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Carob-fruit-extract-enriched meat modulates lipoprotein metabolism and insulin signaling in diabetic rats induced by high-saturated-fat diet

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ABSTRACT

Insulin resistance (IR) produces qualitative, quantitative and kinetic changes of lipoproteins in Type 2 Diabetes Mellitus (T2DM). Carob-fruit-extract (CFE) has demonstrated antidiabetic properties but barely studies have evaluated the effect of CFE functional-meat consumption on early stages of T2DM. The current study was designed to analyze the effect of CFE-enriched meat-consumption on lipemia, lipoprotein profile and its relation with IR in T2DM model. Sixteen Wistar rats were divided in: C group, fed high-saturated-fat diet with commercial meat; and CE group, fed high-saturated-fat diet containing CFE-enriched meat. After 8-weeks, C rats showed hyperglycemia, hyperinsulinemia, hypertriglyceridemia and increased triglyceride-enriched-VLDL compared to CE animals ($p < 0.05$). CE group was able to partially block diabetic dyslipidemia and reduce HOMA-IR by showing higher PI3K, $pAKT^{ser473}$, GLUT2 and LDL-receptor levels *versus* their C counterparts ($p \leq 0.020$). In conclusion, CFE-enriched meat-consumption strongly counterbalanced diabetic dyslipidemia by improving insulin signaling, suggesting its being an adequate functional ingredient for T2DM.

1. Introduction

Type 2 Diabetes Mellitus (T2DM) prevalence has continued increasing in recent years, implying great clinical and social impacts. However, some pathophysiological facts remain poorly understood (Forouhi & Wareham, 2014). Insulin-resistance (IR) is the most frequent endogenous cause of lipid metabolism disorder contributing to higher cardiovascular disease (CVD) risk in T2DM patients (Laakso & Kuusisto, 2014). This pathology produces alterations in plasma lipids levels and consequently in lipoprotein metabolism (Verges, 2015). Fundamentally, T2DM presents quantitative, qualitative and kinetic lipoprotein alterations, which are clearly influenced by IR. The quantitative abnormalities consist in chylomicrons and VLDL increase, as a consequence of hypertriglyceridemia, and plasma HDL-level reduction (Parhofer, 2011; Tomkin & Owens, 2017; Verges, 2015). On the other hand, among qualitative changes, the most relevant are the presence of large VLDL particles, small and dense LDL, and triglyceride-enriched HDL. Due to alterations in chylomicrons, VLDL and LDL catabolism, kinetic changes can also be observed, which contributing to increase CVD risk (Mbue, Mbue, & Anderson, 2017; Parhofer, 2011; Tomkin & Owens, 2017). All these T2DM modifications seem to be related to hepatic insulin signaling alterations. The insulin receptor (InsR)/phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which is highly involved in the metabolic actions of insulin, is altered in IR (Leavens & Birnbaum, 2011; Tangvarasittichai, 2015). It has been demonstrated that disturbances in this pathway influence liver VLDL assembly and lipoprotein kinetic as a result, among other factors, of LDL-receptor (LDLr) expression reduction. Therefore, achieving better IR seems to be

Abbreviations: AE, arylesterase; AI, atherogenic index; CVD, cardiovascular disease; CFE, carob fruit extract; GSK3, glycogen synthase kinase 3; HDL, high-density lipoprotein; HSF, high-saturated-fat; IDL, intermediate-density lipoproteins; InsR, Insulin receptor; LDL, low-density lipoproteins; LDLr, LDL receptor; MUFA, monounsaturated fatty acid; PI3K, phosphatidylinositol 3-kinase; PON1, paraoxonase 1; PUFA, Polyunsaturated fatty acids; RM, restructured meat; SFA, saturated fatty acids; T2DM, Type 2 Diabetes Mellitus; VLDL, very low-density lipoproteins

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an adequate therapeutic strategy to manage dyslipidemia in T2DM (Tomkin & Owens, 2017; Verges, 2015).

At present, a direct relationship between meat intake, especially red meat, and T2DM-risk has been suggested (Schwingshackl et al., 2017). Other studies support ensuring protein-consumption to maintain or improve insulin secretion in T2DM patients (Tian et al., 2017). Our group research has been working for decades on introducing bioactive compounds into meat matrix with the objective of reducing the potential negative effects attributed to meat consumption (Garcimartín, Santos-López, Bastida, Benedí, & Sánchez-Muniz, 2015; Olivero-David et al., 2011; Santos-López et al., 2018). Recent studies have demonstrated beneficial effects of fiber and polyphenols on carbohydrates and fats metabolisms and are highly recommended for the diabetic population (McRae, 2018; Xiao & Hogger, 2015). Taking this into account, we have developed a functional meat incorporating carob fruit extract (CFE). The consumption of this new meat product should counterbalance the low intake of fiber and polyphenols observed in diabetic patients (Kaline, Bornstein, Bergmann, Hauner, & Schwarz, 2007; McRae, 2018). CFE is a great source of insoluble fiber and proanthocyanidins, to which has been related to powerful antidiabetic properties. Specifically, we have already reported a reduction in both postprandial glycemia and lipemia in healthy rats fed CFE (Macho-González et al., 2017, 2018). Likewise, we have also observed a lipoprotein profile improvement after CFE-enriched meat consumption in later-stage T2DM rat model (Macho-González et al., 2019). Due to the scarcity of studies evaluating the effect of functional meat-consumption in the early stages of T2DM, we have tested the effect of CFE-enriched meat-consumption on an established rat model (Fang, Lin, Huang, & Chuang, 2019; Sah, Singh, Choudhary, & Kumar, 2016), in which this early stage of T2DM was induced by a high-saturated-fat (HSF) diet.

Therefore, we hypothesized that CFE inclusion into a meat matrix reduces dyslipidemia and IR in diabetic rats fed the HSF diet. The aims of this study were to analyze the effect of CFE-enriched meat consumption in diabetic rats induced by HSF diet on, a) the qualitative, quantitative and kinetic lipoprotein changes; b) the InsR/PI3K/AKT pathway modifications; and c) the relationship between lipoproteins, main insulin pathway markers, and IR changes.

2. Materials and methods

2.1. Carob fruit extract (CFE)

CFE is a natural product with 71–81% of insoluble dietary fiber, that contains 34–40% of high molecular weight proanthocyanidins, according to Patent WO2006/000551 (Ruíz-Roso, Requejo, Pérez-Olleros, & Holguin, 2006). This extract was obtained from the carob pulp following the procedure described in Patent WO2004/014150 (Ruíz-Roso, Requejo, Pérez-Olleros, Martín-Casero, & Haber, 2004) and Macho-González et al. (2018).

2.2. Restructured meat, diet preparation and experimental design

Diets and restructured meat (RM) were formulated according to the protocol described by Macho-González et al. (Macho-González et al., 2019) (Supplementary Table S1). Briefly, for each diet kilogram, 30% of RM and 70% of a purified formulated diet were mixed and subsequently sieved 3 times until a completely homogenous powder was obtained. Two experimental semisynthetic diets were prepared: a) containing a control-RM (C) and b) containing CFE-enriched meat (CE), whose composition is detailed in Table 1. Both diets contained 49.42%, 36.43 and 14.15% of the total Kcal from fats, carbohydrates and proteins, respectively. Diets were calculated to accomplish micronutrient requirements at their final concentration.

Two-month old male Wistar rats were obtained from the Harlan Laboratories models (Harlan S.L., Barcelona, Spain) and housed in couples under controlled temperature (22.3 ± 1.9 °C) and light (12-h light/dark cycle) in the Animal Experimentation Center of the Alcalá University, Madrid, Spain (register no. ES280050001165). Tap water and food were provided *ad libitum*. Two rat groups of 8 rats each were distributed according to the diet, C and CE. Each group received the same diet for 8 weeks. Each group received the same diet for 8 weeks. Food consumption was measured daily and body weight once a week. Feces were collected the last week and fecal fat was extracted and quantified as previously reported (Macho-González et al., 2018). In order to avoid inter-assay variations, overnight fasted rats were taken, anesthetized with isoflurane (5% v/v) and euthanized. Blood was collected from the descending aorta with a heparinized syringe in cold tubes and placed in ice until processing. Livers were removed, weighed, and prepared properly.

