

Dietary α -lactalbumin induced fatty liver by enhancing nuclear liver X receptor $\alpha\beta$ /sterol regulatory element-binding protein-1c/PPAR γ expression and minimising PPAR α /carnitine palmitoyltransferase-1 expression and AMP-activated protein kinase α phosphorylation associated with atherogenic dyslipidaemia, insulin resistance and oxidative stress in Balb/c mice

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Abstract

The effect and the role played by dietary α -lactalbumin (α -LAC) on hepatic fat metabolism are yet to be fully elucidated. We reported previously that α -LAC intake induced atherogenic dyslipidaemia in Balb/c mice. The aim of the present study was to investigate if this atherogenic effect could be due to a possible α -LAC-induced hepatic steatosis. We examine the ability of dietary α -LAC to induce liver steatosis, identifying the molecular mechanisms underlying hepatic lipid metabolism in association with the lipid profile, peripheral insulin resistance (IR) and changes in the hepatic oxidative environment. Male Balb/c mice (n 6) were fed with diets containing either chow or 14% α -LAC for 4 weeks. The α -LAC-fed mice developed abdominal adiposity and IR. Moderate liver steatosis with increased TAG and NEFA contents was correlated with atherogenic dyslipidaemia. There was increased nuclear expression of liver X receptor $\alpha\beta$ (LXR $\alpha\beta$), sterol regulatory element-binding protein-1c (SREBP-1c) and PPAR γ transcription factors and of the cytosolic enzymes acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase involved in the hepatic *de novo* lipogenesis. The opposite was found for the nuclear receptor PPAR α and the mitochondrial enzyme carnitine palmitoyltransferase-1 (CPT-1), leading to reduced fatty acid β -oxidation (FAO). These changes were associated with a significant decrease in both p-Thr¹⁷²-AMP-activated protein kinase α (AMPK α) (inactivation) and p-Ser⁷⁹-ACC1 (activation) and with a more oxidative liver environment increasing lipid peroxidation and protein oxidation and reducing GSH:GSSG ratio in the α -LAC-fed mice. In conclusion, 4 weeks of 14% α -LAC feeding induced liver steatosis associated with atherogenic dyslipidaemia, IR and oxidative stress by enhancing nuclear LXR $\alpha\beta$ /SREBP-1c/PPAR γ expression and diminishing PPAR α /CPT-1 expression and AMPK α phosphorylation shifting the hepatic FAO toward fatty acid synthesis in Balb/c mice.

Key words: Dietary α -lactalbumin: Atherogenic dyslipidaemia: Fatty liver: Oxidative stress

Liver lipid overload has deleterious effect on the hepatocyte viability and functionality. Non-alcoholic fatty liver disease (NAFLD) is a wide spectrum of fatty liver ranging from simple steatosis, which is previously considered as the benign form of NAFLD, to non-alcoholic steatohepatitis (NASH) which can progress to liver inflammation, fibrosis and malignancy in absence of alcohol consumption⁽¹⁾. NAFLD is known as the ‘hepatic manifestation’ of the metabolic syndrome (MS)⁽²⁾ and fatty liver *per se* has been linked to insulin resistance (IR) and progression of atherosclerosis and thus by itself may be a major aggravating factor in the pathogenesis of the MS and its

associated disorders⁽³⁾. The mechanisms underlying steatosis occurrence and progression to NASH remains incompletely understood but IR, mitochondrial dysfunction, oxidative stress and inflammatory cascades are believed to play a central role in the pathogenesis of NAFLD⁽⁴⁾. Hepatic steatosis originates mainly from the excessive NEFA delivery from insulin resistant adipose tissue into the liver⁽⁵⁾; of the induction of hepatic *de novo* lipogenesis (DNL) by IR⁽⁶⁾ and of the inhibition of fatty acids β -oxidation (FAO) by hyperinsulinaemia⁽⁷⁾. Decreased TAG secretion in the form of VLDL or abnormalities of adipokines metabolism can also contribute to fatty liver⁽⁸⁾. In DNL,

Abbreviations: ACC1, acetyl-CoA carboxylase 1; AMPK α , AMP-activated protein kinase α ; AOE, antioxidant enzymes; BFG, body fat gain; BPG, body protein gain; BW, body weight; CAT, catalase; ChREBP, carbohydrate responsive element-binding protein; CPT-1, carnitine palmitoyltransferase-1; Cu,ZnSOD, copper/zinc superoxide dismutase; DNL, *de novo* lipogenesis; FAO, fatty acids β -oxidation; FASN, fatty acid synthase; GPx, glutathione peroxidase; GR, glutathione reductase; HOMA-IR, homoeostasis model assessment-estimated insulin resistance; IR, insulin resistance; α -LAC, α -lactalbumin; LPO, lipid peroxidation; LXR $\alpha\beta$, liver X receptor $\alpha\beta$; MnSOD, manganese superoxide dismutase; NAFLD, non-alcoholic fatty liver disease; NR, nuclear receptor; p-AMPK α , phosphorylated AMPK α ; PC, protein carbonyl; PI, protein intake; SREBP-1c, sterol regulatory element-binding protein-1c; TC, total cholesterol; TF, transcriptional factor.

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the lipogenic transcriptional regulators sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate responsive element-binding protein (ChREBP) are transactivated by liver X receptor $\alpha\beta$ (LXR $\alpha\beta$), a nuclear receptor (NR) that regulates the metabolism of cholesterol and NEFA. LXR $\alpha\beta$, SREBP-1c and ChREBP regulate coordinately the expression of lipogenic genes, including acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FASN) in response to nutritional signals⁽⁹⁾ and seem to play a major role in the pathogenesis of NAFLD^(10–12). The NR PPAR γ is another transcriptional factor (TF) which has been linked to exacerbated hepatic steatosis by mechanism involving activation of DNL and increased TAG concentration⁽¹³⁾. A role in the development and treatment of fatty liver has been also shown for the NR PPAR α , principal regulator of the fatty acid oxidative metabolism through the transcriptional induction of several enzymes as carnitine palmitoyltransferase-1 (CPT-1), – that catalyses the rate-limiting step in mitochondrial FAO⁽¹⁴⁾, – and for the critical sensor of cellular energy charge the AMP-activated protein kinase α (AMPK α) that regulates the partitioning of NEFA between oxidative and biosynthetic pathway controlling the hepatic lipid homeostasis⁽¹⁵⁾. It has been suggest that a redox control may be involved in the early development of fatty liver⁽¹⁶⁾, as reactive oxygen species (ROS) could modulate liver lipid metabolism acting as NR and TF ligands or alter their DNA binding or nuclear import⁽¹⁷⁾ and modifying post-translationally some proteins involved in NEFA metabolism⁽¹⁸⁾.

Increasing evidence suggests that the dietary protein can affect body lipid metabolism, as the type and the amount of protein in the diet seems to play an important role on the development of obesity and its metabolic consequences⁽¹⁹⁾. Long-term consumption of a high animal/vegetable protein ratio and a low-protein intake (PI) leads to TAG adipocyte accumulation⁽²⁰⁾ and fatty liver infiltration in absence of alcohol⁽²¹⁾. However, recently it has been shown that consumption of whey proteins and/or milk protein fractions lessens several risk factors for metabolic diseases⁽²²⁾, although the data reported are controversial. Thus, while a protective effect of dietary whey protein reducing steatosis and oxidative stress has been found in high-carbohydrate fat-free-fed rats⁽²³⁾, the consumption of 25% whey protein showed an increase in insulinaemia and insulin intolerance in mice compared with dietary casein⁽²⁴⁾. It has been suggested that dietary α -lactalbumin (α -LAC) is the most hypercholesterolaemic and atherogenic animal protein in rabbits⁽²⁵⁾ whereas promotes⁽²⁶⁾ or unmodifies⁽²⁷⁾ lipid liver accumulation in rats compared with dietary casein. We reported previously, that the consumption of 14% dietary α -LAC as the sole protein source *v.* chow-based diet improves body fat weight and induces an atherogenic dyslipidaemia in adult and old male Balb/c mice⁽²⁸⁾. As the presence of atherogenic dyslipidaemia has been reported to be associated with NAFLD⁽²⁹⁾, we reason if the atherogenic effect elicited by dietary α -LAC could be related to a possible hepatic steatosis development. The main purpose of this study was to assess the ability of the dietary α -LAC (14%) to induce liver steatosis identifying the molecular mechanisms underlying hepatic lipid metabolism and its relationship with the peripheral IR and the hepatic redox status. The protein expression of regulators and enzymes involved in DNL (LXR $\alpha\beta$,

SREBP-1c, ChREBP, PPAR γ , ACC1 and FAS), and FAO (PPAR α and CPT-1) as well as the role played by the energy sensor AMPK α were studied. The possible influence of a prooxidant/antioxidant imbalance in the liver of male Balb/c mice capable of modulating lipid metabolism was also investigated.

Methods

Experimental design

A total of twelve male Balb/c mice aged 6 months with an average body weight of 30 (SEM 1) g were obtained from the breeding centre *Nutrición y Fisiología* (Spanish Government licence: ES280790000085), Faculty of Pharmacy, Universidad Complutense Madrid, Spain. The mice matched by weight were housed in individual metabolic cages and kept in a room at 22° ± 1°C, 60% humidity and with a 12 h light–12 h dark cycle. After a 7-d adaptation period, mice were assigned (*n* 6 each) at random and blinded to either the control group fed a standard chow for mouse maintenance (A04; PanLab) or the α -LAC group fed an experimental diet containing α -LAC (α -LAC diet) for an experimental period of 4 weeks. Food and water were freely available. Food intake (FI) and PI and body weight (BW) were measured daily. The experimental diet was formulated according to AIN-93M standards⁽³⁰⁾ substituting α -LAC for casein while maintaining all other components. The composition of diets is shown in Table 1. At the end of study, six mice from each group were starved for 12 h and killed under anaesthesia with ketamine (30 mg/kg) and xylazine (5 mg/kg). Blood sampling were collected from retro-orbital plexus and serum was separated from whole blood by centrifugation (1500 g 10 min; 4°C) and analysed immediately or placed into aliquots and stored at –80°C. Livers were removed after blood was collected, rinsed with cold PBS and then weighed. Parts of each liver were fixed in 10% formaldehyde/PBS or frozen in liquid N₂, and stored at –80°C until used. Abdominal adipose tissues (mesenteric, retroperitoneal and peri-intestinal fat pads), and the gonadal fat pad were carefully removed, weighed and used to determine the adiposity index, calculate as abdominal and/or gonadal (g)/BW(g) × 100). The eviscerated bodies of mice were frozen in liquid nitrogen and ground to a

Table 1. Composition of experimental diets

Chemical composition (%)	Chow	α -LAC
Proteins	16	14
Fat	3	4
Carbohydrates	59.5	72.5
Water	12	–
Fibre	4	5
Minerals*	4.5	3.5
Vitamins†	1	1
Energy intake (kJ/100 g)	1377.5	1599.3

α -LAC, α -lactalbumin.

