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A combined healthy strategy for successful weight loss, weight maintenance and improvement of hepatic lipid metabolism☆

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Abstract

Obesity is critically related with the development of metabolic and pathophysiological alterations among which non-alcoholic fatty liver disease (NAFLD) is of especial relevance. Although there are numerous strategies to successfully treat obesity, the prevention of weight regain still remains challenging for individuals who have undergone weight loss programs. In such context, diet and physical activity are considered essential for the regulation of body weight and lipid metabolism. In this study, rats were fed a high-fat diet (HFD) to induce obesity and alterations in hepatic lipid metabolism. Obese rats were then treated with single or combined strategies of caloric restriction, physical exercise, and/or pharmacological treatment with an appetite suppressant, to lose weight, reverse the obesity-related alterations in hepatic morphology and lipid metabolism and maintain the beneficial effects of the interventions used. HFD induced excess body weight, hepatic steatosis, altered fatty acid profile, dysregulated gene expression of lipogenic and lipolytic enzymes, as well as plasma markers of liver damage, and modifications in liver antioxidant enzyme activity. Such alterations were ameliorated by caloric restriction in combination with a mixed training protocol and/or food-intake inhibitor administration during a weight loss intervention period of 3 weeks, and the beneficial effects remained after 6 weeks of weight maintenance, with some interesting interactions observed. In conclusion, weight loss strategies assayed were efficient at correcting the obesogenic action of a HFD and related alterations in hepatic functionality through different molecular mechanisms. The beneficial effects were also evident along the post-intervention maintenance period to avoid body weight regain.

Keywords: High-fat diet; Obesity; NAFLD; Caloric restriction; Physical exercise; CB1 receptor blockade

1. Background

Obesity is defined as abnormal or excessive fat accumulation that may impair health, since it is a risk factor for other metabolic diseases such as type II diabetes mellitus and non-alcoholic fatty liver disease (NAFLD) [1]. Environmental factors that affect energy balance are mainly related to the development of obesity, although genetic factors are also involved [2]. Energy imbalance is influenced by a diet high in saturated fat and refined sugars as well as a sedentary lifestyle [3]. NAFLD is characterized by fat accumulation, especially triglycerides, in the hepatocytes [4], and by important morphological alterations such as hepatomegalia, steatosis or hepatocyte ballooning, accompanied by inflammation and oxidative stress derived from changes in the hepatic antioxidant defense systems [5], leading to impaired liver function. The relationship between obesity and NAFLD appears of especial relevance since a high percentage of obese individuals are prone to develop NAFLD which may in turn evolve to non-alcoholic steatohepatitis or hepatic cancer [6].

Although numerous strategies centered on weight loss have been designed for obesity treatment [3], it is still challenging for subjects who successfully complete a weight loss program to avoid the rebound effect that has been studied both in human subjects and experimental animals [7,8]. The regain usually takes place right after the end of weight loss intervention as, for most people, weight loss programs are just transient and not definitive changes in lifestyle due to the difficulties in incorporating them into daily habits [9].

Since obesity development is favored by the consumption of unbalanced and hypercaloric diets [10], caloric restriction combined

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with intake of a balanced diet providing adequate amounts of nutrients is highly recommended to treat or prevent its appearance. Regarding the macronutrient prescriptions for weight-loss interventions, it is widely accepted that a reduction in fat is often recommended. In addition, high-protein diets are implemented due to the greater dietary-induced thermogenesis of protein compared to that of carbohydrates and fat, to the benefits of these diets on bone metabolism, and to their minimal disturbance of glucose metabolism [11]. Soluble fiber intervention is also recommended based on the satiating action of this compound and its beneficial action in lipid metabolism parameters that are usually altered in obese subjects [12]. As a result, it has been described as an effective therapeutic agent to treat many of the metabolic syndrome components associated to obesity [13].

Physical activity also plays an essential role in the prevention and treatment of obesity, contributing to generate a negative energy balance via sympathetic action, thus facilitating weight loss and counteracting its biological adaptations that may lead to the rebound effect and subsequent body weight regain [7,8]. Such exercise practice would have benefits against cardiovascular diseases, hypertension, diabetes, obesity, and cancer. Interestingly, physical exercise acts more efficiently on visceral adiposity, a stronger predictor for morbidity and mortality [14]. In this regard, we have shown the beneficial effects of mixed intervallic aerobic training, as it decreases body weight and fat mass in rats while improving their glucose and lipid plasma profile [15,16], as well as on plasma and liver parameters related to NAFLD [17].