This study was approved by the Spanish Science and Technology Advisory Committee (project AGL2014-53207-C2-2-R) and by the Ethics Committee of the Complutense University of Madrid (Spain). Experiments were performed in compliance with Directive 86/609/EEC of 24 November 1986 (amended by EU Directive 2010/63/EU) on the protection of scientific research animals.

Table 1
Composition of the Restructured Meat (RM) incorporated to the experimental diets fed male Wistar rats.

Restructured meat components	Control-RM	CFE-RM
Protein, %	13.1	13.1
Fat, %	38.3	38.3
Water, %	46.9	46.9
Cholesterol, g/kg	0.74	0.74
SFA/MUFA/PUFA ratio	41.2/43.5/8.5	41.2/43.5/8.5
Ingredients		
Lean pork, g/kg	663.1	663.1
Lard, g/kg	331.1	331.1
Na, g/kg	0.5	0.5
STP, g/kg	0.1	0.1
Sodium nitrite, g/kg	1.2	1.2
CFE, g/kg	0.0	4
Cellulose	4	0.0

Control Restructured Meat (Control-RM), CFE-enriched meat (CFE-RM). STP, sodium tripolyphosphate. SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, Polyunsaturated fatty acids.

2.3. Glycemia and insulin determinations

Blood samples were collected in fasting conditions in heparinized tubes at the middle of the third week and at the end of the experiment. Plasma was isolated by centrifugation for 10 min at 986*g*, and glycemia was quantified immediately in a plate reader (SPECTROstar Nano, BMG LABTECH, Offenburg, Germany) at 492 nm, using the GOD kit (Spinreact, Barcelona, Spain) (Macho-González et al., 2017). Insulin was measured using ELISA kit (Rat insulin ELISA Kit, ELR-Insulin, RayBiotech, Inc., USA) according to the manufacturer's manual. The colour intensity was evaluated at 450 nm using a microplate reader (SPECTROstar Nano). QUICKI index was calculated according to the formula 1/[(log insulin)(mIU/l) + (log glucose) mg/dl)] and HOMA-IR was calculated as: glucose (mmol/l) × insulin (mIU/l)/22.5 (Bowe et al., 2014; Gesteiro, Rodríguez Bernal, Bastida, & Sánchez-Muniz, 2012).

2.4. Lipoprotein isolation

Plasma was separated from the whole blood by centrifugation for 20 min at 615*g* and kept at 4 °C until lipoprotein isolation. A SW 40.1 rotor was used to separate the different lipoprotein fractions in 2 mL plasma samples by saline gradient ultracentrifugation during 21 h 40 min at 272,000*g* (40,000 rpm) at 4 °C (Beckman L8-70 M, California, USA) following a modification of the Terpstra et al. method (Terpstra,

Woodward, & Sánchez-Muniz, 1981) as previously described (Garcimartín et al., 2015). Lipoproteins were isolated according to conventional density range for rats [VLDL ($\rho_{20} < 1.0063$ g/mL), IDL ($1.0063 < \rho_{20} < 1.019$), LDL ($1.019 < \rho_{20} < 1.057$), and HDL ($1.057 < \rho_{20} < 1.21$ g/mL)] (Bocanegra et al., 2009).

2.5. Plasma lipid analysis and lipoprotein composition

Total cholesterol, triglycerides and phospholipids were determined in plasma and lipoprotein fractions (VLDL, IDL, LDL, and HDL) using standard enzymatic colorimetric tests (Spinreact) according to the manufacturer's manual in a plate reader at 492 nm (SPECTROstar Nano). Total lipids were calculated as the sum of total cholesterol, triglycerides, and phospholipids. The protein content of each lipoprotein fraction was analyzed by the Bradford method (Bradford, 1976). The total mass of each lipoprotein fraction was calculated as the sum of proteins and total lipids (cholesterol + triglycerides + phospholipids) (both in mg/dL) (Macho-González et al., 2019). The atherogenic index (AI) was calculated as described by Garcimartín et al. (Garcimartín et al., 2015).

2.6. Liver and VLDL oxidation (TBARS assay)

The Mihara and Uchiyama method (Uchiyama & Mihara, 1978) was used to quantify liver and VLDL oxidation (liver-ox and VLDL-ox, respectively). Malondialdehyde (MDA) formed after adding thiobarbituric acid to VLDL samples and liver extracts were measured in a fluorescence plate reader (FLUOstar Omega, BMG LABTECH, Offenburg, Germany) at 485/20 nm excitation and 528/20 nm emission wavelengths. MDA standard curve was performed to quantify liver and VLDL oxidations. Results are expressed as mg MDA mg/L of plasma for VLDL-ox and MDA/mg protein for liver-ox.

2.7. Arylesterase activity measurement

Plasma arylesterase (AE) activity was measured using simulated body fluid as a buffer and phenylacetate as a substrate according to Nus, Sánchez-Muniz, Gago, López-Oliva, and Sánchez-Montero (2008). Reaction rates of AE were followed at 270 nm in thermostatically controlled 10-mm Lightpath quartz cuvettes using a spectrophotometer (SPECTROstar Nano). Liver AE activity was measured in liver extracts according to Macho-González et al. (Macho-González et al., 2019) and results expressed in AE units/mg protein. One unit of AE was defined as the phenol (mmol) formed from phenylacetate per minute.

2.8. Western blotting

Immunoblots were performed using liver lysates. Protein extracts (DC Protein Assay Kit, Bio-Rad, Madrid, Spain) from each sample were separated by SDS–PAGE and transferred to polyvinylidene fluoride membranes (GE Healthcare, Madrid, Spain). Membranes were incubated overnight at 4 °C with the following primary antibodies: anti-LDLr, anti-insulin receptor β, anti-PI3K, anti-AKT2, anti-pAKT^{ser473}, anti-GSK3β, anti-pGSK3β^{ser9} and anti-GLUT2 (Santa Cruz Biotechnology, Quimigen, Madrid, Spain). Anti-β-actin and anti-α-tubulin were used as the loading control. Antibodies hybridization was revealed by incubating the membranes with the appropriate secondary antibodies conjugated with peroxidase for 1 h at room temperature. The chemiluminescence signal of membranes was read in an ImageQuant LAS 500 (GE Healthcare, Madrid, Spain) using the ECL kit Select-kit (GE Healthcare, Madrid, Spain).

2.9. Immunohistochemical staining

Paraffin-embedded liver sections were deparaffinized and rehydrated in a graded ethanol series. After retrieving citrate antigen and quenching endogenous peroxidase, sections were incubated with different primary antibodies overnight at 4 °C to determine the presence of: anti-LDLr, anti-insulin receptor β, anti-PI3K, anti-AKT2, anti-pAKT^{ser473}, anti-GSK3β and anti-pGSK3β^{ser9} (Santa Cruz Biotechnology). The color reaction was developed with a polymerized horseradish peroxidase-conjugated secondary antibody and counterstained with hematoxylin. The intensity of immunostaining for each antibody was measured using ImageJ free software (U. S. National Institutes of Health, Bethesda, USA). A total of 15 fields per section per rat (200× magnification for image analysis) were selected and analyzed. The positive staining intensity was calculated as percentage of the ratio of the stained area to the total field assessed.

2.10. Statistical analysis

Results were expressed as mean ± standard deviation (SD). Group results were compared by the unpaired Student's *t*-test. The Chi-squared test was used to compare the prevalence of hypertriglyceridemia in both diet groups. Pearson product-moment correlations were applied to correlate GLUT2, insulin, HOMA-IR, QUICKI, VLDL total mass, VLDL total lipids, PI3K, pAKT2^{ser473} and LDLr. Differences were considered significant at $p < 0.05$. Statistical analysis was performed using SPSS version 25.0 (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. Growth rate, feed consumption and fecal excretion

Table 2 summarizes the feed intake, growth rate and fecal excretion of both groups. CE animals showed significant differences respect to C group in the growth rate, fecal excretion, fecal moisture, fecal fat and dietary digestibility ($p \leq 0.014$). CE rats displayed lower growth rate and dietary digestibility than C animals. Fecal excretion, fecal fat and fecal moisture were significantly higher in CE rats respect to C group (10.1%, 31.4% and 72.4%, respectively).