* 1 kg of diet contains the following minerals (g/kg): chow diet: Ca, 8.8; P, 5.9; Mg, 1.40; K, 6.25; sodium, 2.5; Cl, 3.1. α -LAC diet: mineral mix (AIN-93M).

† 1 kg of diet contains the following vitamins: chow diet: vitamin A, 2.0 mg (6600 IU); vitamin D₃, 22.5 µg (900 IU); vitamin E, 30.0 mg; vitamin K₃, 2 mg; thiamine, 1 mg; riboflavin 4 mg; niacin, 20 mg; pantothenic acid, 15 mg; pyridoxine, 4 mg; biotin, 0.1 mg; folic acid, 1 mg; cyanocobalamin, 20 mg; choline 500 mg. α -LAC diet: vitamin mix (AIN-93VX).



homogeneous mixture and used to the body composition measure. Aliquots were used to determine DM, diethyl ether extract (representing the fat content), protein and ash⁽³¹⁾. Results were used to calculate relative content of BW. Linear relationships between body protein gain (BPG) and body fat gain (BFG) *v.* PI were fitted to study the efficiency of PI utilisation to BPG and BFG. All animal care and experimental protocols conformed to the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and were approved by the Institutional Animal Care and Use Committee of Madrid Complutense University.

Serum measurements

Serum total cholesterol (TC), HDL-cholesterol, TAG and NEFA concentrations were assayed using enzymatic/colorimetric assay kits (Biolabo SA. Materlab). The non-HDL-cholesterol ((LDL + VLDL)-cholesterol) was calculated according the formula: (LDL + VLDL)-cholesterol = TC – HDL-cholesterol. The TC:HDL-cholesterol ratio was employed as atherogenic index. Serum glucose was measured using the glucose oxidase method (Spinreact S.A.). Serum insulin concentration was determined using an ELISA for mouse insulin (Merckodia AB). The homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated using the following formula: fasting blood glucose (mmol/l) × fasting insulin (mIU/l)/22.5. The greater the HOMA-IR value, the higher the level of IR. The absorbance of enzyme alanine aminotransferase (ALT) was read at 505 nm and the enzyme activity was calculated as IU/l. Serum adiponectin, leptin and TNF α levels were measured using ELISA kits (Diagnostic Systems Laboratories Inc.).

Liver lipids determinations

The liver of each mouse was homogenised and total lipids were extracted with a mixture of chloroform–methanol (2:1, v/v) according to the Folch *et al.*⁽³²⁾ method. The hepatic TAG and NEFA contents were assayed using enzymatic/colorimetric assay kits.

Histological and immunohistochemical examination

Liver tissues were sectioned (4–5 μ m), alcohol-dehydrated, and embedded in paraffin. Sections were stained with haematoxylin–eosin to evaluate severity of histological changes. For each mouse, assessment of the area occupied by the lipid vacuoles was performed on five different fields at 100 \times magnification and it was expressed as a percentage of the total surface of these different fields, using Image J version 1.44 software (<http://rsb.info.nih.gov/ij/>). Hepatic steatosis was graded according to the percentage of hepatocytes containing fat, as 0: no steatosis (fatty hepatocytes occupying <5%), 1: minimal steatosis (fatty hepatocytes occupying 5–33%), 2: moderate steatosis (fatty hepatocytes occupying 34–66%) or 3: severe steatosis (fatty hepatocytes occupying >66%)⁽³³⁾. The liver sections were observed under a light microscope using a Leica DM LB2 microscope and a digital Leica DFC 320 camera (Leica).

Standard immunostaining procedures were performed to assess the effect of the α -LAC feeding on the expression of lipogenic enzymes (ACC1; FASN) in the liver. Before the immunostaining, the sections were deparaffinised, rehydrated and then treated in 3% hydrogen peroxide in methanol to inhibit peroxidase activity. They were then boiled in a microwave oven in 0.01 M sodium citrate buffer (pH 6.0) for 20 min. To avoid background staining, blocking serum was derived from same specie in which the secondary antibody had been raised. After, the sections were incubated overnight at 4°C with the following rabbit polyclonal primary antibodies: anti-ACC1 (1:50) and anti-FASN (1:50) (Santa Cruz Biotechnology). After washes with PBS the sections were covered for 30 min at room temperature with biotinylated goat anti-rabbit (1:500) as secondary antibody (Santa Cruz Biotechnology). Immunochemical staining was performed for 30 min using streptavidin-biotin conjugated horseradish peroxidase (Sigma Aldrich) and visualised by incubation with 3, 3'-diaminobenzidine (Sigma Aldrich) for 10 min at room temperature. The sections were counterstained by Harris's haematoxylin, dehydrated and mounted. Protein expression was evaluated by their staining pattern: weak, moderate, diffuse or intense.

Tissue preparation for biochemical analysis

Mouse liver tissue was homogenised in a glass homogeniser in 1:10 (w/v) of ice-cold buffer (mannitol 210 mM, sucrose 70 mM, HEPES 5 mM, EDTA 1 mM and 0.01% protease inhibitor cocktail, pH 7.4) for assessment of protein expression. For oxidative status and lipid parameters assessments, liver samples were homogenised in 10 volumes of ice-cold PBS. Tissue homogenates were centrifuged at 800 **g** for 15 min at 4°C. The supernatants and the pellets were collected. The nuclear pellets were washed twice and re-suspended in 20 mM HEPES, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.1 mM EGTA, 25% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 0.5 mM spermidine and protease inhibitors at pH 7.9. After frequent vortexing for 15 min, nuclear homogenates were stored at –80°C until analysis.

The supernatant fractions were centrifuged at 14 000 **g** for 25 min at 4°C. The resulting supernatants representing the mitochondria-free cytosolic fraction were collected, sampled and stored at –80°C for later biochemical analyses. The mitochondrial pellets were re-suspended in homogenising buffer, and centrifuged at 14 000 **g** for 25 min at 4°C. The supernatant fractions were decanted and the mitochondrial pellets re-suspended in storage buffer and stored at –80°C. The protein concentration of the liver fractions was quantified using the Lowry method (DC^(TM) Protein Assay Kit; Bio-Rad). Bovine serum albumin in a concentration range of 0–50 μ g/ml was used as a standard.

Western blotting

The protein content of the LXRA β , SREBP-1c, ChREBP transcription factors, as well as the PPAR α and PPAR γ NR were assessed in the nuclear liver fraction. The protein expression of the enzymes ACC1, FASN, and CPT-1, the protein kinase AMPK α , the phospho-derivatives phosphorylated-AMPK α (p-Thr¹⁷²-AMPK α)



and phosphorylated-ACC1 (p-Ser⁷⁹-ACC1) as well as the anti-oxidant enzymes (AOE) Mn superoxide dismutase (MnSOD), Cu, Zn superoxide dismutase (Cu,ZnSOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) were assessed in mitochondrial and/or cytosolic fractions of liver.

For each sample 50–80 μ g protein were boiled in Laemmli sample buffer and loaded into each well of 10 or 15% polyacrylamide gel (SDS-PAGE) and after migration, proteins were transferred to a polyvinylidene fluoride membrane (GE Healthcare). All membranes were blocked with 5% non-fat dry milk for 1 h at room temperature. For immunodetection, membranes were incubated overnight at 4°C with the following rabbit polyclonal primary antibodies: anti-SREBP-1c (1:200); anti-ChREBP (1:500); anti-ACC1(1:500), anti-FASN (1:1000), anti-CPT-1 (1:1000); anti-AMPK α (1:500); anti-p-Ser⁷⁹-ACC1 (1:700); anti-p-Thr¹⁷²-AMPK α (1:1000); anti-MnSOD (1:1000); anti-Cu,ZnSOD (1:1000); anti-GPx (1:500); anti-GR (1:1000) and anti-CAT (1:2000) (Santa Cruz Biotechnology). Goat polyclonal anti-PPAR α (1:1000) and anti-PPAR γ (1:2000), and mouse monoclonal anti-LXR α (1:100) primary antibodies were also used (Santa Cruz Biotechnology). Membranes were then washed in 0.05% Tween-20, incubated with appropriate peroxidase-conjugated secondary antibodies for 1 h at room temperature, and then washed with PBS and the antigen-antibody complexes were visualised by chemiluminescence (ECL Select-kit; GE Healthcare). Anti- β -actin, anti-TFIID and anti-Tom20 antibodies (Santa Cruz Biotechnology) were used as loading control for cytosolic, nuclear and mitochondrial proteins respectively. p-AMPK α and ACC1 were quantified by densitometric analysis and normalised to the levels of endogenous AMPK α and ACC1 proteins respectively. Relative levels of immunoreactive proteins were quantified using Image-Quant LAS 500 system (GE Healthcare) and the Quantity One version 4.62 for Windows as densitometric analysis software (Bio-Rad).

Lipid peroxidation assay

The assay used by lipid peroxidation (LPO) measure is based on the reaction of a chromogenic reagent *N*-methyl-2-phenylindole in acetonitrile, with malonyldialdehyde (MDA) and 4-hydroxynonenal (4-HNE) at 45°C (Bioxytech LPO-586 kit; Oxis International). Mitochondrial and cytosolic MDA + 4-HNE concentrations were expressed as nmol/mg protein.

Protein oxidation assay

Carbonyl groups were measured as indicative of liver proteins damage according to the method of Levine *et al.*⁽³⁴⁾. The liver homogenates were incubated in 0.25 M TRIS (pH 7.4) and 0.2 M sucrose and 10 mM 2, 4-dinitrophenylhydrazine (DNTP) in 2 M HCl at room temperature for 1 h. Blanks were run without DNTP. Afterward, proteins were precipitated with TCA and centrifuged (3000 **g**; 20 min; 4°C). The protein pellet was washed three times with ethanol–ethylacetate (1:1) and incubated at 37°C until complete re-suspension. The mitochondrial and cytosolic carbonyl content was measured spectrophotometrically at 370 nm (molar extinction coefficient 22000 M⁻¹ × cm⁻¹) and expressed as nmol per mg protein.