One more crucial factor related to the control of energy balance is the regulation of food intake in which the hypothalamic peptides play a critical role. The endocannabinoid system is also responsible for food intake control, especially through CB1 receptors. Furthermore, an overactivation of the endocannabinoid system has been described in diabetic and obese rats [18]. Therefore, modulation of this system through the blockade of CB1 receptors could contribute to the control of obesity and related metabolic alterations such as NAFLD [19,20].

In this experiment, we have developed an animal model of diet-induced obesity (DIO) aimed to i) design and implement adequate intervention strategies to lose weight and avoid the post-intervention body weight regain by means of an adequate dietary pattern combined or not with a mixed training protocol and/or the administration of a food intake inhibitor through CB1 receptor blockade, ii) act on the prevention and treatment of metabolic and hepatic pathophysiological alterations derived from obesity, and iii) study gene expression of enzymes involved in lipogenic and lipolytic pathways to extend our knowledge on the potential molecular mechanisms that support our experimental findings.

2. Materials and methods

2.1. Animals and experimental design

The experiment used 112 male Sprague–Dawley rats with an average body weight of 184±10 g (6 weeks old, Charles Rives, Barcelona, Spain) that were allocated to fourteen different experimental groups (n=8). The experiments lasted for 21 weeks and were divided in three stages (0–12, 12–15 and 15–21 weeks) (Fig. 1). Two control experiments that involved three groups of animals in each of them were organized with the following design:

1. Control CNOR groups fed a normocaloric standard rodent diet (Teklad Global Diet 2014; 2.4 kcal/g) along the whole experiment:
   - G1. CNOR 12 weeks
   - G2. CNOR 15 weeks
   - G3. CNOR 21 weeks
2. Control CHC groups fed a hypercaloric obesogenic diet containing 60% of kcal as fat (Research diets D12492; 5.2 kcal/g) along the whole experiment:
   - G4. CHC 12 weeks
   - G5. CHC 15 weeks
   - G6. CHC 21 weeks