3.2. Plasma analysis

Table 3 shows glycemia, insulinemia, HOMA-IR, QUICKI, plasma lipid concentrations, atherogenic index (AI) and the cholesterol-to-phospholipid and cholesterol-to-HDL cholesterol ratios in the two experimental groups. C rats showed a typical T2DM status with fasting hyperglycemia, hyperinsulinemia and high levels of HOMA-IR. Insulin, HOMA-IR, QUICKI, triglycerides, total lipids and AI were significantly affected by CFE-RM diet ($p \leq 0.028$). CE rats revealed significantly lower insulin (−11.5%), HOMA-IR (−15.7%), triglycerides (−15.2%), total lipids (−10.8%) and AI (−34.4%) than C animals, while QUICKI

Table 2
Feed intake, growth rate and fecal excretion of animals fed different diets.

	C	CE	Student's *t*-test
Dietary intake (g/wk)	115.8 ± 8.52	101.0 ± 6.69	p = 0.002
Growth rate*	0.20 ± 0.01	0.18 ± 0.01	p < 0.001
Final weight (g)	388.3 ± 38.9	377.6 ± 32.2	p > 0.05
Fecal excretion (g/wk)**	7.99 ± 0.56	8.80 ± 0.29	p = 0.014
Fecal moisture (%)	14.35 ± 1.12	18.86 ± 1.21	p < 0.001
Fecal fat (mg/g faeces)**	80.00 ± 6.66	137.89 ± 7.15	p < 0.001
Dietary digestibility***	0.92 ± 0.01	0.90 ± 0.01	p = 0.001

Data are expressed as the mean ± standard deviation (n = 8).
* Growth rate: g food converted in g body weight.
** Data are dry matter weights.
*** Dietary digestibility = (feed intake − faeces)/feed intake. Dietary intake was measured daily and body weight, once a week; feces were collected the last week of the study. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

Table 3
Glycemia, insulin, plasma lipids, cholesterol:phospholipid and cholesterol:HDL-cholesterol ratio and atherogenic index of animals fed different diets.

	C	CE	Student's *t*-test
Glycemia 3rd week (mmol/L)	6.00 ± 0.44	6.44 ± 0.47	p > 0.05
Glycemia 8th week (mmol/L)	13.92 ± 0.91	13.23 ± 0.71	p > 0.05
Insulin (μIU/mL)	15.84 ± 0.73	14.02 ± 1.04	p = 0.002
HOMA-IR*	9.79 ± 0.64	8.25 ± 0.63	p < 0.001
QUICKI**	0.278 ± 0.002	0.284 ± 0.003	p < 0.001
Total cholesterol (mmol/L)	2.16 ± 0.18	2.06 ± 0.18	p > 0.05
Phospholipids (mmol/L)	1.35 ± 0.32	1.23 ± 0.24	p > 0.05
Triglycerides (mmol/L)	1.78 ± 0.25	1.51 ± 0.13	p = 0.017
Total lipids (mg/dL)+	332 ± 35.3	296 ± 18.5	p = 0.028
Cholesterol:phospholipids (mol/mol)	1.57 ± 0.29	1.65 ± 0.29	p > 0.05
Cholesterol:HDL cholesterol (mol/mol)	1.43 ± 0.22	1.28 ± 0.14	p > 0.05
Atherogenic index++	0.32 ± 0.06	0.21 ± 0.09	p = 0.014

Data are expressed as the mean ± standard deviation (n = 8).
* HOMA-IR: (fasting insulin (μIU/mL) × fasting glucose (mmol/L)/22.5).
** QUICKI: 1/[log (fasting insulin (μIU/mL) + log (fasting glucose (mg/dL)]).
+ Total lipids: cholesterol + triglycerides + phospholipids.
++ Atherogenic index = (total cholesterol − HDL cholesterol)/HDL cholesterol. To transform mmol/L into mg/dL of glucose, cholesterol, TGs, and phospholipids, multiply data by 18.0, 38.68, 89.0, and 75.0, respectively. All parameters were evaluated at the end of the study (8th week) with the exception of glycemia (3rd week). C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

index was significantly higher in CE *versus* C group (p < 0.001). Hypertriglyceridemia (≥1.7 mmol/L) was found in 62.5% of C rats, but 0% of CE rats (chi-square test, p < 0.001), showing a marked reduction in CE groups *versus* their C counterparts (−15.2%).

3.3. Lipoprotein composition

The composition of the different lipoprotein fractions in absolute values (mmol/L or mg/dL) is shown in Table 4. HDLs were the major carriers of cholesterol (75.7 *versus* 77.1%), phospholipids (72.7 *versus* 73.04%) and apolipoproteins (91.0 *versus* 85.7%) in C *versus* CE rats; whereas VLDLs were the major carrier of TGs (86.2 *versus* 70.8% in C and CE, respectively).

CE rats showed significantly (p < 0.05) lower content of cholesterol, triglycerides, phospholipids, total lipids and total mass in VLDL fraction with respect to C group. CE animals displayed higher triglycerides, phospholipids, total lipids, proteins and total mass in IDL and LDL lipoprotein fractions (p = 0.015) than their C counterparts. HDL lipoprotein fraction only exhibited differences in phospholipids, showing lower levels in CE rats respect to C (p = 0.014).

CE animals exhibited significantly lower total lipids/proteins and cholesterol/proteins ratios in VLDL and LDL fractions (p = 0.001); and lower total lipids/proteins of IDL than C rats. Non-significant differences (p > 0.05) were observed between C and CE groups in the IDL cholesterol/protein ratio and HDL of lipids and cholesterol ratios (data not-shown).

3.4. Percentage contribution of lipids to lipoprotein composition and liver LDLr levels and immunolocalization

Fig. 1(a)–(d) displays the percentage contribution of lipids and proteins to the total mass of plasma VLDL, IDL, LDL, and HDL. CE rats had less triglycerides (−3.4%) and more proteins (55.4%) in VLDL fraction than C group (p = 0.041). Cholesterol (99.0%) and proteins (169.6%) contributed more while phospholipids less (−40.1%) to the IDL total mass in CE *versus* C rats (p = 0.002). CE animals showed LDL

Table 4
Lipoprotein component concentrations in plasma of animals fed different diets.

Lipoproteins	C	CE	Student's *t*-test
Cholesterol, mmol/L			
VLDL	0.44 ± 0.05	0.35 ± 0.09	p = 0.005
IDL	0.02 ± 0.00	0.10 ± 0.02	p < 0.001
LDL	0.03 ± 0.00	0.03 ± 0.01	p > 0.05
HDL	1.53 ± 0.14	1.62 ± 0.14	p > 0.05
Triglycerides, mmol/L			
VLDL	1.56 ± 0.05	0.97 ± 0.06	p < 0.001
IDL	0.08 ± 0.04	0.19 ± 0.05	p < 0.001
LDL	0.03 ± 0.01	0.05 ± 0.01	p = 0.014
HDL	0.14 ± 0.01	0.16 ± 0.02	p > 0.05
Phospholipids, mmol/L			
VLDL	0.31 ± 0.03	0.20 ± 0.04	p < 0.001
IDL	0.05 ± 0.01	0.09 ± 0.01	p < 0.001
LDL	0.02 ± 0.00	0.09 ± 0.01	p < 0.001
HDL	1.01 ± 0.15	0.84 ± 0.11	p = 0.014
*Total lipids, mg/dL**			
VLDL	178.60 ± 6.12	113.22 ± 10.20	p < 0.001
IDL	11.56 ± 3.69	27.50 ± 4.87	p < 0.001
LDL	4.93 ± 1.43	6.63 ± 0.96	p = 0.015
HDL	145 ± 12.10	137 ± 11.58	p > 0.05
Proteins, mg/dL			
VLDL	5.07 ± 0.81	5.05 ± 0.56	p > 0.05
IDL	0.57 ± 0.26	3.88 ± 0.87	p < 0.001
LDL	0.40 ± 0.14	1.08 ± 0.45	p = 0.003
HDL	60.9 ± 5.43	60.1 ± 3.12	p > 0.05
*Total lipoprotein mass, mg/dL***			
VLDL	184 ± 6.44	118 ± 10.50	p < 0.001
IDL	12.05 ± 3.91	30.83 ± 5.78	p < 0.001
LDL	5.32 ± 1.49	7.72 ± 1.08	p = 0.003
HDL	206 ± 15.96	197 ± 14.14	p > 0.05

Data are expressed as the mean ± standard deviation (n = 8).
* Total lipids: cholesterol + triglycerides + phospholipids.
** Total mass: total lipids + proteins. All samples were taken at the end of 8th week experiment. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

with less cholesterol (−38.1%) and phospholipids (−24.8%) but more proteins (82.7%) than C counterparts (p = 0.010). HDL total mass revealed more cholesterol (10.5%) and less phospholipids (−12.9%) contributions in CE *versus* C rats (p = 0.003).