Measurement of GSH and GSSG

GSH and GSSG concentrations were measured by a commercial kit supplied by Cayman Chemical. This kit utilises an optimised enzymatic GR recycling method for quantification of GSH⁽³⁵⁾. Just after homogenisation of the liver, 100 μ l aliquots of the cytosolic and mitochondrial homogenates were added to an equal volume of 5% (w/v) meta-phosphoric acid to remove protein. After 30 min of incubation on ice, samples were centrifuged (2000 **g**; 20 min; 4°C). Then, 50 μ l of 4 M triethanolamine (catalogue no. T5830-0; Sigma-Aldrich) were added for each millilitre of homogenate to increase the pH. For total GSH assay, 50 μ l of sample was added to 150 μ l of a reaction mixture containing 0.4 M 2-(*N*-morpholino) ethane-sulfonic acid, 0.1 M phosphate (pH 6.0), 2 mM EDTA, 0.24 mM NADPH, 0.1 mM 5,5'-dithiobis-2-nitrobenzoic acid and 0.1 unit GR. The reaction was carried out at 37°C for 25 min, and then total glutathione was determined by absorbance at 405 nm using GSSG as standard. For the measurement of GSSG, GSH was removed from the reaction by adding 10 ml of 1 M 2-vinylpyridine/ml of homogenate. Then, the remaining GSSG in the reaction was quantified as total GSH assay. The amount of reduced GSH was obtained by subtracting GSSG from total glutathione. Each sample was assessed in duplicate, and the levels of GSH and GSSG were expressed as nmol/mg protein. The ratio of GSH:GSSG was used to indicate redox status that infers the detoxification capacity.

Antioxidant enzyme activities

MnSOD, Cu,ZnSOD, GPx, GR and CAT activities were assessed in cytosolic and/or mitochondrial fractions of the liver. In brief, cytosolic Cu,ZnSOD activity was determined by using the hypoxanthine–xanthine oxidase system to generate O₂. The capacity to scavenge superoxide radicals by suppression of nitrotriazolium reduction was monitored at 550 nm⁽³⁶⁾. Enzyme activity was expressed in units/mg protein (1 unit of SOD is defined as the amount of enzyme required to inhibit the rate of nitrotriazolium reduction by 50%). MnSOD activity was determined in the mitochondrial fraction under the same conditions as the Cu,ZnSOD assay, with the addition of 1 mM-KCN to inhibit the Cu,ZnSOD isoform. The GPx activity assay was based on the oxidation of GSH to GSSG, catalysed by GPx. NADPH oxidation at 340 nm and 25°C was measured when GSSG is reduced back by GR with cumene hydroperoxide as the substrate⁽³⁷⁾. Cytosolic and mitochondrial GPx activities were expressed as nmol NADPH oxidised to NADP/min per mg protein. GR catalyses the NADPH-dependent reduction of GSSG to GSH. GR activity was also determined by monitoring NADPH oxidation at 340 nm and was expressed as nmol NADPH oxidised/min per mg protein at 25°C⁽³⁸⁾. Cytosolic CAT activity was measured by monitoring the decomposition of H₂O₂ at 240 nm⁽³⁹⁾. The reaction consisted of 50 mM sodium phosphate buffer (pH 7.2) and 10 mM H₂O₂. CAT activity was expressed as nmol formaldehyde formed/min per mg protein at 25°C.

ATP measurements

ATP content was measured in cytosolic and/or mitochondrial fractions of the liver by using an ATP colorimetric assay kit



according to the manufacturer's protocol (Biovision, Deltaclon S.L.). ATP content in liver homogenates was determined in a reaction mix that supported the phosphorylation of added glycerol as a substrate. The reaction mix was incubated in a ninety-six-well plate for 30 min at room temperature. The OD was measured at 570 nm in a microplate reader, and ATP content was calculated by applying sample to a standard curve.

Statistical analysis

The sample size calculation was based on observed variations in lipid profile parameters from our previously published study⁽²⁸⁾, and was conducted through power analysis method by using the software G*Power version 3.1.9.2 (Faul F., Universitat Kiel). For a value of $\alpha = 0.05$ and 80% power ($\beta = 0.20$) and using the means of serum TC (2.65 for chow control group and 4.78 for α -LAC group with an average sd of 1.1), we calculated an effect size of 1.936. Hence, conducting a power analysis for a two-tailed *t* test, this effect size revealed a sample size of six mice per experimental group. Results were expressed as means with their standard errors. Differences were assessed by unpaired Student's *t* test, and were considered statistically significant at the 5% level on two-sided testing. The data obtained from the semi-quantitative steatotic score were comparatively analysed using the Kruskal–Wallis test. Linear correlation analysis was used to explore the relationships between the continuous variables studied. Correlation coefficients (*R*) and *P* values were evaluated to judge the fit of the correlation; two-sided $P < 0.05$ and $P < 0.001$ values of correlations were considered significant and highly significant, respectively. The statistical analyses were conducted using SPSS 22.1 for Windows (SPSS).

Results

α -Lactalbumin diet increased body weight and abdominal adiposity in the Balb/c mice

Obesity is a hallmark in the pathogenesis of the MS and the fatty liver. As shown in Table 2, the BW gain in the α -LAC-fed mice

increased significantly (5-fold; $P = 0.0001$) compared with the control group, leading to a BW 14% greater at end of experiment. The increase of BW was associated with a significantly elevated relative body fat (12.63 (SEM 1.27) *v.* 8.63 (SEM 0.40); $P = 0.01$) together to a reduction in relative body water (4%; $P = 0.009$) and protein (7.75%; $P = 0.013$) as compared *v.* the control group. Specially, the α -LAC group exhibit higher abdominal (57.6%; $P = 0.005$) and gonadal fat pads weights (27%; $P = 0.007$) than the chow-fed mice but only the abdominal fat adiposity index was significantly greater (34.89% (0.58 (SEM 0.03) *v.* 0.43 (SEM 0.05); $P = 0.033$).

α -Lactalbumin diet enhanced the protein intake efficiency towards body fat gain in the Balb/c mice

Although daily FI and PI did not differ among groups ($P > 0.05$), the efficiency of PI utilisation on body substrate partitioning was significantly modified by the α -LAC diet. As evidenced in Fig. 1(a), the control mice utilised their PI towards BPG as indicates the significantly correlation found between PI and BPG ($R = 0.891$; $P = 0.0046$), whereas any correlation for the α -LAC-fed mice was observed ($P > 0.05$). On the contrary, only the α -LAC-fed mice directed the higher proportion of their PI to BFG as it was showed by the significantly correlation coefficient of BFG relative to PI ($R = 0.705$; $P = 0.036$) (Fig. 1(a)), whereas no significant correlation ($P > 0.05$) was found in the control mice. These results indicate that the α -LAC intake induced anomalous body substrate partitioning and improved body fat stores.

α -Lactalbumin diet produced dyslipidaemia and insulin resistance in the Balb/c mice

Dyslipidaemia is one of the most striking features in the development of fatty liver. In our work serum TC (64.82%; $P = 0.0001$), TAG (65.47%; $P = 0.0001$) and NEFA (70%; $P = 0.0001$) levels in the α -LAC group were significantly higher than those of the control mice (Table 3). Also, the α -LAC intake increased serum (LDL + VLDL)-cholesterol (113.45%; $P = 0.0001$) level, whereas serum HDL-cholesterol level was

Table 2. Feed and protein intake, body weight (BW), body weight gain, body composition, abdominal and gonadal fat pad weights and abdominal and gonadal adiposity index of chow and α -lactalbumin (α -LAC)-fed Balb/c mice (Mean values with their standard errors; *n* 6)

	Chow		α -LAC		<i>P</i> *
	Mean	SEM	Mean	SEM	
Feed intake (g/d)	3.92	0.19	3.56	0.16	NS
Protein intake (g/d)	0.55	0.04	0.50	0.02	NS
Start weight (g)	26.96	1.34	27.65	0.91	NS
Final weight (g)	27.79	1.45	31.80	0.61	0.042
Body weight gain (g)	0.83	0.18	4.15	0.57	0.0001
Body protein (%)	18.73	0.26	17.28	0.42	0.013
Body fat (%)	8.63	0.40	12.63	1.27	0.01
Body water (%)	67.82	0.35	65.10	0.80	0.009
Body ash (%)	4.82	0.06	4.98	0.17	NS
Abdominal fat pad weight (g)	0.118	0.013	0.186	0.012	0.005
Gonadal fat pad weight (g)	0.222	0.012	0.282	0.013	0.007
Abdominal adiposity index (g/BW g × 100)	0.43	0.05	0.58	0.03	0.033
Gonadal adiposity index (g/BW g × 100)	0.81	0.05	0.89	0.04	NS

* Differences were considered significant at $P < 0.05$ between α -LAC and chow groups (two-sided unpaired *t* test).