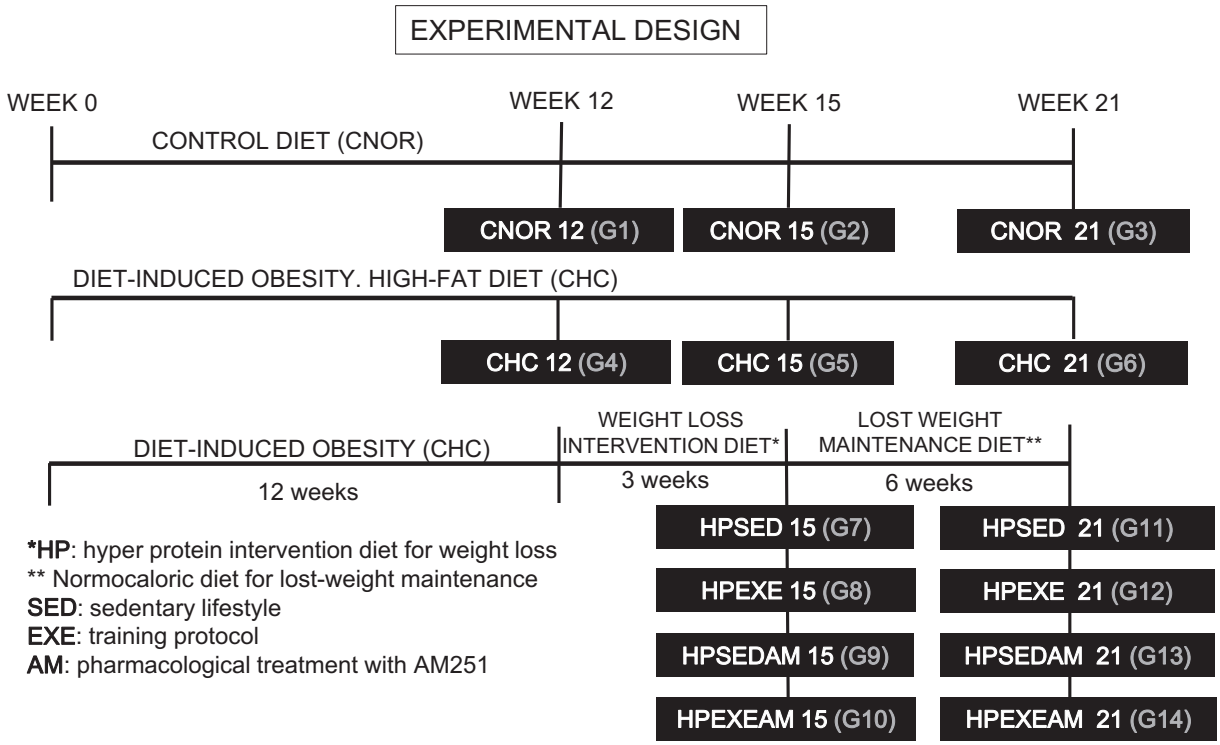

Fig. 1. Experimental design. Six control experiments were carried out during 21 weeks using a standard rat chow diet (CNOR12, CNOR15 and CNOR21) or a hypercaloric diet to induce obesity (CHC12, CHC15 and CHC21). For intervention trials, rats were divided into 8 groups that were fed the hypercaloric diet to induce obesity during 12 weeks, followed by three weeks of intervention with a hyperproteic diet for weight loss (HP15) combined or not with the training protocol (SED or EXE, respectively) and the pharmacological treatment (AM). The intervention period was followed by an additional six weeks of dietary treatment with a standard rat chow diet (HP21) combined or not with the training protocol (SED or EXE, respectively) and the pharmacological treatment (AM) in order to avoid the post-intervention weight regain.

After the first stage of 12 weeks of DIO by ingestion of the hypercaloric diet (CHC), in a second 3-week stage of the experiment (up to week 15), four experimental groups were subjected to the following interventions in order to lose weight and ameliorate metabolic alterations:

G7. A caloric restriction intervention was implemented using an experimental diet designed to induce greater satiety combining the effects of high protein and soluble dietary fiber content (HPSED15) (2.9 kcal/g; Table 1). In addition to its satiating action, soluble dietary fiber is an effective therapeutic agent to treat many of the metabolic syndrome components associated to obesity [13].

G8. Similar dietary intervention combined with a mixed training protocol implemented 5 days per week during the 3-week weight loss intervention (HPEXE15).

G9. Similar dietary intervention combined with the intraperitoneal administration of a CB1 receptor blocker (AM251) thrice a week at a dose of 3 mg/kg body weight as reported by [21] during the 3-week weight loss intervention (HPSEDAM15).

G10. Similar dietary intervention combined with both the training protocol and CB1 receptor blocker during the 3-week weight loss intervention (HPEXEAM15).

Finally, for another four experimental groups, a third stage of the experiment ran for a period between weeks 15 and 21 and was designed for maintenance of lost body weight avoiding rebound effect:

G11. Ingestion of a normocaloric diet after the 3-week weight-loss dietary intervention of caloric restriction with a high-protein/high-fiber diet (HPSED21). The amount of food ingested during that 6-week period was *pair fed* to 23 g/d in order to achieve a 12–15% caloric reduction compared to the same period in control CNOR group as part of the strategy to maintain the lost-weight during post-intervention stage avoiding the rebound effect.

G12. Similar dietary treatment combined during the intervention and lost-weight maintenance period with a mixed training protocol implemented 5 days per week (HPEXE21).

G13. Similar dietary treatment combined with the intraperitoneal administration of a CB1 receptor blocker thrice a week during weight loss intervention and once a week during the lost-weight maintenance stage at a dose of 3 mg/kg body weight (HPSEDAM21).

G14. Similar dietary treatment combined with both the mixed training protocol and the administration of a CB1 receptor blocker (HPEXEAM21).