The CFE-enriched meat effects on liver LDLr levels and immunolocalization are shown in Figs. 2 and 3(a) and (b). CE rats revealed higher levels (12.9%) and immunohistochemical staining (37.8%) than C animals (p < 0.05). Immunoblots from CE rats displayed higher LDLr levels (12.9%) compared to C animals (p < 0.05). Immunohistochemistry revealed a diffuse and very faint cytoplasmic staining pattern in sections from C liver using a monoclonal anti-LDLr antibody. Punctate most intense and stronger LDLr-specific staining was seen in the CE liver sections, showing a panlobular pattern. CE induced a higher LDLr immunoreactivity (37.8%) compared with C liver sections.

3.5. Arylesterase activity, VLDL and liver oxidation

Table 5 shows the plasma AE activity, the AE activity-to-plasma cholesterol ratio, liver AE activity and VLDL and liver oxidations. Plasma AE activity and AE activity-to-plasma cholesterol ratio were significantly reduced by CE diet (−19.5 and −16.7%, respectively; p = 0.024). Instead, a significant increase was observed in the liver AE activity (23.6%; p = 0.037) in CE compared to C rats. Non-significant differences were observed (p > 0.05) in VLDL-ox and liver-ox between groups.

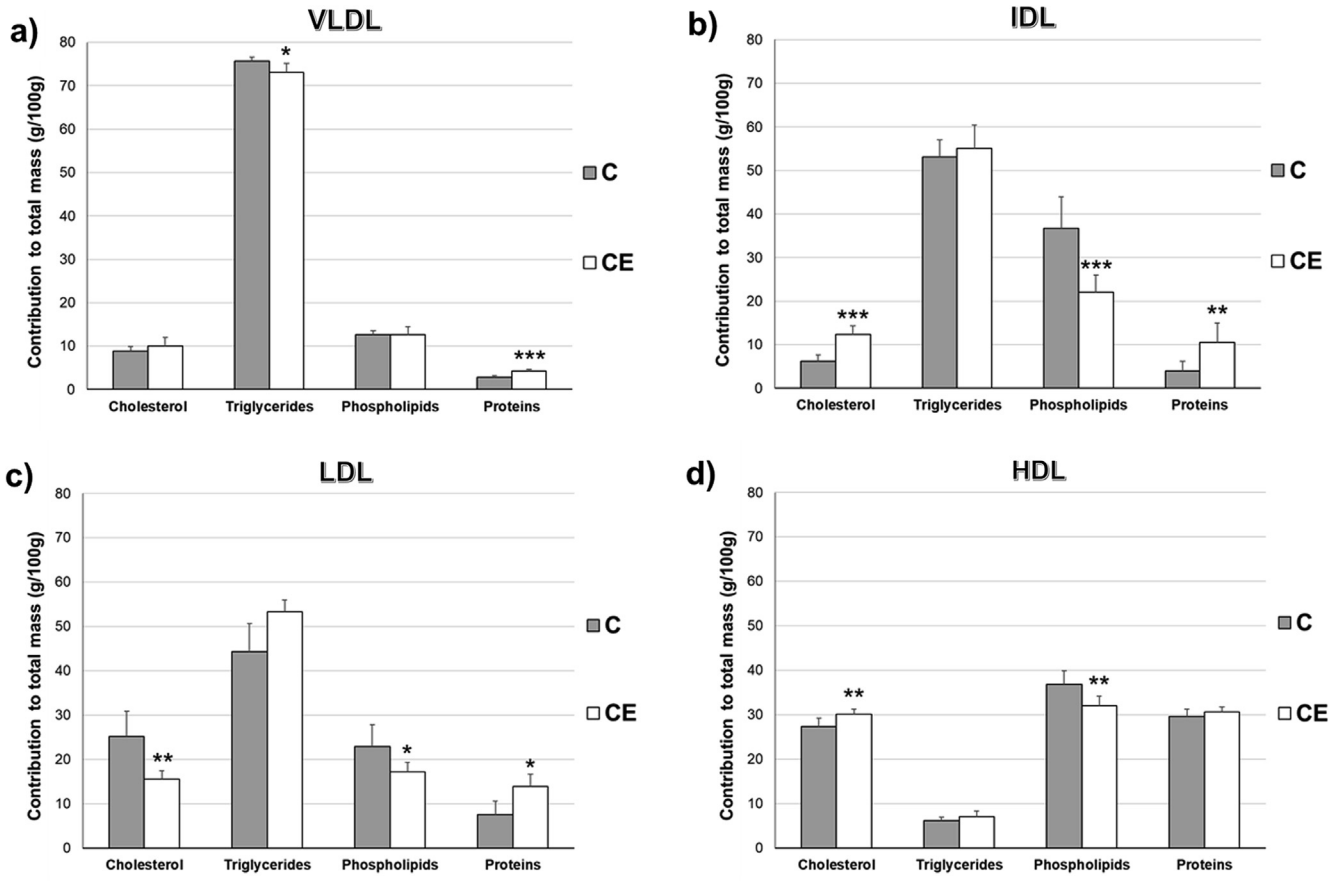

Fig. 1. Percentage contribution (%) of proteins and lipids to the total mass of plasma VLDL (a), IDL (b), LDL (c) and HDL (d) fractions in the C and CE groups. Data are expressed as the mean ± standard deviation (n = 8). *p < 0.05; **p < 0.01; ***p < 0.001. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

3.6. Insulin signaling (InsR/PI3K/AKT/GSK3 pathway) analyzed by western blot and immunohistochemistry

Fig. 3(a)–(d) displays the levels and distribution of the main proteins related to liver insulin signaling pathway (InsR/PI3K/AKT/GSK3 pathway). CE rats showed higher protein expression levels of PI3K (WB = 29.3%), pAKT2^{ser473} (WB = 13.1%) and GLUT2 (WB = 33.9%) than C animals. The immunohistochemistry analysis data supported the quantitative western blot results. A homogeneously distributed staining for InsR, PI3K, pAKT2^{ser473} and pGSK3β^{ser9}, which was slightly stronger

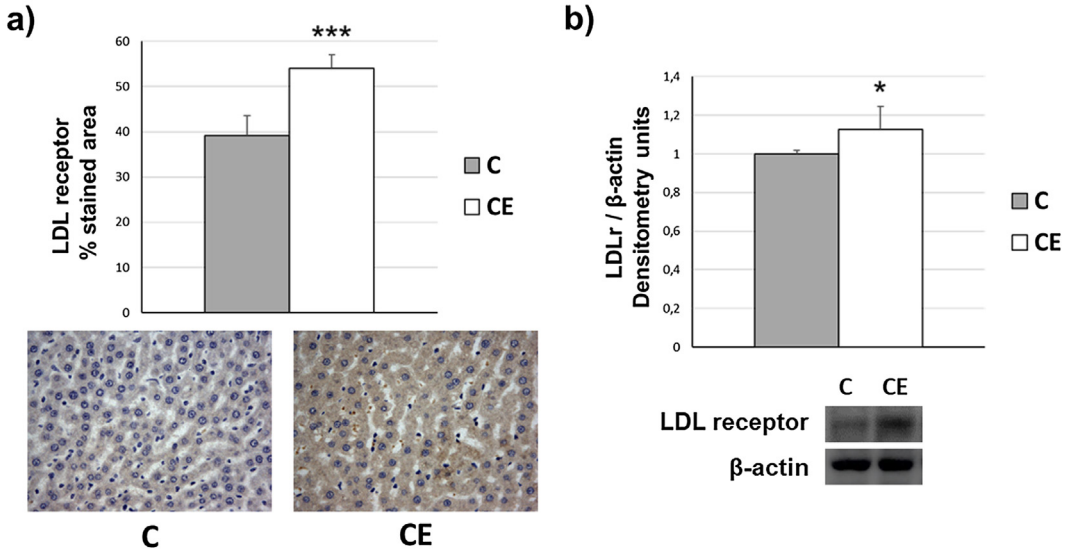

Fig. 2. Liver LDL-receptor levels and localization in the different groups. Data are expressed as the mean ± standard deviation (n = 8). (a) LDLr immunohistochemical staining quantification and representative images of the different groups; (b) LDLr levels quantification by western blot. *p < 0.05; **p < 0.01; ***p < 0.001. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