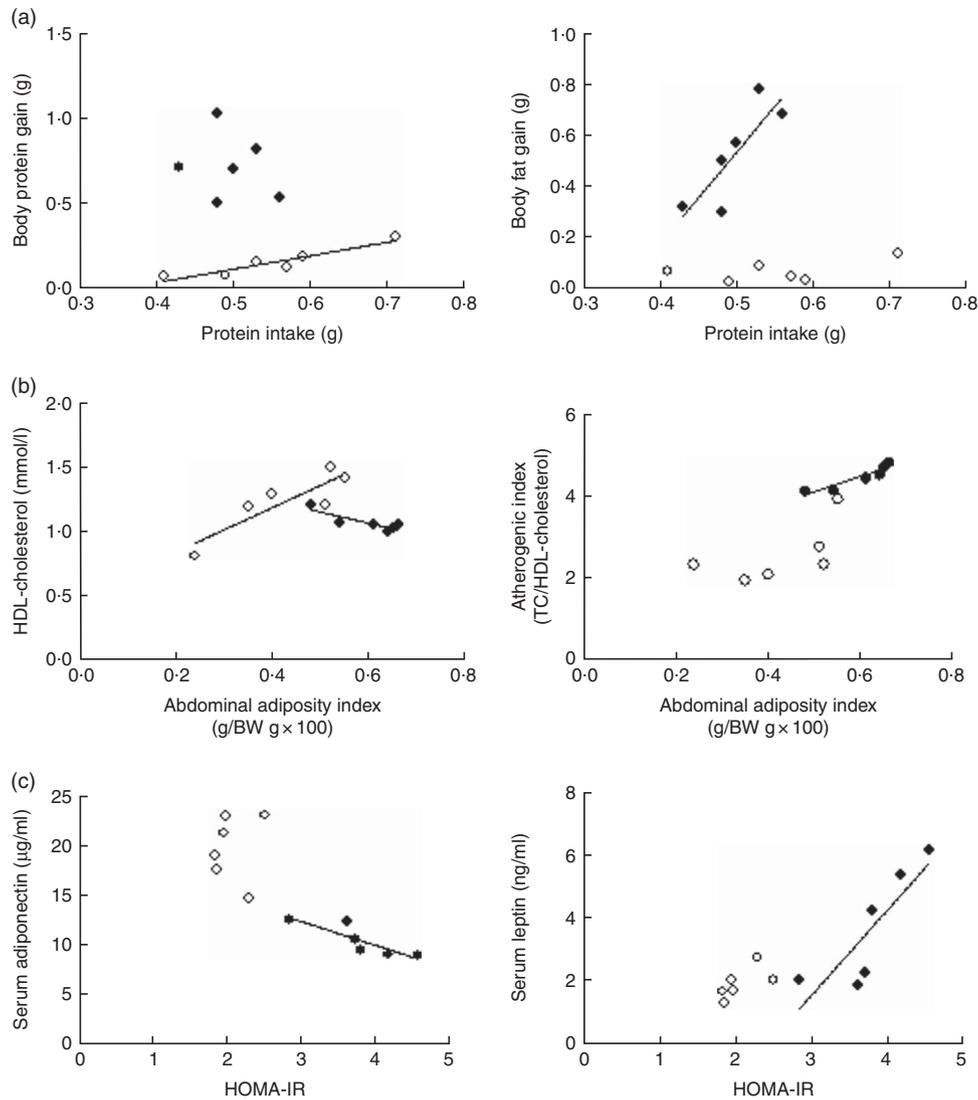


Fig. 1. Scatterplots of the relationships: (a) between protein intake and both body protein gain and body fat gain; (b) between abdominal adiposity index and both serum HDL-cholesterol and atherogenic index; (c) and between homoeostasis model assessment-estimated insulin resistance (HOMA-IR) and both serum adiponectin and serum leptin of chow (\circ) and α -lactalbumin (α -LAC)-fed (\bullet) Balb/c mice. Each linear regression analysis is represented by its correlation coefficient (R), P value and the fitted regression line ($P < 0.05$). BW, body weight; TC, total cholesterol.

reduced (13%; $P = 0.04$). Thus, the atherogenic index was enhanced (82%; $P = 0.001$) in the α -LAC-fed mice compared with the chow-fed mice. The higher abdominal adiposity index of the α -LAC-fed mice was negatively correlated with HDL-cholesterol ($R = -0.875$; $P = 0.021$) and positively with the atherogenic index ($R = 0.932$; $P = 0.006$) whereas the chow-fed mice correlated positively ($R = 0.873$; $P = 0.023$) with HDL-cholesterol (Fig. 1(b)). These results suggest that the abdominal fat content plays a role in the α -LAC-induced atherogenic dyslipidaemia.

Mean serum insulin levels of the α -LAC-fed mice were significantly higher (77%; $P = 0.0001$) than the control mice, whereas mean serum glucose levels did not differ among the groups ($P > 0.05$) (Table 3). Furthermore, the HOMA-IR was significantly higher in the α -LAC group (79%; $P = 0.002$) than the control group, suggesting that the α -LAC intake induced an insulin sensitivity decline in the Balb/c mice.

α -Lactalbumin diet modulated serum adiponectin, leptin, and TNF α levels and alanine aminotransferase activity in the Balb/c mice

Serum adiponectin, leptin and TNF α levels as IR and fatty liver biomarkers were evaluated (Table 3). Our results showed that the α -LAC diet induced hypoadiponectinaemia (45%; $P = 0.0001$) whereas serum leptin (110%; $P = 0.0001$) and serum TNF α (60%; $P = 0.0001$) levels significantly increased compared with the control diet. Serum ALT activity, a marker of hepatocyte damage, was also significantly increased (33%; $P = 0.0001$) in the α -LAC group compared with the chow group. Significantly negative and positive correlations between serum adiponectin ($R = -0.860$; $P = 0.027$) and between serum leptin ($R = 0.843$; $P = 0.034$) and HOMA-IR were found in the α -LAC-fed mice (Fig. 1(c)). These results suggest that these adipokines derived of adipose tissue could be involved in the IR α -LAC induced.

Table 3. Serum lipid profile, atherogenic index, glucose, insulin, adiponectin, leptin and TNF α levels, homoeostasis model assessment-estimated insulin resistance (HOMA-IR) and alanine aminotransferase (ALT) activity of chow and α -lactalbumin (α -LAC)-fed Balb/c mice (Mean values with their standard errors; n 6)

	Chow		α -LAC		P^*
	Mean	SEM	Mean	SEM	
Total cholesterol (mmol/l)	2.90	0.12	4.7	0.26	0.0001
TAG (mmol/l)	0.84	0.05	1.39	0.08	0.0001
NEFA (mmol/l)	0.67	0.04	1.14	0.05	0.0001
HDL-cholesterol (mmol/l)	1.23	0.09	1.07	0.003	0.04
LDL + VLDL (mmol/l)	1.71	0.18	3.65	0.23	0.0001
Atherogenic index (TC/HDL-cholesterol)	2.40	0.31	4.37	0.11	0.001
Glucose (mg/l)	813.8	24.6	837.4	35.3	NS
Insulin (mIU/ml)	10.44	0.14	18.50	0.19	0.0001
HOMA-IR	2.10	0.11	3.76	0.29	0.002
ALT (IU/l)	24.50	1.59	32.80	2.29	0.0001
Adiponectin (μ g/ml)	19.71	1.35	10.74	0.72	0.0001
Leptin (ng/ml)	1.89	0.20	3.97	0.84	0.0001
TNF α (ng/ml)	276.58	8.46	443.27	38.49	0.0001

* Differences were considered significant at $P < 0.05$ between α -LAC and chow groups (two-sided unpaired t test).

α -Lactalbumin diet increased hepatic weight, TAG and NEFA contents inducing fatty liver in the Balb/c mice

Hepatic fat accumulation defines fatty liver. The liver weight was significantly increased in the α -LAC group (11%; $P = 0.045$) (Fig. 2(c)) but the liver:BW ratio (4.24 (SEM 0.11) *v.* 4.33 (SEM 0.19); $P > 0.05$) did not differ among the groups, indicating that the liver grows in proportion to BW. Hepatic TAG (7.81 (SEM 0.24) *v.* 4.87 (SEM 0.19) μ mol/g liver; $P = 0.0001$) and NEFA (1.02 (SEM 0.12) *v.* 0.45 (SEM 0.03) μ mol/g liver; $P = 0.001$) contents were increased also by the α -LAC diet, attaining at 60% (TAG) and at 126% (NEFA) over control values (Fig. 2 (d and e)).

Histological sections of liver tissue from the α -LAC group showed macrovesicular intracellular lipid droplet, predominantly in pericentral zone of the liver compared with the chow-fed mice (Fig. 2(a)). The average steatosis grade score was significantly higher in the α -LAC liver (2.1 (SEM 0.01) *v.* 0.42 (SEM 0.02)) than control mice (Fig. 2(b)), suggesting that the α -LAC diet developed a moderate steatosis, consistent with the results of biochemical evaluation. The higher steatosis score elicited by α -LAC-fed mice was correlated negatively with serum HDL-cholesterol ($R = -0.905$; $P = 0.013$) and positively with the atherogenic index ($R = 0.879$; $P = 0.020$) (Fig. 2(f)). Neither signs of inflammation nor fibrosis were histopathologically observed. Liver sections from the control group showed normal lobular architecture. These results suggest that the α -LAC intake induced liver fat infiltration associated to atherogenic dyslipidaemia.

α -Lactalbumin diet increased hepatic *de novo* lipogenesis in the Balb/c mice

We investigated the pattern of expression of the lipogenic TF, LXR α , SREBP-1c, ChREBP and PPAR γ and of their enzymes target ACC1 and FASN in the nucleus and the cytosol respectively, of the liver of control and α -LAC mice.

Hepatic LXR α (15%; $P = 0.042$), SREBP-1c (34%; $P = 0.001$) and PPAR γ (23%; $P = 0.003$) nuclear proteins were overexpressed

in the α -LAC group compared with the control group when examined by immunoblot. The protein expression of cytosolic ACC1 and FASN, the key enzymes of the DNL and targets of LXR α , SREBP-1c and PPAR γ , also exhibited a significant up-regulation in liver of α -LAC-fed mice increasing at 17% ($P = 0.019$ (ACC1)) and 37% ($P = 0.006$ (FASN)) respectively of the control mice (Fig. 3(a)). On the contrary, nuclear ChREBP protein did not differ among the groups ($P > 0.05$) (Fig. 3(a)). These results seem to indicate that the α -LAC diet induced DNL activation in liver of the Balb/c mice. Immunostaining of hepatic ACC1 and FASN proteins in α -LAC mice showed an intense staining in the cytoplasm of the hepatocytes especially for FASN, whereas there was a weak staining of both enzymes in control mice. These data confirm that α -LAC intake promotes greater expression of ACC1 and FASN in liver of the Balb/c mice (Fig. 3(b)).

Interestingly, nuclear LXR α and SREBP-1c protein expression appears positively associated with both HOMA-IR (LXR α , $R = 0.895$; $P = 0.015$; SREBP-1c, $R = 0.948$; $P = 0.0037$) and hepatic NEFA (LXR α , $R = 0.926$; $P = 0.008$; SREBP-1c, $R = 0.921$; $P = 0.009$), whereas nuclear PPAR γ protein was associated positively with hepatic NEFA ($R = 0.928$; $P = 0.007$) in α -LAC liver (Fig. 3(c)). On the contrary, α -LAC intake elicits a significantly negative correlation between nuclear LXR α ($R = -0.822$; $P = 0.044$); SREBP-1c ($R = -0.897$; $P = 0.016$) and PPAR γ ($R = -0.938$; $P = 0.005$) proteins and serum adiponectin (Fig. 3(c)). No significant correlations were found in control mice. These results suggest that the DNL activation α -LAC-induced could be controlled by IR, of the hypoadiponectinaemia and of the NEFA overload in the Balb/c mice.

α -Lactalbumin diet reduced hepatic mitochondrial fatty acid β -oxidation in the Balb/c mice

Fat accumulation in liver of α -LAC mice could also result of decreased FAO. The NR PPAR α and the enzyme CPT-1 are involved in mitochondrial FAO pathways. Thus, immunoblotting was performed to evaluate the pattern of nuclear PPAR α and of mitochondrial CPT-1 protein contents in liver of the α -LAC and control groups.