Table 1
Formulation and composition of the experimental diets

	CHC	HP
Casein	258.5	-
Whey protein	-	198.4
Pea protein	-	170.0
L-Cystine	3.88	1.13
Wheat starch	-	198.4
Maltodextrin 10	161.5	141.7
Sucrose	88.9	-
Cellulose	64.6	103.9
***Plantago ovata* fiber**	-	103.9
Soybean oil	32.3	-
Lard	316.0	-
Olive oil	-	37.8
Mineral mix S10026/AIN-93M	12.92	33.1
Dicalcium phosphate	16.8	-
Calcium carbonate	7.11	-
Potassium citrate	21.3	-
Vitamin Mix V10001/AIN-93M	12.92	9.45
Choline bitartrate	2.58	2.36
FD&C blue dye #1	0.065	-
Nutrient composition		
kcal/kg diet	4560	2920
Protein (g/kg)	222	267
Fiber (g/kg)	50	231
Ash (g/kg)	41.1	42.0
Fat (g/kg)	312.0	42.3
Fatty acid profile (%)		
Miristic (C14:0)	0.25	-
Palmitic (C16:0)	21.3	7.2
Stearic (C18:0)	7.10	1.30
Palmitoleic (C16:1n9)	0.36	-
Oleic (C18:1n9)	41.6	78.2
Octadecenoic (C18:1n7)	0.36	-
Linoleic (C18:2n6)	27.9	12.4
Linolenic (C18:3n3)	1.13	0.92

CHC, hypercaloric diet for dietary induction of obesity; **HP**, hyperprotein intervention diet for weight loss. **CNOR**, standard rat chow, formulation according to manufactured Teklad global 14% protein rodent maintenance diet in descending order of inclusion: wheat middlings, ground wheat, ground corn, corn gluten meal, calcium carbonate, soybean oil, dicalcium phosphate, iodized salt, L-lysine, vitamin E acetate, DL-methionine, magnesium oxide, choline chloride, manganous oxide, ferrous sulfate, menadione sodium bisulfite complex (source of vitamin K activity), zinc oxide, copper sulfate, niacin, calcium pantothenate, calcium iodate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, vitamin B12 supplement, folic acid, cobalt carbonate, biotin, vitamin D3 supplement; Nutrient composition, Protein (g/kg): 135, Fiber (g/kg): 223, Ash (g/kg): 45.3, Fat (g/kg): 44.1 https://www.envigo.com/p/teklad/laboratory-animal-diets/natural-ingredient/rodent/2014-diets.aspx;

The weight-loss intervention and body weight maintenance periods have been designed based on the information provided by Sengupta [22] who reported that laboratory rats live about 2–3.5 years (average 3 years), while the worldwide life expectancy of humans is 80 years. Thus, one human year almost equals two rat weeks (13.8 rat days) while correlating their entire life span. Under our experimental conditions, 3 weeks equal to 1.5 years of human life, which is a long enough period to achieve an efficient weight loss. The maintenance period of 6 weeks equals to 3 years which is enough to demonstrate the success of our combined strategy against body weight regain.

The composition of different experimental diets is shown in Table 1. The animals were housed in 4-rat group cages to ensure animal welfare and located in a well-ventilated, thermostatically controlled room (21±2°C) (UEA, CIC, Universidad de Granada). A reversed 12:12 light/dark cycle was implemented so the animals would perform the training protocol in darkness. Throughout the trial, animals had free access to Type 2 water (resistivity 15 $M\Omega^{-cm}$) and consumed the diet *ad libitum*, with the exception of the intervention groups in the last stage of the experiment that were adapted to slightly lower food intake (23 g/d) compared to that of the normocaloric control during the same period (28 g/d) in order to keep a certain degree of caloric adaptation to avoid body weight rebound, as recommended by weight control programs [23]. The diet was provided for all four animals in each cage but the body weight control was registered individually. Caloric intake was recorded daily whereas body weight was measured once a week. At the end of every experimental period, the animals were fasted for 8 h, anesthetized with ketamine/xylazine and sacrificed. Blood was collected by puncture of the abdominal aorta (with heparin as anticoagulant) and centrifuged at 1458×*g* for 15 min to separate plasma that was subsequently frozen in liquid nitrogen and stored at -80°C. The liver was extracted, weighed, divided into various portions and immediately frozen in liquid nitrogen and stored at -80°C with the exception of 100 mg that were immersed in RNA preserving solution (RNAlater, Ambion). All experiments were undertaken according to Directional Guides Related to Animal Housing and Care [24] and all procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada, Spain (Project Reference DEP2014-58296R).