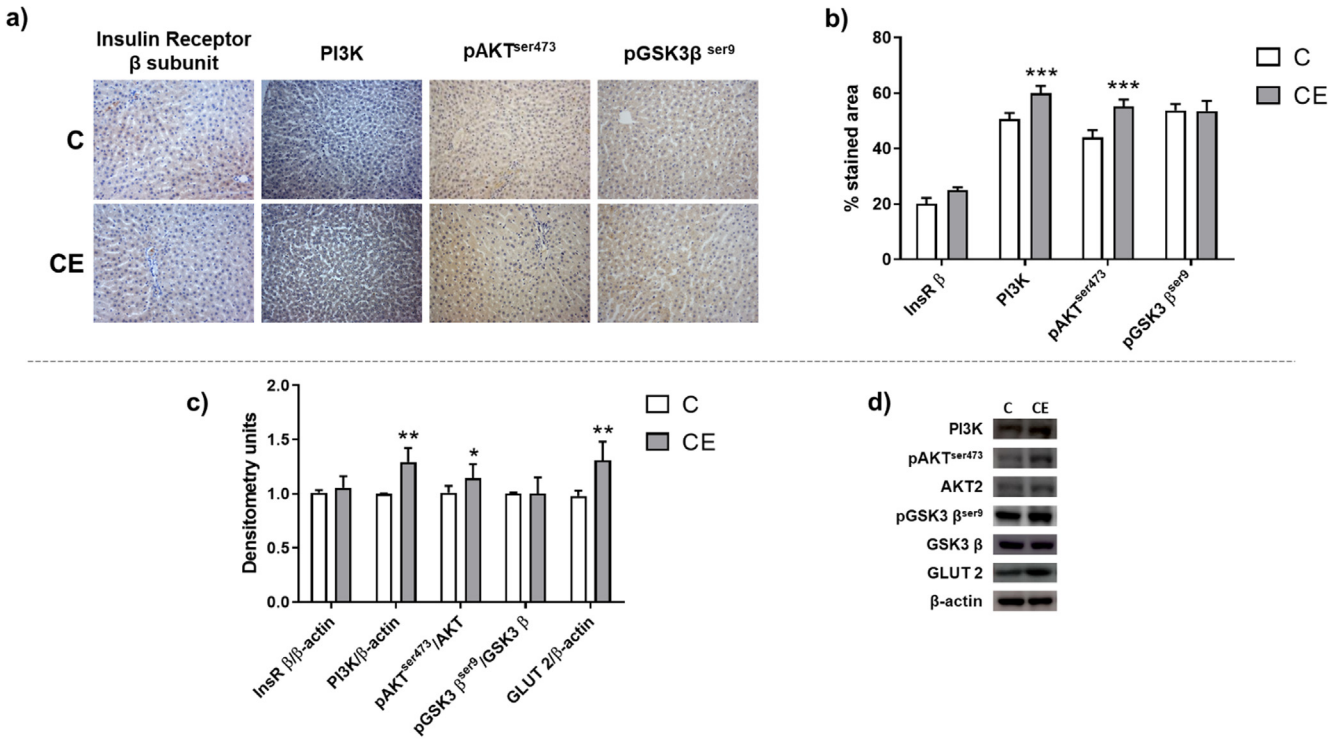

Fig. 3. Levels and localization of the main proteins related to InsR/PI3K/AKT/GSK3 pathway of animals fed the experimental diets. Data are expressed as the mean ± standard deviation (n = 8). (a) representative images of liver immunohistochemical staining for the InsR/PI3K/AKT/GSK3 pathway; (b) staining area quantification for each of the analyzed proteins; (c) quantification of InsR/PI3K/AKT/GSK3 levels in liver extracts by western blot; (d) representative images of the different western blot realized. *p < 0.05; **p < 0.01; ***p < 0.001. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

Table 5
LDL receptor levels, plasma and liver AE activity and VLDL and liver oxidation of animals fed the experimental diets.

	C	CE	Student's *t*-test
Plasma AE (U/L)	185 ± 38.9	149 ± 15.8	p = 0.006
Plasma AE:cholesterol (U/mg)*	0.24 ± 0.06	0.20 ± 0.02	p = 0.024
Liver AE (U/g protein)	6.11 ± 1.05	7.55 ± 1.24	p = 0.037
VLDL-ox (TBARS, mg MDA/L)	2.18 ± 0.24	2.23 ± 0.22	p > 0.05
Liver-ox (TBARS, mg MDA/mg protein)	2.62 ± 0.44	2.52 ± 0.26	p > 0.05

Data are expressed as the mean ± standard deviation (n = 8).

* Units AE (U/L)/total cholesterol (mg/L). One unit of AE was defined as mmols of phenol formed from phenylacetate per minute. All samples were taken at the end of 8th week experiment. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

in centrilobular areas, was observed in liver sections from both groups. The CE livers showed a stronger PI3K (18.4%) and pAKT2^{ser473} (25.3%) staining and bigger surface area in hepatocytes around periportal and pericentral areas, suggesting PI3K and pAKT2^{ser473} over-expression. Non-significant differences between C and CE groups were found for InsR and pGSK3β^{ser9} proteins.

4. Discussion

These results shed new light on the role of CFE and its utilization to develop functional meats to reduce qualitative, quantitative and kinetic lipoprotein alterations by improving insulin signaling in T2DM rats fed a high-saturated-fat diet. CFE-enriched meat partially blocked T2DM alterations, reduced insulinemina, HOMA-IR, triglyceridemia and VLDL levels and composition; and increased fecal fat excretion, LDLr levels and insulin signaling in the liver (Fig. 4). These results are relevant given the high consumption of meat in Western countries and the low fiber and polyphenols intakes observed in diabetic population. Therefore, it can be suggested the possibility of reducing T2DM alterations and increasing fiber intake, by including the CFE as a functional ingredient in meat products.

Experimental diets were well accepted and agreed with previous results of our group on functional meat products (Macho-González et al., 2019; Santos-López et al., 2018). Although the final weight of rats was very similar in both groups, the lower dietary intake and dietary digestibility of CE group justify the lower growth rate of those rats. These results seem primarily due to a satiating effect and increase of fecal bolus mediated by fiber (Macho-González et al., 2018; McRae, 2018). Likewise, differences in fecal amount and composition should be related to CFE proanthocyanidins. Similar results were previously found in a digestibility study in healthy and diabetic rats (Macho-González et al., 2018, 2019). Proanthocyanidins have also been related to gastric emptying delay. Together with the fiber satiating effect, these two components would increase satiety and therefore a lower diet intake, as observed in CE animals (Cires, Wong, Carrasco-Pozo, & Gotteland, 2016; Serrano et al., 2016).

Feeding with a HSF diet induces IR in different animal models (Sah et al., 2016; Skovsø, 2014). As observed in our results, the C rats present hyperglycemia, hyperinsulinemia, and typical dyslipemia at early T2DM stage (Asrafuzzaman et al., 2017; Fang et al., 2019). CE animals displayed lower HOMA-IR and higher QUICKI mainly due to their insulinemia being significantly lower than that of C group and linked to the proanthocyanidins effect on carbohydrates metabolism, as reported by Yang et al. (Yang & Chan, 2017). Furthermore, proanthocyanidin-consumption, by diminishing both glucose digestion and absorption, improves IR by reducing glycemia and insulinemia (Cires et al., 2016; Salvado, Casanova, Fernandez-Iglesias, Arola, & Blade, 2015; Yang & Chan, 2017). In fact, a lower glycemia increase (−15%) was observed between the third and eighth week in CE rats (6.79 *versus* 7.92 mmol/L), which was consistent with the hypoglycemic effect observed in streptozotocin-nicotinamide diabetic rats (Macho-González et al., 2019)

