 Diabetes.



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