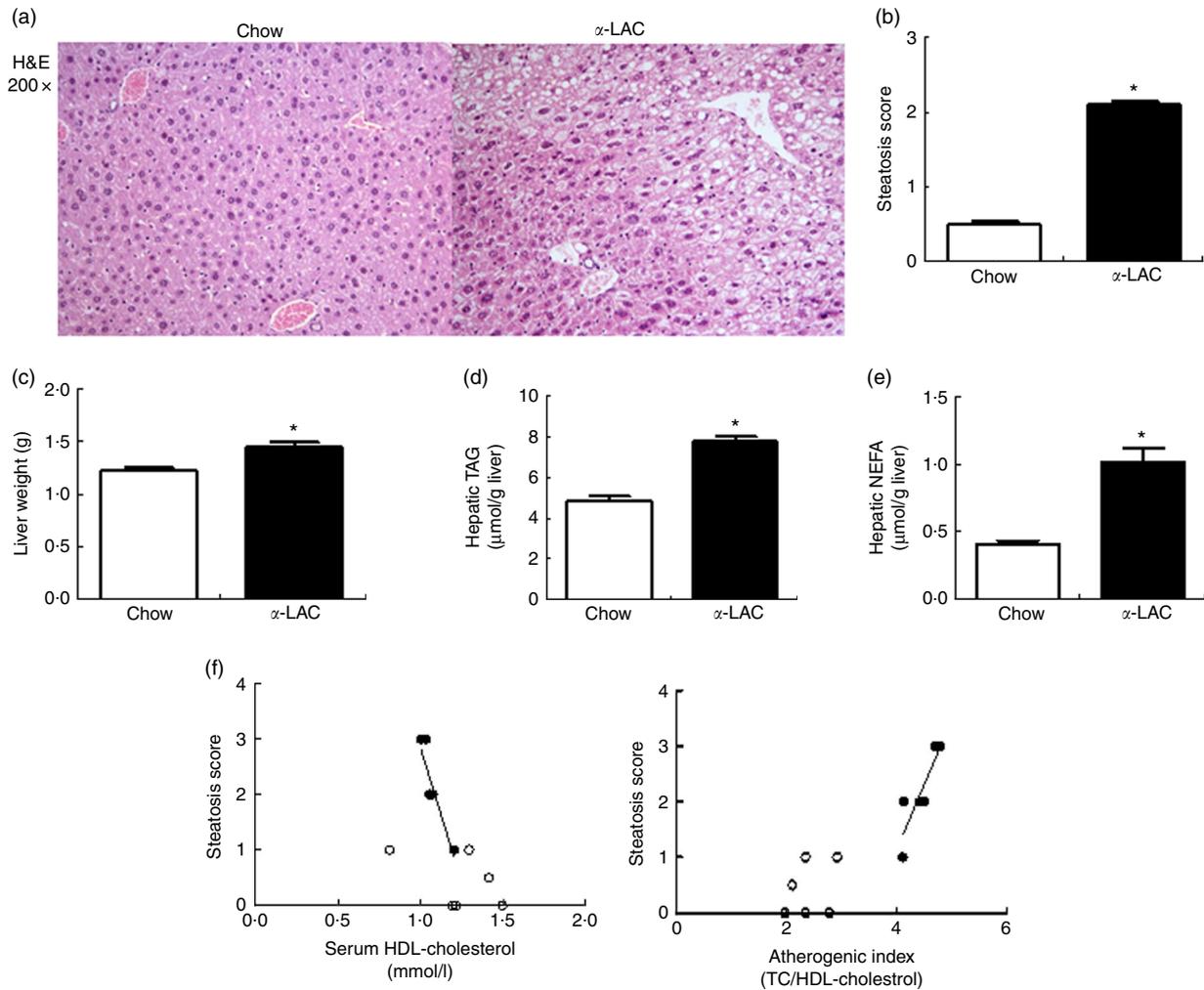


Fig. 2. Dietary α -lactalbumin (α -LAC) induced non-alcoholic fatty liver in Balb/c mice. (a) Haematoxylin–eosin (H&E) stain shows macrovesicular lipid accumulation in liver of α -LAC-fed mice compared with normal liver histology of the chow-fed mice (light microscope, magnification 200 \times). (b) Steatosis score (c) liver weight; (d) hepatic TAG and (e) hepatic NEFA in the chow and α -LAC-fed mice. Values are means (n 6), with their standard errors represented by vertical bars. * Mean value was significantly different from that of the control group ($P < 0.05$); two-sided unpaired t test). (f) Scatterplots of the relationships between the steatosis score and both serum HDL-cholesterol and the atherogenic index in the liver of α -LAC (\bullet) and chow-fed (\circ) mice. Each linear regression analysis is represented by its correlation coefficient (R), P value and the fitted regression line ($P < 0.05$). TC, total cholesterol.

As shown in Fig. 4(a), both nuclear PPAR α (22%; $P = 0.006$) and mitochondrial CPT-1 (21%; $P = 0.0001$) protein expression were significantly reduced in the liver of the α -LAC-fed mice compared with the chow-fed mice. These results suggest that the reduction of the mitochondrial FAO is involved in the fatty liver α -LAC-induced.

The dysregulation of the liver lipid metabolism α -LAC-induced is linked to inactivation of AMP-activated protein kinase α

To determine whether the prolipogenic effects of the α -LAC intake could be mediated by AMPK α inactivation, the phosphorylation of AMPK α and of its target the enzyme ACC1 were analysed by Western blot in the cytosolic fraction of liver of the experimental mice.

As shown in Fig. 4(a), the α -LAC diet led to a significantly decrease in both p-Thr¹⁷²-AMPK α (22%; $P = 0.005$) and p-Ser⁷⁹-ACC1 (30%; $P = 0.0001$) respectively, resulting in lower p-AMPK α :

AMPK α and p-ACC1:ACC1 ratios compared with the control mice. p-AMPK α :AMPK α ratio changes were positively correlated with nuclear PPAR α (α -LAC group; R 0.918; $P = 0.009$; control group; R 0.968; $P = 0.001$) and with mitochondrial CPT-1 (α -LAC group; R 0.895; $P = 0.015$; control group; R 0.941; $P = 0.005$) proteins in both α -LAC and control groups. Also, the p-AMPK α :AMPK α ratio was negatively correlated with the nuclear PPAR γ protein in α -LAC liver (R -0.830; $P = 0.048$) (Fig. 4(b)). These results seem to indicate that the impaired AMPK α and ACC1 phosphorylation in liver of the α -LAC mice could contribute to fatty liver by inhibiting FAO and enhancing DNL.

α -Lactalbumin diet increased lipid peroxidation and protein oxidation and decreased cytosolic and mitochondrial ATP levels of the Balb/c mice liver

Increased oxidative stress, a condition associated with fatty liver, can raise the level of LPO (MDA + 4-HNE) and/or protein

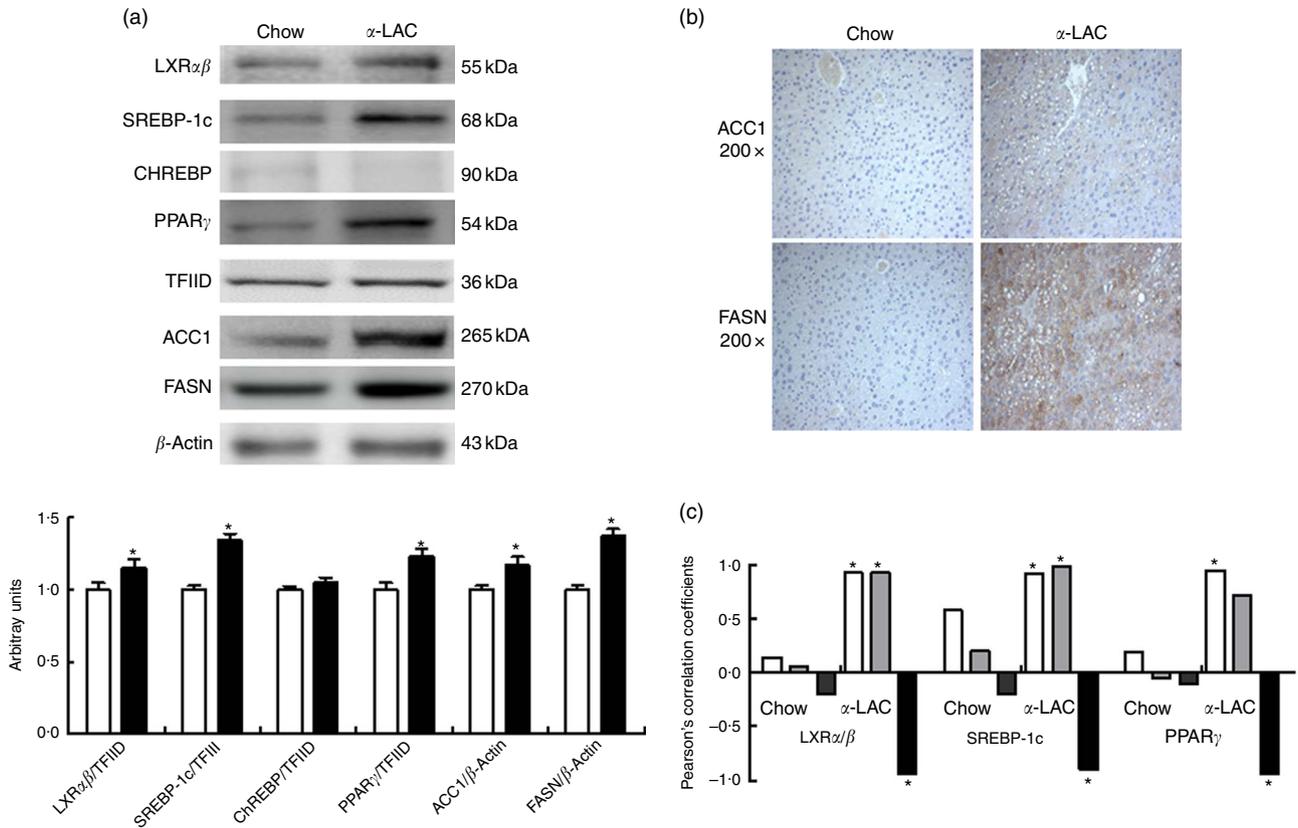


Fig. 3. Effect of dietary α -lactalbumin (α -LAC) on *de novo* lipogenesis in liver of Balb/c mice. (a) Immunoblotting analysis was performed to measure the protein expression of the transcription factors liver X receptor $\alpha\beta$ (LXR $\alpha\beta$), sterol regulatory element-binding protein-1c (SREBP-1c), carbohydrate responsive element-binding protein (ChREBP) and PPAR γ and of the enzymes acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FASN) in hepatic nuclear and cytosolic fractions of α -LAC (■) and chow-fed (□) mice. A representative blot is shown from three independent experiments with identical results. Values are means (n 6), with their standard errors represented by vertical bars. TFIIID, transcriptional factor II D. * Mean value was significantly different from that of the control group ($P < 0.05$; two-sided unpaired t test). (b) Representative photomicrograph of cytosolic ACC1 and FASN protein expression in liver of chow and α -LAC-fed mice (light microscope, magnification 200 \times). (c) Linear regression analysis between nuclear LXR $\alpha\beta$, SREBP-1c and PPAR γ proteins and hepatic NEFA (□), homoeostasis model assessment-estimated insulin resistance (■) and serum adiponectin (■). The Pearson's correlation coefficient (R) of each linear regression is represented by vertical bars. * Significant correlations ($P < 0.05$).