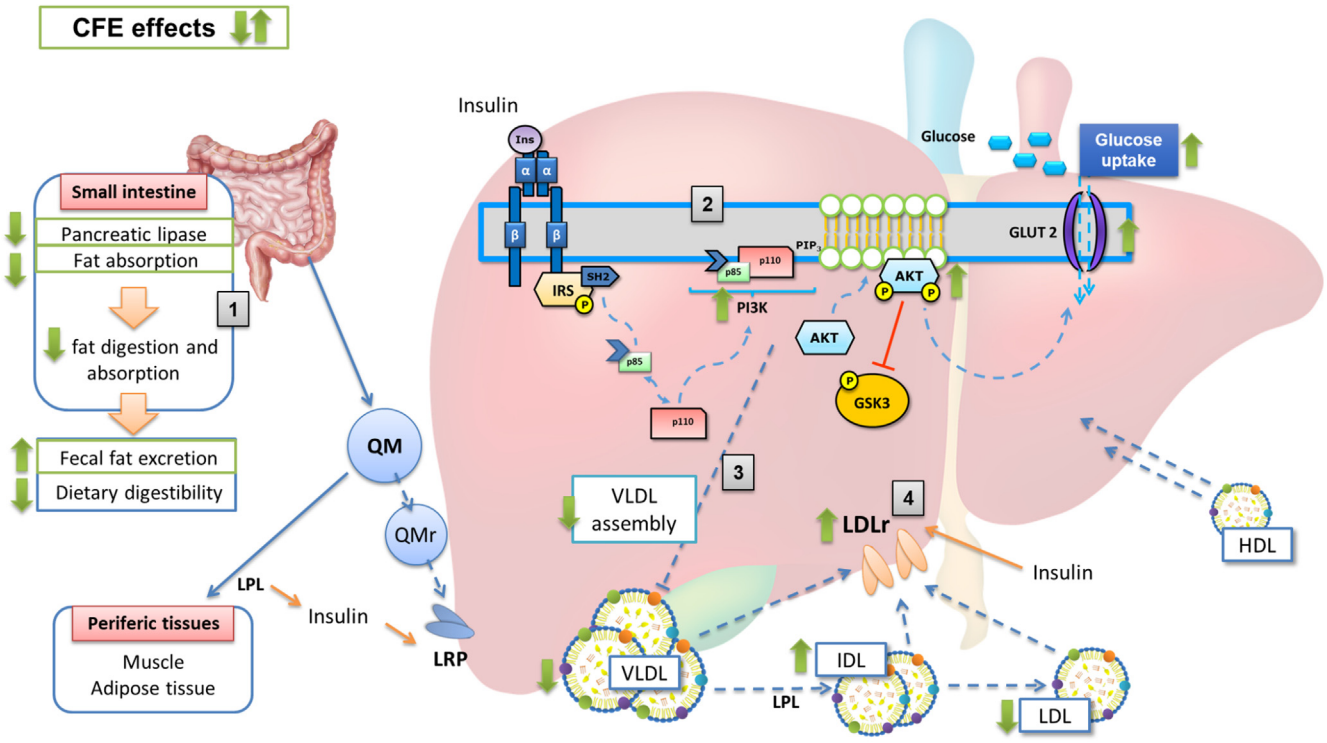

Fig. 4. Proposed mechanism of the carob fruit extract (CFE) effects on lipoprotein metabolism and insulin signaling. The T2DM alterations induced by high-saturated-fat diet consumption are based on hyperglycemia, hyperinsulinemia and insulin resistance. (1) The CFE-enriched meat consumption increases fat excretion by decreasing digestion and absorption of lipids; (2) the main proteins levels involved in insulin signaling increased in the group fed with CFE, which would improve glucose uptake; (3) the lower plasma triglycerides availability to form VLDL, together with the InsR/PI3K/AKT pathway inhibition on the VLDL assembly and maturation, justify the lower levels and size of these lipoproteins; (4) the CFE increases LDLr levels, promoting the uptake of IDL and LDL. AKT; CFE, carob fruit extract; HDL, high-density lipoproteins; GLUT2, glucose transporter 2; GSK3, glycogen synthase kinase 3; IDL, intermediate-density lipoproteins; Ins, insulin receptor; IRS, insulin receptor substrate; LDL, low-density lipoproteins; LDLr, low density lipoprotein receptor; LPL, lipoprotein lipase; LRP, low density lipoprotein receptor-related protein; PI3K, phosphoinositide 3-kinase (formed by p85 and p110 subunits); QM, chylomicrons; QMr, remnant chylomicrons; VLDL, very low-density lipoproteins.

and in chow fed rats during a postprandial study (Macho-González et al., 2017).

Diabetic dyslipidemia is the main and more frequently lipid abnormality observed in T2DM patients (Verges, 2015). Hypertriglyceridemia is considered one of the basic T2DM dyslipidemia pillars, being the promoter of quantitative and qualitative lipoprotein changes (Verges, 2015). Using 1.7 mmol/L as a cut-off point of hypertriglyceridemia (Berglund et al., 2012), 62.5% of the C rats while 0% of CE animals presented hypertriglyceridemia. These results agree with previous studies of our group in healthy rats, where CFE-consumption reduced postprandial hypertriglyceridemia (Macho-González et al., 2018), and with studies carried out in humans (Goulas, Stylos, Chatziathanasiadou, Mavromoustakos, & Tzakos, 2016).

The lower plasma triglyceride, total lipid and AI observed in CE rats seem to be due to the conjoint action of proanthocyanidins (reducing fat digestion and absorption), and the drag effect of fiber (Kaline et al., 2007; Salvado et al., 2015). Thus, the inclusion of CFE-enriched meat in the diet seems to be an adequate nutritional strategy to ensure the fiber and polyphenols intakes, usually reduced in T2DM patients (McRae, 2018). Together with the hypertriglyceridemia, C rats showed higher total lipids and AI than CE rats. Results of C group on lipoprotein fractions indicate VLDL qualitative and quantitative changes in comparison with previous studies in healthy rats (Schultz-Moreira et al., 2014). C animals showed the typical lipoprotein profile of diabetic rats with the presence of $VLDL_1$, characterized by a larger size and by being triglycerides-enriched (Haas, Attie, & Biddinger, 2013; Verges, 2015). In fact, isolated VLDL in C rats contain 4.2 and 3.2 times more triglycerides and their total lipid masses were 3.8 and 2.6 times higher than those found by Schultz-Moreira et al. and Viejo, respectively (Schultz-Moreira et al., 2014; Viejo, 1992), suggesting a VLDL overproduction or lower VLDL catabolism. On the other hand, CE rats display 36% less total VLDL mass than C rats. In addition, these VLDL particles seem to be smaller and in a more advanced catabolism, because of their lower content in triglycerides while higher in proteins (Fig. 1). Related to this, an increase in total IDL and LDL mass was clearly observed in CE group. A detailed study of IDL composition suggests that they were enriched in cholesterol and impoverished in phospholipids. These results may be due to lipoprotein kinetic modifications, proper of the diabetic situation in which a decrease in lipoprotein remnant catabolism has been suggested (Parhofer, 2011; Verges, 2015). In order to reduce this remnant lipoprotein amount, higher LDLr levels were found in CE rats, a fact that has also been observed in a previous study with diabetic and hypercholesterolemic rats (Macho-González et al., 2019). Finally, diabetic dyslipidemia is also related to quantitative and qualitative changes in the HDL fraction (Tomkin & Owens, 2017). However, in our study we barely observed relevant modifications in the HDL fraction, just a relative cholesterol-enrichment and phospholipid-impoverished of HDL particles in CE group with respect to C, which could be associated with greater cholesterol elimination from the tissues (Barter, 2002).

The PON-1 has been defined as a suicide enzyme with pleiotropic antioxidant action (Aviram, 2004). According to Olivero-David et al. (Olivero-David et al., 2011) this enzyme acts first to protect the presence of other antioxidant enzymes. As CFE shows antioxidant activity (Bastida et al., 2009) it can be speculated that less AE is demanded to protect the antioxidant status in CE rats *versus* C rats. Estrada-Luna et al. (Estrada-Luna et al., 2018) found that rats fed antioxidants show a decrease in the AE activity. The higher liver AE activity of CE animals seems related to the enhanced lipoprotein catabolism of this group, in

order to reduce the radical oxygen species formation during fatty acids oxidation. However, more research has to be performed to ascertain this potential relationship.