2.2. Training protocol

Rats trained on a specially designed treadmill (Panlab, LE 8710R) following a protocol based on interval aerobic training combined with strength exercise in the same session as previously described by Coll-Risco et al. [15] and Martínez et al. [16]. Training sessions were performed 5 days/week and during the dark cycle of the animals (active period). To establish the velocity that would correspond to the VO_2 max of each rat, a maximal incremental test was performed at the start of the study following the protocol described by Wisløff et al. [25] and Clemente et al. [26].

2.3. Intraperitoneal administration of AM251

The CB1 receptor blocker, N-(Piperidin-1-yl)-5 (4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) (Tocris Cookson, Bristol, UK) was dissolved in the vehicle tween-80: dimethyl sulfoxide (DMSO): 0.9% NaCl (1:2:97) for intraperitoneal administration in rats at a dose of 3 mg/kg body weight.

2.4. Total hepatic lipid content

A liver portion was lyophilized in order to determine the percentage of water. Hepatic lipids were extracted with hexane from an aliquot of the freeze-dried liver portion using the method described by Folch et al. [27] with slight modifications [28]. Total liver lipids were measured gravimetrically after solvent extraction under N_2 stream.

2.5. Microscopic liver study

A portion of liver was fixed in 10% phosphate-buffered formalin, dehydrated in ethanol, embedded in paraffin, and sectioned for histological examination using hematoxylin-eosin (HE) staining for general microscopy morphology. Four different preparations of each staining were analyzed for each animal and eight animals were evaluated in each experimental group (n= 32 samples per group). Histological alterations were evaluated in zones 1, 2 and 3 of the acinus and the Non-Alcoholic Steatohepatitis (NASH) semi-quantitative scoring system of Kleiner et al. [29] as

adapted by Chen et al. [30] was used to evaluate the degree of NASH development. According to the former authors, a validated histological feature scoring system that addresses the full spectrum of lesions of NAFLD based on HE and Masson stains was implemented together with a NAFLD activity score (NAS). The scoring system comprised 14 histological features, four of which were evaluated semi-quantitatively: steatosis (0–3), lobular inflammation (0–2), hepatocellular ballooning (0–2), and fibrosis (0–4). Another nine features were recorded as present or absent. NAS score was calculated by the sum of steatosis grade, lobular inflammation and ballooning. NAS of >5 correlated with a diagnosis of "NASH", and scores of <3 were diagnosed as "not NASH" [29].

2.6. Fatty acid profile of liver

A freeze-dried liver portion were extracted and methylated according to Lepage and Roy [31] for gas chromatography analysis of fatty acid profile using an Agilent 7890A chromatograph equipped with CTC Pal combi-xt model sampler and a Waters Quattro micro GC mass spectrometer detector. Individual fatty acid methyl esters (FAMEs) were separated with a 30 m × 0.25 mm ZB Fame capillary column (0.2 μm thickness) (Phenomenex, Torrance, CA, USA). The gas chromatography conditions were as follows: injector temperature 250°C, injection volume 2 μl Split (proportion 10:1), temperature gradient from 100°C to 210°C with a rate of 4°C/min, hold time 5 min. The flow rate of the carrier gas (Helium) was 1 mL/min. Analysis time was 40 min and measurement range 45–450 uma (scan mode). Chromatographic data were recorded and integrated using Masslynx, version 4.1 software. FAMEs were identified using analytical standards and mass spectral library. Peak areas were measured and used to calculate the percentage of each individual fatty acid related to the total sum of all the fatty acid areas in the sample. Some product to precursor fatty acid ratios were used as indices of desaturase or desaturase-elongase enzyme activities in the liver as described by Gonzalez-Torres et al. [32] using the following formulas. Delta-6-elongase-desaturase: arachidonic acid/linoleic acid. Oleoyl-coA activity (SCD): oleic acid/stearic acid.