carbonylation. They are the two most common biomarkers of oxidative lipid and protein damage and contribute to the development of hepatocyte steatosis⁽¹⁶⁾. Thus, liver cytosolic and mitochondrial LPO and protein carbonyl (PC) concentrations were determined to assess the α -LAC diet-related lipids and proteins oxidative changes (Fig. 5(a)). The present results showed that mitochondrial (2.45-fold ($P = 0.000$)) and cytosolic (1.65-fold ($P = 0.000$)) LPO and mitochondrial (2.04-fold ($P = 0.001$)) and cytosolic (2.4-fold ($P = 0.000$)) PC levels were higher respectively in the α -LAC liver than control mice. Also, LPO and PC biomarkers were positively correlated with nuclear LXR $\alpha\beta$ (LPO: R 0.971; $P = 0.0012$; PC: R 0.890; $P = 0.016$), SREBP-1c (LPO: R 0.931; $P = 0.007$; PC: R 0.975; $P = 0.0009$) and PPAR γ (LPO: R 0.920; $P = 0.009$; PC: R 0.976; $P = 0.0009$) proteins and negatively with nuclear PPAR α (LPO: R -0.867; $P = 0.025$; PC: R -0.867; $P = 0.013$), mitochondrial CPT-1 (LPO: R -0.816; $P = 0.045$; PC: R -0.928; $P = 0.007$) proteins and the pAMPK α :AMPK α ratio (LPO: R -0.820; $P = 0.045$; PC: R -0.814; $P = 0.045$) (Fig. 5(b)). These results suggest that the α -LAC intake induced oxidative stress in liver which in turn could to modulate the hepatic lipid metabolism of Balb/c mice.

In addition, as a consequence of excessive ROS the efficiency of mitochondrial ATP synthesis was reduced. As shown in Fig. 5(a), the α -LAC intake reduced significantly the hepatic

cytosolic (37%; ($P = 0.001$)) and mitochondrial (24%; ($P = 0.03$)) ATP levels compared with the control mice. A significantly positive correlation between mitochondrial CPT-1 protein and mitochondrial ATP levels in both α -LAC (R 0.865; $P = 0.003$) and control groups (R 0.956; $P = 0.026$) were found (Fig. 4(c)). It could indicate that the α -LAC intake elicited a hepatic mitochondrial dysfunction related to decrease of mitochondrial FAO.

α -Lactalbumin diet decreased the cytosolic GSH redox status of the Balb/c mice liver

GSH constitutes the major intracellular low molecular weight thiol. GSH is responsible for the maintenance of an intracellular redox balance and detoxification of electrophilic xenobiotics and ROS. GSH and GSSG levels were measured to evaluate the effect of the α -LAC feeding on the cytosolic GSH:GSSG ratio in the liver, as the reduction in the redox status has been observed in human NAFLD.

Both, cytosolic GSH and GSSG contents in liver showed a significant decline in α -LAC mice although the GSH fall was higher (33%; ($P = 0.001$)) than the GSSG (14%; ($P = 0.014$))

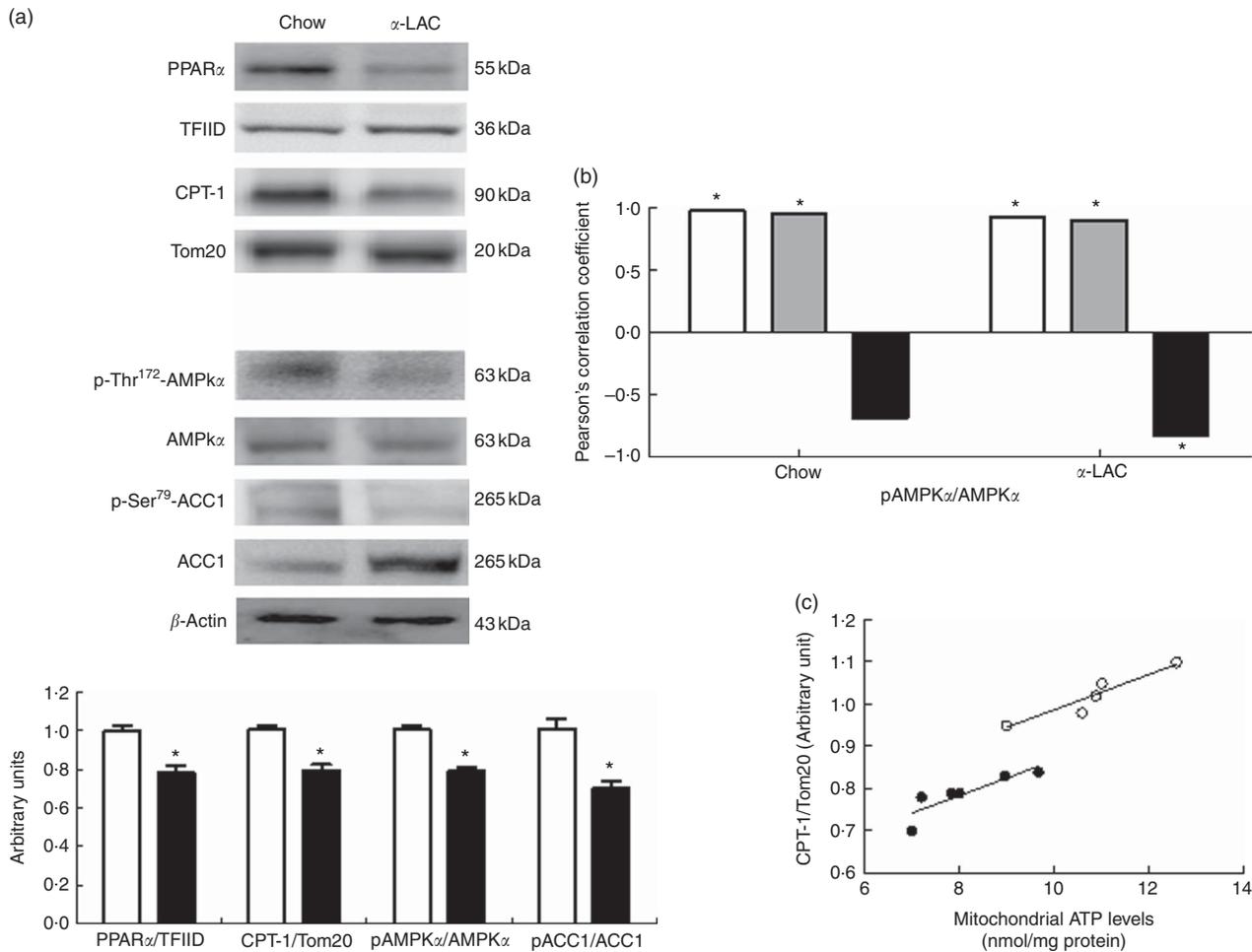


Fig. 4. Effect of dietary α -lactalbumin (α -LAC) on fatty acid β -oxidation in liver of Balb/c mice. (a) Immunoblotting analysis was performed to measure the protein expression of nuclear PPAR α and mitochondrial carnitine palmitoyltransferase-1 (CPT-1) as well as the cytosolic phosphorylated AMP-activated protein kinase α (AMPK α), total AMPK α , phosphorylated acetyl-CoA carboxylase 1 (ACC1) and total ACC1 proteins in liver of chow (□) and α -LAC-fed (■) mice. A representative immunoblot is shown from three independent experiments with identical results. Values of PPAR α and CPT-1 and the p-AMPK α :AMPK α and p-ACC1:ACC1 ratios are means (n 6), with their standard errors represented by vertical bars. * Mean value was significantly different from that of the control group ($P < 0.05$; two-sided unpaired t test). (b) Linear regression analysis between phosphorylated (p)-AMPK α :AMPK α ratio and nuclear PPAR α (□) and PPAR γ (■) proteins and mitochondrial CPT-1 (▣) protein. The Pearson's correlation coefficient (R) of each linear regression is represented by vertical bars. * Significant correlations ($P < 0.05$). (c) Scatterplots of the relationship between mitochondrial ATP levels and CPT-1 protein of chow (○) and α -LAC-fed (●) mice. Each linear regression analysis is represented by its correlation coefficient (R), P value and the fitted regression line ($P < 0.05$).

compared with the control group (Table 4). Consequently, the GSH:GSSG ratio decreased significantly (17.2%; $P = 0.05$) in the α -LAC group (Table 4), indicating that the α -LAC intake elicited a more oxidised GSH redox status in the liver of the Balb/c mice.

α -Lactalbumin diet-related changes in the cytosolic and mitochondrial antioxidant enzyme system of the Balb/c mice liver

The AOE system, including the GSH-dependent enzymes, constitutes an important defense mechanism against the oxidative stress and it has been reported that impairment of AOE functionality was associated with progression of hepatocyte damage⁽⁶⁷⁾. To evaluate changes in the AOE system by the α -LAC intake, we determine the activities and the protein expression of SOD (MnSOD and Cu,ZnSOD), GPx, GR and CAT in the cytosolic and/or mitochondrial fractions of the liver.

A significant decline in the antioxidant enzyme activities except for GPx was induced by the α -LAC diet (Table 4). Thus, the activities of cytosolic Cu,ZnSOD (40%; $P = 0.01$) and mitochondrial MnSOD (58%; $P = 0.000$) decreased significantly in the α -LAC liver compared with the control mice (Table 4) but the protein content was unmodified (Fig. 5(c)). This suggests that α -LAC diet induced a lower capacity for dismutation of O_2^- to H_2O_2 in liver. Similarly, the activities and the protein contents of both CAT and GR in the α -LAC liver (ranging between 33 and 39%) were significantly lower compared with the control mice (Table 4 and Fig. 5(c)), suggesting that the α -LAC intake diminished both the H_2O_2 scavenging of CAT function and GSH reduction capacity from GSSG of GR in liver.