In order to find the relationship between diabetic dyslipidemia and insulinemia, we evaluated hepatic insulin-signaling as the central axis of the altered lipoprotein metabolism (Haas et al., 2013; Verges, 2015). Several studies indicate that the Ins/PI3K/AKT pathway modulates the insulin effect on lipoproteins metabolism, and its alteration may produce a VLDL overproduction (Brown & Gibbons, 2001; Taghibiglou et al., 2002) and hepatic LDLr reduction (Duvillard et al., 2003), results that explain, at least partially, C rat data. CE rats presented higher PI3K and pAKT2^{ser473} levels than their C counterparts, suggesting that a CE diet promotes a more effective insulin signals transduction than the C diet. The insulin binding to its receptor causes the recruitment of proteins from the insulin receptor substrate and the subsequent PI3K translocation, inducing many of the known metabolic insulin effects. PI3K activates AKT, which phosphorylates and inhibits GSK3, favoring glucose uptake and glycogen synthesis (Lee & Kim, 2007). This signaling pathway is affected by IR and the proanthocyanidins consumption may improve its activation in order to manage glucose homeostasis (Salvado et al., 2015). The Ins/PI3K/AKT pathway participates in the assembly and VLDL maturation, by inhibiting the lipids transfer to the VLDL precursors (Sparks, Sparks, & Adeli, 2012) and justifies the lower VLDL amount and size in CE rats. Likewise, the improvement in insulin signaling induces the LDLr expression, as observed and previously discussed in rats fed CFE-enriched meat (Macho-González et al., 2019). On the other hand, the PI3K and pAKT2^{ser473} higher levels in CE rat liver are also related to better glucose uptake. Proanthocyanidin-consumption has shown beneficial effects in glycemia by improving glucose uptake in different tissues through the AKT2 phosphorylation (Salvado et al., 2015; Yang & Chan, 2017). Proanthocyanidin consumption has been associated with an increase in liver GLUT2 levels (Cao et al., 2016). In the present study CE rats showed a 31% higher GLUT2 levels *versus* those C animals, being a clear indication of improved glucose uptake and insulin-sensitivity. In fact, a strong negative and significant correlation ($r = -0.696$; $p < 0.01$) was found between GLUT2 and HOMA-IR. A detailed study of data suggests a very high relationship between insulin-signaling, glucose homeostasis and VLDL levels. Thus, PI3K and pAKT2^{ser473} quantified by immunohistochemistry were negatively and very significantly correlated ($r = -0.700$; $p < 0.01$) with VLDL mass, VLDL lipids, insulin and HOMA-IR; and positively and significantly correlated with LDLr, GLUT2 and QUICKI ($r = 0.648$; $p < 0.05$).

Despite the positive results observed, this study presents some possible limitations: (1) only the study has been carried out in males, (2) healthy control could be added to the study, and (3) the study lasted only 8 weeks. As T2DM is a chronic degenerative disease, future researches should analyze the CFE-enriched meat effect in animal model longitudinal studies. In addition, as CFE has been found to be safe in healthy and diabetic rats (Macho-González et al., 2017, 2018) and in hypercholesterolemic human (Ruiz-Roso, Quintela, de la Fuente, Haya, & Perez-Olleros, 2010), it seems desirable to conduct a clinical trial in prediabetic and/or T2DM patients.

In conclusion, present results show for the first time that a diet containing meat formulated with CFE improves both lipoprotein metabolism and InsR/PI3K/AKT pathway as a consequence of insulin sensitivity-ameliorating. Fundamentally, CFE reduces VLDL and plasma triglyceride levels by increasing fecal fat excretion, and permitting to increase the effectiveness of the insulin signaling. At the same time, it favors the clearance of atherogenic lipoproteins, by increasing hepatic LDLr (Macho-González et al., 2019). Therefore, we can suggest that CFE-enriched meat is adequate functional food to be consumed by T2DM patients to control diabetic dyslipidemia and IR.

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Declaration of Competing Interest

No conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103600.

References

Asrafuzzaman, M., Cao, Y., Afroz, R., Kamato, D., Gray, S., & Little, P. J. (2017). Animal models for assessing the impact of natural products on the aetiology and metabolic pathophysiology of Type 2 diabetes. *Biomedicine & Pharmacotherapy, 89*, 1242–1251.

Aviram, M. (2004). Introduction to the serial review on paraoxonases, oxidative stress, and cardiovascular diseases. *Free Radical Biology and Medicine, 37*(9), 1301–1303.

Barter, P. J. (2002). Hugh sinclair lecture: The regulation and remodelling of HDL by plasma factors. *Atherosclerosis Supplements, 3*(4), 39–47.

Bastida, S., Sánchez-Muniz, F. J., Olivero, R., Pérez-Olleros, L., Ruíz-Roso, B., & Jiménez-Colmenero, F. (2009). Antioxidant activity of Carob fruit extracts in cooked pork meat systems during chilled and frozen storage. *Food Chemistry, 116*(3), 748–754.

Berglund, L., Brunzell, J. D., Goldberg, A. C., Goldberg, I. J., Sacks, F., Murad, M. H., & Stalenhoef, A. F. (2012). Evaluation and treatment of hypertriglyceridemia: An Endocrine Society clinical practice guideline. *The Journal of Clinical Endocrinology & Metabolism, 97*(9), 2969–2989.

Bocanegra, A., Bastida, S., Benedí, J., Nus, M., Sánchez-Montero, J. M., & Sánchez-Muniz, F. J. (2009). Effect of seaweed and cholesterol-enriched diets on postprandial lipoproteinaemia in rats. *British Journal of Nutrition, 102*(12), 1728–1739.

Bowe, J. E., Franklin, Z. J., Hauge-Evans, A. C., King, A. J., Persaud, S. J., & Jones, P. M. (2014). Metabolic phenotyping guidelines: Assessing glucose homeostasis in rodent models. *Journal of Endocrinology, 222*(3), G13–G25.

Bradford, M. M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254.

Brown, A. M., & Gibbons, G. F. (2001). Insulin inhibits the maturation phase of VLDL assembly via a phosphoinositide 3-kinase-mediated event. *Arteriosclerosis, Thrombosis, and Vascular Biology, 21*(10), 1656–1661.

Cao, B. Y., Li, R., Tian, H. H., Ma, Y. J., Hu, X. G., Jia, N., & Wang, Y. Y. (2016). PI3K-GLUT4 signal pathway associated with effects of EX-B3 electroacupuncture on hyperglycemia and insulin resistance of T2DM rats. *Evidence-Based Complementary and Alternative Medicine, 2016*, 7914387.

Cires, M. J., Wong, X., Carrasco-Pozo, C., & Gotteland, M. (2016). The gastrointestinal tract as a key target organ for the health-promoting effects of dietary proanthocyanidins. *Frontiers in Nutrition, 3*, 57.

Duvillard, L., Florentin, E., Lizard, G., Petit, J. M., Galland, F., Monier, S., ... Verges, B. (2003). Cell surface expression of LDL receptor is decreased in type 2 diabetic patients and is normalized by insulin therapy. *Diabetes Care, 26*(5), 1540–1544.

Estrada-Luna, D., Martinez-Hinojosa, E., Cancino-Diaz, J. C., Belefant-Miller, H., Lopez-Rodriguez, G., & Betanzos-Cabrera, G. (2018). Daily supplementation with fresh pomegranate juice increases paraoxonase 1 expression and activity in mice fed a high-fat diet. *European Journal of Nutrition, 57*(1), 383–389.

Fang, J.-Y., Lin, C.-H., Huang, T.-H., & Chuang, S.-Y. (2019). In vivo rodent models of type 2 diabetes and their usefulness for evaluating flavonoid bioactivity. *Nutrients, 11*(3).

Forouhi, N. G., & Wareham, N. J. (2014). Epidemiology of diabetes. *Medicine (Abingdon, England: UK ed.), 42*(12), 698–702.

Garcimartín, A., Santos-López, J. A., Bastida, S., Benedí, J., & Sánchez-Muniz, F. J. (2015). Silicon-enriched restructured pork affects the lipoprotein profile, VLDL oxidation, and LDL Receptor gene expression in aged rats fed an atherogenic diet. *Journal of Nutrition, 145*(9), 2039–2045.

Gesteiro, E., Rodríguez Bernal, B., Bastida, S., & Sánchez-Muniz, F. J. (2012). Maternal diets with low healthy eating index or Mediterranean diet adherence scores are associated with high cord-blood insulin levels and insulin resistance markers at birth. *European Journal of Clinical Nutrition, 66*(9), 1008–1015.

Goulas, V., Stylos, E., Chatziathanasiadou, M. V., Mavromoustakos, T., & Tzakos, A. G.