2.7. RNA extraction and quantitative RT-PCR

Total RNA was isolated from liver. One hundred milligrams of tissue were homogenized in 1 mL of Tri-Reagent® (Sigma-Aldrich). The RNA was solubilized in RNAse-free H_2O and treated with DNase (Applied Biosystems) to remove any DNA present in the sample. A total of 100–250 ng of RNA was reverse-transcribed according to standard protocols using a Lifepro Thermal Cycler (Bioer Serves Life, P. R. China). Quantitative RT-PCR was performed with 7900 HT Fast Real-Time PCR system (Applied Biosystems), using primer/probes for genes involved in lipid metabolism and signaling pathways or antioxidant defense system, *Fasn*, fatty acid synthase; *Ldlr*, low density lipoprotein receptor; *G6pd*, glucose 6 phosphate dehydrogenase; *Scd1*, stearoyl-CoAdesaturase-1; *Gpat2*, glycerol-3-phosphate acyl transferase 2; *Hmgcr*, 3-hydroxy-3-methyl-glutaryl-CoA reductase; *Cpt1a*, carnitine palmitoyl transferase 1A; *Lpl*, lipoprotein lipase; *Lipc*, hepatic triacylglycerol lipase; *Cyp7a1*, cholesterol 7 alpha-hydroxylase; *Acox1*, peroxisomal acyl-CoA oxidase; *Ucp2*, uncoupling protein 2; *Ppara*, peroxisome proliferator activated receptor alpha; *Srebf1*, sterol regulatory element binding transcription factor 1; *Nfe2l2*, nuclear factor erythroid 2 like2 (Applied Biosystems). The PCR master mix reaction included the first strand cDNA template, primers and 2X TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems). Expression of the test gene was related to that of *Actb* measured in parallel in the same sample using the ΔCt method. Data are presented as $2^{-\Delta\Delta Ct}$, in reference to the CNOR group in each experimental period. A heatmap including expression data of all the genes analyzed was built. The data are shown as log 2 of the $2^{-\Delta\Delta Ct}$ in relation to the CNOR 12 weeks group.

2.8. Plasma biochemical analysis

Plasma biochemical parameters were analyzed using a Shenzhen Midray BS-200 Chemistry Analyzer (Bio-Medical Electronics) at the Bioanalysis Unit of the CIC (PTS, Universidad de Granada) in order to determine aspartate amino transferase (AST), alanine amino transferase (ALT), γ glutamyl transferase (γ-GT), and alkaline phosphatase (ALP) activities.

2.9. Hepatic antioxidant activity assays

A fresh liver aliquot was homogenized (1:10 w/v) in 50 mM phosphate buffer (pH 7.8) containing 0.1% Triton X-100 and 1.34 mM of DETAPAC using a Micra D-1 homogenizer (ART modern labortechnik) at 18.000 rpm for 30 s followed by treatment with Sonoplus HD 2070 ultrasonic homogenizer (Bandelin) at 50% power three times for 10s. Liver homogenates were centrifuged at 13000 × g, 4°C for 45 min and the supernatant was used to determine the activity of antioxidant enzymes. Catalase activity was measured by the method of Cohen et al. [33], total cellular glutathione peroxidase (GPX) activity was determined by the coupled assay of NADPH oxidation [34] using cumene hydroperoxyde as substrate, and total superoxide dismutase (SOD) activity was measured as described by Ukeda et al. [35]. Mn-SOD activity was determined by the same method after treating the samples with 4 mM KCN for 30 min, whereas CuZn-SOD activity resulted from subtracting the Mn-SOD activity from the total SOD activity. One unit of SOD activity was defined as the enzyme needed to inhibit 50% 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction. Protein concentration was assayed by the method of Bradford [36].

2.10. Hepatic lipid peroxidation

Hepatic lipid peroxidation was determined in liver homogenates according to the methodology of Ohkawa et al. [37]. Thiobarbituric acid reactive substances (TBARS) were assayed spectrophotometrically at 532 nm. The amount of TBARS was expressed as nanomoles per milligram of protein.