GPx showed a different pattern: α -LAC diet did not affect GPx activity of the liver (Table 4) but the GPx protein expression was significantly increased in the mitochondrial (15%; $P = 0.05$) and especially in the cytosolic (33%; $P = 0.0001$) (Fig. 5(c))

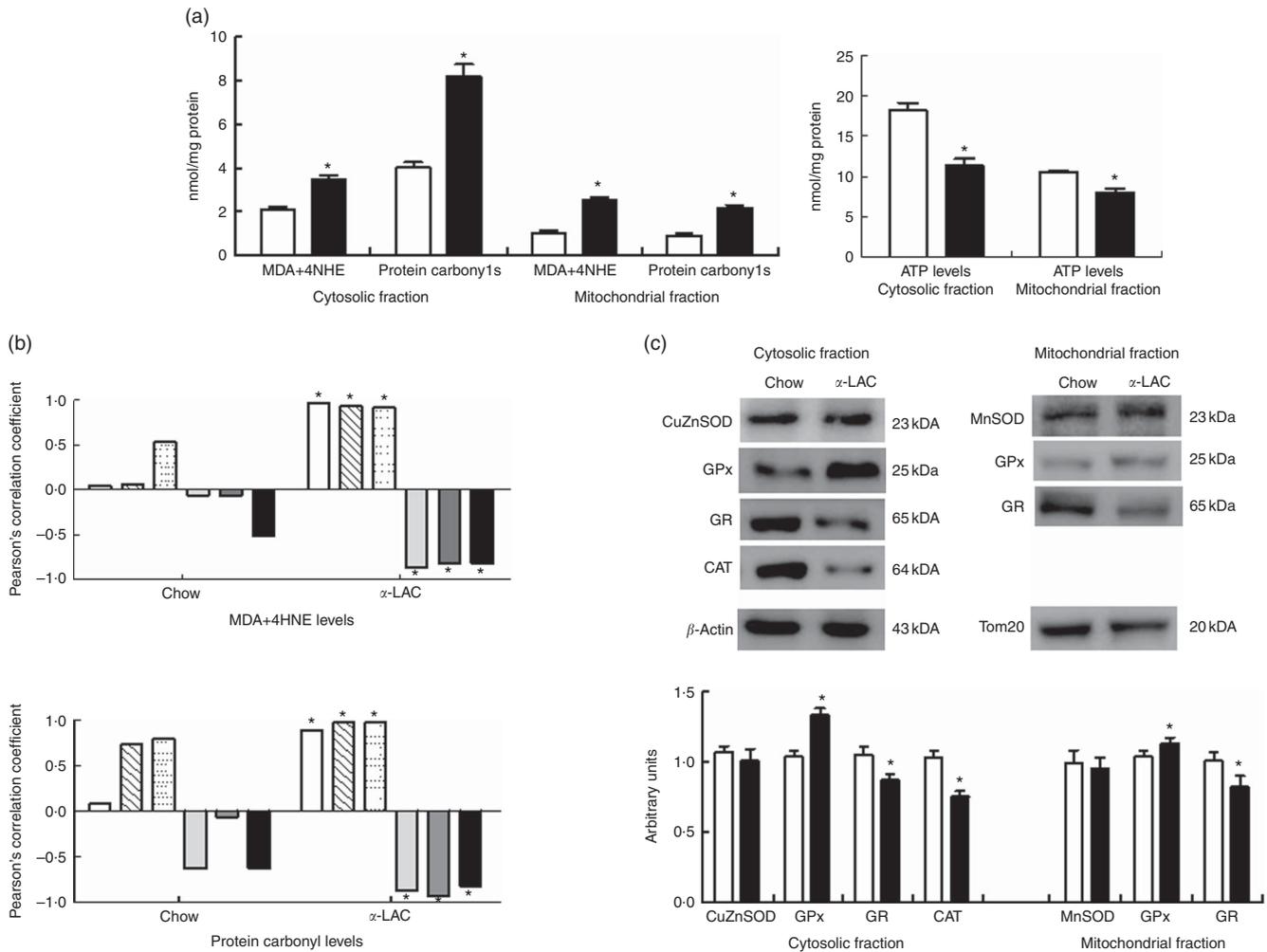


Fig. 5. Effect of dietary α -lactalbumin (α -LAC) on lipid peroxidation (LPO), protein carbonylation, antioxidant enzyme system and ATP levels in liver of chow and α -lactalbumin (α -LAC)-fed Balb/c mice. (a) Malonyldialdehyde + 4-hydroxynonenal (MDA + 4-HNE) (LPO), protein carbonyls and ATP levels in the hepatic cytosolic and/or mitochondrial fractions of α -LAC (■) and chow-fed (□) mice. Values are means (n 6), with their standard errors represented by vertical bars. * Mean value was significantly different from that of the control group ($P < 0.05$; two-sided unpaired t test). (b) Linear regression analysis between both MDA + 4-HNE and protein carbonyl levels and nuclear liver X receptor $\alpha\beta$ (LXR $\alpha\beta$, □), sterol regulatory element-binding protein-1c (SREBP-1c, ▨), PPAR γ (▤), PPAR α (▥) and mitochondrial carnitine palmitoyltransferase-1 (CPT-1) (▧) proteins and the phosphorylated (p)-AMPK α :AMPK α (■) ratio. The Pearson's correlation coefficient (R) of each linear regression is represented by vertical bars. * Significant correlations ($P < 0.05$). (c) Immunoblotting analysis was performed to measure the protein levels of hepatic cytosolic and/or mitochondrial superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) of α -LAC (■) and chow-fed (□) mice. A representative blot is shown from three independent experiments with identical results. β -Actin (cytosolic fractions) and Tom20 (mitochondrial fractions) were used as internal controls to monitor equal loading of the proteins. Values are means with their standard errors represented by vertical bars. * Mean value was significantly different from that of the control group ($P < 0.05$; two-sided unpaired t test).

fraction of liver compared with the control values. Thus, the α -LAC diet seems to up-regulate GPx, increasing the enzyme expression but maintaining GPx activity. The significant decrease of the SOD:GPx ratio compared with the control mice (Table 4) in the hepatic cytosolic and/or mitochondrial fractions (45%; $P = 0.001$ and 66%; $P = 0.000$ respectively) suggests an enhanced capacity of liver to reduce H_2O_2 or hydroperoxides.

Discussion

α -LAC a whey protein, has been reported to have nutritive⁽⁴⁰⁾ and health properties related to immunity, cancer, learning and metabolism⁽⁴¹⁾ but its effects on lipid metabolism are controversial.

We reported previously that feeding male Balb/c mice a diet containing 14% α -LAC as the sole protein source induces an atherogenic lipoprotein profile and an increase in abdominal fat pad, associated with low serum arylesterase activity⁽²⁸⁾. In the present investigation we looked at whether this atherogenic effect could be due to a possible α -LAC-induced hepatic steatosis, identifying the molecular mechanisms that regulate the hepatic lipid metabolism and its relationship with the peripheral IR and the hepatic redox status. The main findings of this study were as follows: (1) 14% α -LAC intake for 4 weeks produced macrovesicular hepatic steatosis related to increased adiposity, atherogenic dyslipidaemia, IR, hyperleptinaemia, hypo adiponectinaemia and enhanced serum TNF α in Balb/c mice; (2) the fatty liver development was due to enhancement of both NEFA influx into the liver and DNL, together with a

Table 4. Effect of dietary α -lactalbumin (α -LAC) on GSH, GSSG and the GSH:GSSG ratio and on superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) activities and both the SOD:GPx and SOD:CAT ratios of hepatic cytosolic and/or mitochondrial fractions of chow and α -lactalbumin (α -LAC)-fed Balb/c mice (Mean values with their standard errors; n 6)

	Cytosolic fraction				P^*	Mitochondrial fraction				P^*
	Chow		α -LAC			Chow		α -LAC		
	Mean	SEM	Mean	SEM		Mean	SEM	Mean	SEM	
GSH (nmol/mg protein)	97.87	5.82	65.22	3.33	0.001	–	–	–	–	–
GSSG (nmol/mg protein)	1.86	0.09	1.49	0.07	0.014	–	–	–	–	–
GSH:GSSG	52.90	3.51	43.98	1.93	0.050	–	–	–	–	–
SOD (IU/mg protein)	89.15	6.31	53.24	3.98	0.01	88.99	5.61	37.38	1.18	0.000
GPx (nmol/min per mg protein)	385.00	34.14	382.97	14.50	NS	20.45	1.22	22.83	1.83	NS
GR (μ mol/min per mg protein)	106.83	9.73	70.00	3.21	0.011	89.93	5.73	55.00	4.05	0.018
CAT (nmol/min per mg protein)	253.33	13.16	166.07	13.95	0.000	–	–	–	–	–
SOD:GPx ratio	0.24	0.01	0.13	0.01	0.001	4.36	0.04	1.48	0.03	0.000
SOD:CAT ratio	0.36	0.02	0.32	0.01	NS	–	–	–	–	–

* Differences were considered significant at $P < 0.05$ between α -LAC and chow groups (two-sided unpaired t test).

decrease in FAO; (3) AMPK α inactivation seems to influence both hepatic DNL and FAO in the α -LAC-induced fatty liver and (4) changes in the α -LAC-induced hepatic lipid metabolism were related to a pro-oxidative redox status.

Increased body fat was the main cause of the change in BW in the α -LAC-fed mice. This was a consequence of an anomalous change in dietary protein efficiency leading to a redistribution of body energy from BPG toward BFG, increasing the BW and the abdominal adiposity index, as evidenced by the positive correlation found between BFG and PI (Fig. 1(a)). This higher abdominal adiposity index was related to reduced HDL-cholesterol levels and an increase in the atherogenic index (Fig. 1(b)). The accumulation of intraabdominal fat has been associated with IR, atherogenic dyslipidaemia as well as atherosclerosis and CHD^(42,43). Improved peripheral IR due to hyperinsulinaemia in the α -LAC mice, as indicated by the increase in HOMA-IR, leads to the release of NEFA from visceral fat into the portal vein and they are therefore cleared mainly by the liver⁽⁴⁴⁾ contributing to liver fat accumulation. Increased inflow of both pro-inflammatory TNF α and antiestrogenic leptin, and decreased inflow of insulin-sensitising adiponectin in blood draining into the liver, suggest a role for these adipokines in the α -LAC-mediated IR development, reducing peripheral insulin sensitivity⁽⁴⁵⁾ as they seem to indicate, the negative and the positive correlation found respectively, between serum adiponectin and leptin and HOMA-IR (Fig. 1(c)). Conversely, beneficial effects of α -LAC improving impaired glucose tolerance via increases in adiponectinaemia in diabetic rats, when they were oral and chronically administered (300 mg/kg) with bovine milk-derived α -LAC for 10 weeks have been reported⁽⁴⁶⁾. In addition, α -LAC seems to have an anti-inflammatory effect in rats⁽⁴⁷⁾. However, in this study excessive NEFA flux to the liver due to diminished insulin responsiveness at the level of the adipocyte, would seem to be an important factor developing fatty liver in α -LAC-fed mice contributing to moderate hepatic steatosis, indicated by high hepatic TAG and NEFA levels and steatotic score which was associated with the atherogenic index (Fig. 2(f)). Also, increased liver fat together with differences in portal flow of amino acids have been reported in 15%