(2016). Functional components of carob fruit: Linking the chemical and biological space. *International journal of molecular sciences, 17*(11), 1875.
Haas, M. E., Attie, A. D., & Biddinger, S. B. (2013). The regulation of ApoB metabolism by insulin. *Trends in Endocrinology and Metabolism, 24*(8), 391–397.
Kaline, K., Bornstein, S. R., Bergmann, A., Hauner, H., & Schwarz, P. E. (2007). The importance and effect of dietary fiber in diabetes prevention with particular consideration of whole grain products. *Hormone and Metabolic Research, 39*(9), 687–693.
Laakso, M., & Kuusisto, J. (2014). Insulin resistance and hyperglycaemia in cardiovascular disease development. *Nature Reviews Endocrinology, 10*, 293.
Leavens, K. F., & Birnbaum, M. J. (2011). Insulin signaling to hepatic lipid metabolism in health and disease. *Critical Reviews in Biochemistry and Molecular Biology, 46*(3), 200–215.
Lee, J. S., & Kim, M. S. (2007). The role of GSK3 in glucose homeostasis and the development of insulin resistance. *Diabetes Research and Clinical Practice, 77*, S49–S57.
Macho-González, A., Garcimartín, A., López-Oliva, M. E., Bertocco, G., Naes, F., Bastida, S., ... Benedí, J. (2017). Fiber purified extracts of carob fruit decrease carbohydrate absorption. *Food & Function, 8*(6), 2258–2265.
Macho-González, A., Garcimartín, A., López-Oliva, M. E., Ruiz-Roso, B., Martín de la Torre, I., Bastida, S., ... Sánchez-Muniz, F. J. (2019). Can carob-fruit-extract-enriched meat improve the lipoprotein profile, VLDL-oxidation, and LDL receptor levels induced by an atherogenic diet in STZ-NAD-diabetic rats? *Nutrients, 11*(2).
Macho-González, A., Garcimartín, A., Naes, F., López-Oliva, M. E., Amores-Arrojo, A., González-Muñoz, M. J., ... Sánchez-Muniz, F. J. (2018). Effects of fiber purified extract of carob fruit on fat digestion and postprandial lipemia in healthy rats. *Journal of Agricultural and Food Chemistry, 66*(26), 6734–6741.
Mbue, N. D., Mbue, J. E., & Anderson, J. A. (2017). Management of lipids in patients with diabetes. *Nursing Clinics of North America, 52*(4), 605–619.
McRae, M. P. (2018). Dietary fiber intake and type 2 diabetes mellitus: An umbrella review of meta-analyses. *Journal of Chiropractic Medicine, 17*(1), 44–53.
Nus, M., Sánchez-Muniz, F. J., Gago, J. V. S., López-Oliva, E., & Sánchez-Montero, M. (2008). Determination of rat and mice arylesterase activity using serum mimetics. *Enzyme and Microbial Technology, 43*(3), 252–256.
Olivero-David, R., Schultz-Moreira, A., Vázquez-Velasco, M., González-Torres, L., Bastida, S., Benedi, J., ... Sánchez-Muniz, F. J. (2011). Effects of Nori- and Wakame-enriched meats with or without supplementary cholesterol on arylesterase activity, lipaemia and lipoproteinaemia in growing Wistar rats. *British Journal of Nutrition, 106*(10), 1476–1486.
Parhofer, K. G. (2011). Pathophysiology of diabetic dyslipidemia: Implications for atherogenesis and treatment. *Clinical Lipidology, 6*(4), 401–411.
Ruíz-Roso, B., Requejo, A.M., Pérez-Olleros, L., Martín-Casero, M., Haber, B., 2004. Denatured Carob Flour (DCF) with a low content of soluble tannins and sugars, meant for human consumption and process to obtain it. WO 2004/014150.
Ruíz-Roso, B., Requejo, A.M., Pérez-Olleros, L., Holguin, J.A., 2006. Product of vegetal origin comprising proanthocyanidines and its preparation process, WO 2006/000551.
Ruiz-Roso, B., Quintela, J. C., de la Fuente, E., Haya, J., & Perez-Olleros, L. (2010). Insoluble carob fiber rich in polyphenols lowers total and LDL cholesterol in hypercholesterolemic sujects. *Plant Foods for Human Nutrition, 65*(1), 50–56.
Sah, S. P., Singh, B., Choudhary, S., & Kumar, A. (2016). Animal models of insulin resistance: A review. *Pharmacological Reports, 68*(6), 1165–1177.
Salvado, M. J., Casanova, E., Fernandez-Iglesias, A., Arola, L., & Blade, C. (2015). Roles of proanthocyanidin rich extracts in obesity. *Food & Function, 6*(4), 1053–1071.
Santos-López, J. A., Garcimartín, A., Bastida, S., Bautista-Ávila, M., González-Muñoz, M. J., Benedí, J., & Sánchez-Muniz, F. J. (2018). Lipoprotein profile in aged rats fed chia oil- or hydroxytyrosol-enriched pork in high cholesterol/high saturated fat diets. *Nutrients, 10*(12).
Schultz-Moreira, A., Olivero-David, R., Vázquez-Velasco, M., González-Torres, L., Benedi, J., Bastida, S., & Sánchez-Muniz, F. J. (2014). Protective effects of sea spaghetti-enriched restructured pork against dietary cholesterol: Effects on arylesterase and lipoprotein profile and composition of growing rats. *Journal of Medicinal Food, 17*(8), 921–928.
Schwingshackl, L., Hoffmann, G., Lampousi, A. M., Knuppel, S., Iqbal, K., Schwedhelm, C., ... Boeing, H. (2017). Food groups and risk of type 2 diabetes mellitus: A systematic review and meta-analysis of prospective studies. *European Journal of Epidemiology, 32*(5), 363–375.
Serrano, J., Casanova-Marti, A., Gil-Cardoso, K., Blay, M. T., Terra, X., Pinent, M., & Ardevol, A. (2016). Acutely administered grape-seed proanthocyanidin extract acts as a satiating agent. *Food & Function, 7*(1), 483–490.
Skovsø, S. (2014). Modeling type 2 diabetes in rats using high fat diet and streptozotocin. *Journal of diabetes investigation, 5*(4), 349–358.
Sparks, J. D., Sparks, C. E., & Adeli, K. (2012). Selective hepatic insulin resistance, VLDL overproduction, and hypertriglyceridemia. *Arteriosclerosis, Thrombosis, and Vascular Biology, 32*(9), 2104–2112.
Taghibiglou, C., Rashid-Kolvear, F., Van Iderstine, S. C., Le-Tien, H., Fantus, I. G., Lewis, G. F., & Adeli, K. (2002). Hepatic very low density lipoprotein-ApoB overproduction is associated with attenuated hepatic insulin signaling and overexpression of protein-tyrosine phosphatase 1B in a fructose-fed hamster model of insulin resistance. *The Journal of Biological Chemistry, 277*(1), 793–803.
Tangvarasittichai, S. (2015). Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. *World Journal of Diabetes, 6*(3), 456–480.
Terpstra, A. H. M., Woodward, C. J. H., & Sánchez-Muniz, F. J. (1981). Improved techniques for the separation of serum-lipoproteins by density gradient ultra-centrifugation – visualization by pre-staining and rapid separation of serum-lipoproteins from small volumes of serum. *Analytical Biochemistry, 111*(1), 149–157.
Tian, S., Xu, Q., Jiang, R., Han, T., Sun, C., & Na, L. (2017). Dietary protein consumption and the risk of type 2 diabetes: A systematic review and meta-analysis of cohort studies. *Nutrients, 9*(9).
Tomkin, G. H., & Owens, D. (2017). Diabetes and dyslipidemia: Characterizing lipoprotein metabolism. *Diabetes, Metabolic Syndrome and Obesity, 10*, 333–343.
Uchiyama, M., & Mihara, M. (1978). Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Analytical Biochemistry, 86*(1), 271–278.
Verges, B. (2015). Pathophysiology of diabetic dyslipidaemia: Where are we? *Diabetologia, 58*(5), 886–899.
Viejo, J., 1992. Doctoral Thesis "Utilización de sardinas fritas en aceite de oliva en el tratamiento de hipercolesterolemia experimental inducida por dieta". Avalibale in: https://dialnet.unirioja.es/servlet/tesis?codigo=15534.
Xiao, J. B., & Hogger, P. (2015). Dietary polyphenols and type 2 diabetes: Current insights and future perspectives. *Current Medicinal Chemistry, 22*(1), 23–38.
Yang, K., & Chan, C. B. (2017). Proposed mechanisms of the effects of proanthocyanidins on glucose homeostasis. *Nutrition Reviews, 75*(8), 642–657.