2.11. Statistical analysis

For a more accurate description and interpretation of the data, the experimental period has been divided in three different phases: i) dietary induction of obesity during Weeks 0–12, ii) individual or combined weight loss interventions: dietary (caloric restriction), lifestyle (training protocol of mixed exercise), and pharmacological interventions (CB1 receptor blocker three days a week) during Weeks 13–15, and iii) post-intervention maintenance stage with normocaloric, diet combined or not with a training protocol and CB1 receptor blocker administration once a week during Weeks 16–21. Significant differences in liver weight, plasma and liver biochemical parameters and indices, hepatic fatty acid profile and indices, hepatic antioxidant enzyme activity, and gene expression data between CNOR and CHC groups were analyzed by t-test at 12 weeks of the experimental period, and by one-way ANOVA on weeks 15 and 21 among all the experimental groups. Tukey's test was used to detect differences between treatment means. T-test was also used to analyze significant differences in liver weight and hepatic fat content between 15 and 21 weeks within the same experimental group (Fig. 3). Statistical analysis was performed with Statistical Package for Social Sciences (IBM SPSS for Windows®, version 22.0, Armonk, NY), and the level of significance was set at $p < 0.05$.

3. Results

3.1. Caloric intake and body weight

Fig. 2 shows the effects of HFD treatment and different weight loss interventions on caloric intake and body weight along the 21-week experimental period. During the 12-week obesity induction period, and due to the higher caloric content provided by HFD, there was a significantly higher caloric intake by the groups of rats fed the HFD (CHC) compared to the normocaloric groups (CNOR) that led to significantly higher body weight in the former groups compared to their CNOR counterparts. In fact, from week five of experimental period, the difference in body weight between normocaloric and HFD-fed animals was equal or greater than 2 standard deviations (data not shown), a well-accepted value to declare obesity in experimental animals. The interventions with high-protein/high fiber diet, mixed training protocol and CB1 receptor blocker administration showed a synergistic action at decreasing the caloric intake during the first week of intervention, with the effect getting stronger sequentially in parallel to the combination of two or three interventions. The above-mentioned decrements in caloric intake were paralleled by concomitant decreases in body weight during the entire intervention period, thus reflecting actual weight loss by the animals that was more noticeable during the first week of intervention. During the post-intervention maintenance period, from 16 to 21 weeks, the dietary intervention switched to a normocaloric standard rat chow, although the daily food intake was pair fed to 23 grams per day in order to keep a slight degree of caloric restriction in accordance with the nutritional recommendations usually given in this period of body weight control treatment [23]. In addition, physical exercise on the trained groups continued with the same frequency as that of the intervention period, whereas the administration of CB1 receptor inverse agonist was reduced to once a week. In general, changes in body weight during this last phase of the experimental period were maintained in nearly null values that were consistently lower than those exhibited by the control normocaloric or hypercaloric groups during the same time period. Specifically, the most efficient post-intervention strategies to maintain body weight were those that involved physical exercise, either combined only with a small degree of caloric restriction or also