lactalbumin-fed rats compared with casein-fed rats⁽²⁶⁾. In addition to NEFA inflow, enhancement of DNL seems to help promote fatty liver in the α -LAC mice, as a consequence of activation of the hepatic TF and enzymes involved in fatty acid synthesis⁽⁴⁸⁾. LXR $\alpha\beta$ is a major regulator of FA synthesis⁽⁴⁹⁾. It has been postulated that LXR $\alpha\beta$ plays a direct and indirect role in the regulation of lipogenesis, controlling the expression of SREBP-1c and of ChREBP as well as of their target genes (i.e. *Acc* and *Fasn*) when they are induced by insulin or glucose respectively⁽⁵⁰⁾. In the present study, we observed that the α -LAC diet enhanced both nuclear LXR $\alpha\beta$ and SREBP-1c protein expression. This suggests that LXR $\alpha\beta$ overexpression simultaneously up-regulates SREBP-1c in the α -LAC liver. As a consequence, both hepatic lipogenic enzymes ACC1 and FASN were up-regulated⁽⁵¹⁾, increasing their cytosolic protein expression in the hepatocyte, wherein they were strongly identified immunohistochemically (Fig. 3(b)). The up-regulation of PPAR γ coincided with up-regulation of LXR $\alpha\beta$ and SREBP-1c as compared with the control mice, suggesting that high expression of nuclear PPAR γ protein in the α -LAC liver induced an important lipogenic effect. This possibly takes place through enhanced uptake and intracellular binding/transport of NEFA, which may reinforce the lipogenic actions associated with LXR $\alpha\beta$ and SREBP-1c, as has been demonstrated in obese patients⁽⁵²⁾ and in male C57Bl/6J mice subjected to continuous intragastric overfeeding with a high-fat liquid diet⁽⁵³⁾. This hepatic DNL enhancement could be related to the insulin action, which seems to persist in IR states and to the detrimental effect of the hepatic NEFA overload, as suggested by the strong positive correlation found between the nuclear LXR $\alpha\beta$ and SREBP-1c proteins and HOMA-IR and hepatic NEFA, and also between the nuclear PPAR γ protein and hepatic NEFA in α -LAC liver (Fig. 3(c)). These relationships have also been reported in obese patients⁽⁵²⁾, in casein and soy protein fed-rats⁽⁵⁴⁾ and in high-fructose fed insulin-resistant rats⁽⁵⁵⁾. In addition, according to Handa *et al.*⁽⁵⁶⁾ who suggested the participation of a reduced adiponectin-AdipoR2/LKB1/AMPK α signalling pathway in the development of NALFD in rat, adiponectin appears as a controller of DNL in α -LAC liver as suggested by the negative

correlation between the nuclear LXR α , SREBP-1c and PPAR γ proteins and serum adiponectin (Fig. 3(c)).

Our results also suggest that the reduction of the mitochondrial FAO contributed to α -LAC-induced hepatic steatosis and that both the NR PPAR α and the energy sensor AMPK α are involved in the process. We observed that the α -LAC diet down-regulates both PPAR α and AMPK α in liver, as evidenced by the significant reduction in both nuclear PPAR α and mitochondrial CPT-1 protein expression and by the fall in the AMPK α phosphorylation at the Thr¹⁷² site (inactivation) as compared with the control (Fig. 4(a)). However, it has been reported that PPAR α gene expression was unmodified in liver of 49% lactalbumin-fed Wistar rats⁽²⁷⁾, whereas soya protein diet up-regulated PPAR α and CPT-1 gene expression in the liver of Zucker rats⁽⁵⁷⁾. ACC1 is a substrate for AMPK α and its activity is negatively regulated by phosphorylation⁽⁵⁸⁾. Thus, the decrease of hepatic p-Thr¹⁷²-AMPK α in the α -LAC liver induced lower ACC1 phosphorylation at the Ser⁷⁹ site, leading to ACC1 activation and consequently to possibly higher production of malonyl-CoA, supporting the notion of mitochondrial FAO inhibition via allosteric regulation of CPT-1. This suggests that α -LAC intake elicits an impairment of long-chain fatty acyl-CoA inflow into mitochondria⁽⁵⁹⁾ through PPAR α /AMPK α signalling pathway inactivation, as the significant association between nuclear PPAR α protein and the p-AMPK α :AMPK α ratio found in our study (Fig. 4(b)) suggests that they were coordinately dysregulated. In addition, the inactivation of the PPAR α /AMPK α signalling pathway could contribute to the enhancement of the DNL in α -LAC liver, not only via ACC1 but also via PPAR γ , as shown by the negative correlation found between the nuclear PPAR γ protein and the p-AMPK α :AMPK α ratio (Fig. 4 (b)). On the contrary, inhibition of PPAR γ transcriptional activity by activated AMPK α has been reported in hepatoma cells⁽⁶⁰⁾.

In the α -LAC liver, reduced FAO via CPT-1 was associated with lower cytosolic and mitochondrial ATP levels, the major product of mitochondrial oxidative phosphorylation (Fig. 4(c)). This was likely as a consequence of the impairment of mitochondrial respiratory chain activity, which could contribute to α -LAC-induced NALFD through increased ROS, leading to an imbalance in pro/antioxidant molecules and to a more oxidative environment⁽⁶¹⁾. In the present study, the increase of by-products of lipid (MDA + 4-HNE) and protein (PC) oxidation indicates the prooxidant potential of the α -LAC diet, which could impair liver lipid metabolism. A role for oxidative stress in α -LAC-induced hepatic lipid metabolic dysregulation was suggested by both the positive and negative correlation found between nuclear LXR α , SREBP-1c and PPAR γ proteins and between nuclear PPAR α and mitochondrial CPT-1 proteins and the p-AMPK α :AMPK α ratio respectively and LPO and PC biomarkers (Fig. 5(b)). For instance, modulation of the lipogenic TF by free radicals during hepatic steatosis in humans⁽⁶²⁾, specific adducts between 4-HNE and CPT-1 in a NAFLD model⁽⁶³⁾ and increased production of ROS and cytotoxic aldehydic derivatives by repression of AMPK α have all been reported⁽⁶⁴⁾. In addition, the hepatic AOE system was altered by the α -LAC diet. The reduction in mitochondrial and cytosolic SOD activities, coupled with a decrease of cytosolic CAT, suggests a functional fall in both the capacity of dismutation of O₂⁻ to H₂O₂

and in the capacity to reduce H₂O₂ in the hepatocyte. Then again, the unexpected maintenance of GPx activity (decrease in SOD:GPx ratio) and the increase in its protein expression, suggests that GPx could help to scavenge H₂O₂ faster than it is formed. Nevertheless, in this study the antioxidant GPx function seems compromised. The maintenance of GPx activity, consistent with sustained capacity to oxidise GSH to GSSG, combined with the decrease in both GR protein and activity, produces a decrease in the cytosolic GSH:GSSG ratio, altering the GSH redox status in the α -LAC liver⁽⁶⁵⁾. In this connection, Merry *et al.*⁽⁶⁶⁾ demonstrated that hepatocyte GPx-1-deficiency improves hepatic glucose metabolism and reduces steatohepatitis in mice despite high hepatic levels of H₂O₂ and evidence of increased systemic oxidative stress. Thus, the α -LAC-associated changes in the GSH:GSSG ratio demonstrate that α -LAC intake induced a pro-oxidising shift, contributing to a more oxidative cellular environment in the hepatic cytosol of Balb/c mice. In this connection, Koek *et al.*⁽⁶⁷⁾ reported that overproduction of ROS in NAFLD may directly deplete antioxidant molecules, leading to apparently insufficient antioxidant capacity to compensate for oxidative stress. In parallel with these oxidative abnormalities, the serum ALT activity was increased suggesting that α -LAC intake promotes hepatocellular injury.

An important issue addressed in the present study was the potential effect of dietary α -lactalbumin upon the metabolic regulators involved in DNL and the FAO in liver, that demonstrates for the first time, to our knowledge the prolipogenic effect of a 14% dietary α -LAC intake. However, the variability among diets could be a limitation in the present study. Although the chow diet is used commonly in many NAFLD-related dietary studies^(68,69), it is possible that differences in macro and micronutrients and in the consumed energy between the diets could contribute to the differences observed in the liver lipid metabolism. Finally, there are several possible causes for the detrimental effects elicited by α -LAC diet on hepatic lipid metabolism: the amount of protein in the diet (14%) could induce NAFLD as a high PI has been shown to reduce hepatocellular lipid deposition and revert steatosis in mice⁽⁷⁰⁾; also the very high chemical score of α -LAC with an essential amino acids (EA), account for 63.2% (specially of tryptophan, cysteine and lysine) against 51.4 or 52.5% of casein or whole milk protein which could justify their lipotoxicity⁽⁷¹⁾. Accordingly, recent studies demonstrate that elevated circulating branched-chain amino acids (BCAA) are strongly associated with NAFLD-related metabolic disorders⁽⁷²⁾. However, contradictory results have been published regarding the role played by EA in lipid metabolism. Thus, both a low BCAA diet⁽⁷³⁾ and a diet high in ketogenic EA⁽⁷⁴⁾ appear to enhance liver lipid metabolism and improve hepatic steatosis through down-regulation the hepatic expression of lipogenic genes particularly ACC1, FASN and stearoyl-coA desaturase 1 in female broiler chickens and in high-fat diet-fed male *ob/ob* mice respectively. Interestingly, this repression in *ob/ob* mice appears to be mediated by the SREBP-1c pathway. Then again, there are differences in the protein absorption kinetics and in the portal flow rates of amino acids⁽²⁶⁾ to be considered. Therefore, given the importance of α -LAC in the human and animal diet further research is required to determine their effects on lipid metabolism.



All together, the present study provides evidence that a 4-week diet containing 14% of α -LAC intake induced liver steatosis in Balb/c mice as a consequence of increased systemic IR and hepatic lipid metabolism dysregulation, shifting the FAO toward fatty acid synthesis. Dietary α -LAC induced LXR α β /SREBP-1c/PPAR γ signalling activation and PPAR α /CPT-1 pathway inhibition and this mechanism was probably mediated by AMPK α inactivation. The prolipogenic effect of dietary α -LAC was associated with a more oxidative liver environment caused by prooxidant/antioxidant imbalance.

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The authors declare that there are no conflicts of interest.

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