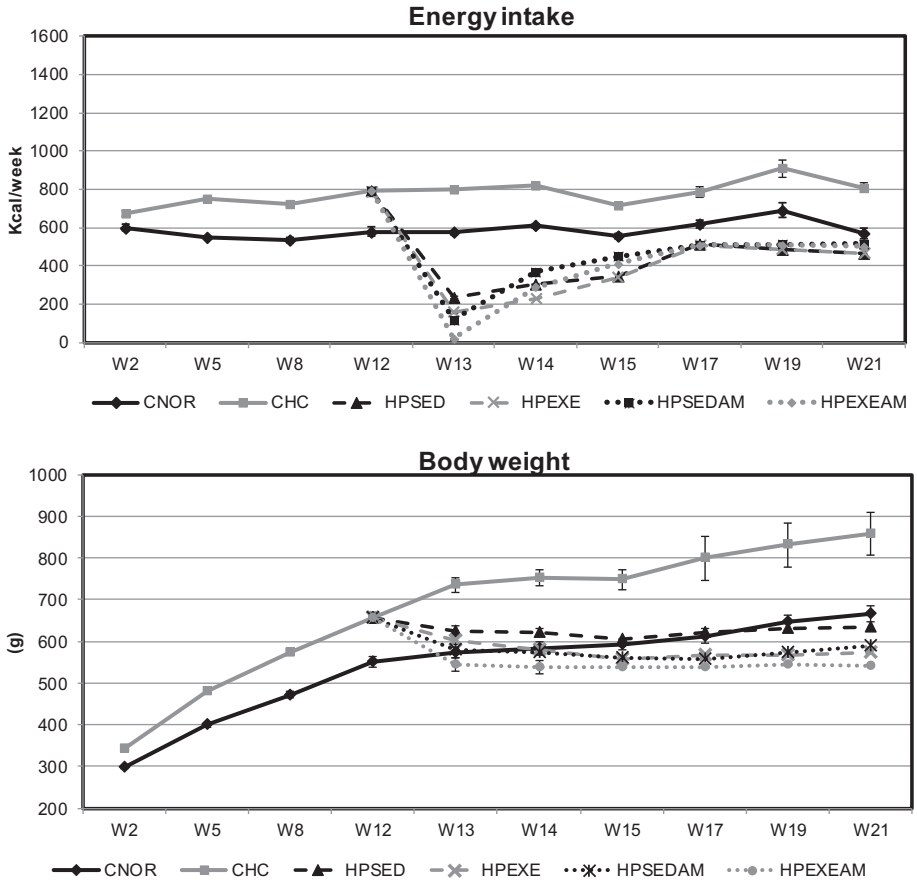

Fig. 2. Weekly caloric intake (kcal) and body weight (grams) of the different experimental groups along the experimental period. To observe and differentiate the results more clearly, only the most representative weeks of the experimental period are included in the graph. CNOR, control standard rat chow diet; CHC, control hypercaloric diet for dietary induction of obesity; HPSED, hyperprotein intervention diet for weight loss with sedentary lifestyle; HPEXE, hyperprotein intervention diet for weight loss with training protocol; HPSEDAM, hyperprotein intervention diet for weight loss with sedentary lifestyle and pharmacological treatment with AM251; HPEXEAM, hyperprotein intervention diet for weight loss with training protocol and pharmacological treatment with AM251. Results are means of eight rats ± SEM depicted by vertical bars.

complemented with once-a-week administration of the CB1-receptor blocker (Fig. 2).

3.2. Liver weight and total fat content

Dietary induction of obesity using a HFD resulted in significant increments in liver weight and liver fat content at 12 or 15 weeks of experimental period, whereas on week 21 a significant increase in hepatic fat contents but not in liver weight was observed (Fig. 3). All the interventions assayed efficiently reversed the hepatomegalia and steatosis induced by obesity. In fact, just the dietary caloric restriction during the intervention period and posterior switch to normocaloric diet was enough to reverse them. Nevertheless, the mixed training protocol showed a trend towards more consistent decrements in liver weight, with especially pronounced action in the AM251-administered group at 21 weeks. Regarding within group comparisons between weeks 15 and 21, a small decrease in liver weight and fat content was observed in the CHC group, whereas an increase in liver fat was observed in the CNOR group. As for the intervention groups, a small increase in liver weight was observed in HPSED and HPEXE animals at the end of post intervention period (21 weeks) compared to 15 weeks, although no differences were found for any of the experiment on liver fat content between the former time periods, thus pointing out to an overall maintenance of beneficial effects achieved by the weight loss treatments after the 6-week post-intervention period.

3.3. Liver histological study

Our experimental model of DIO led to the development of hepatic steatosis, featured by important microvesicular fat accumulation in the liver together with a lower degree of macrovesicular changes and ballooning (Table 2, Fig. 4). The extent of alterations developed increased throughout the experimental period, attaining an upper grade in animals treated with HFD over 15 or 21-weeks compared to those treated during 12-weeks. The different interventions reduced significantly these histological alterations and even led to the disappearance of ballooning and microvesicular fat changes as well as to NAS score values of 2 on weeks 15 and 21 of experimental period. On the other hand, the development of liver steatosis did not lead to an increase of the histological inflammatory markers observed compared with the livers of rats fed the CNOR diet.

3.4. Fatty acid profile of the liver

The influence of obesity and different weight-loss interventions on hepatic fatty acid profile is presented in Table 3. Obese animals exhibited a decrease in the percentage of total saturated and polyunsaturated fatty acids that was especially evident for stearic, arachidonic or docosahexaenoic (DHA). According to these changes, the Δelongase/desaturase ratio (arachidonic acid/linoleic acid) was significantly lower in these rats. In contrast, a significant increase in monounsaturated fatty acids was found that was especially

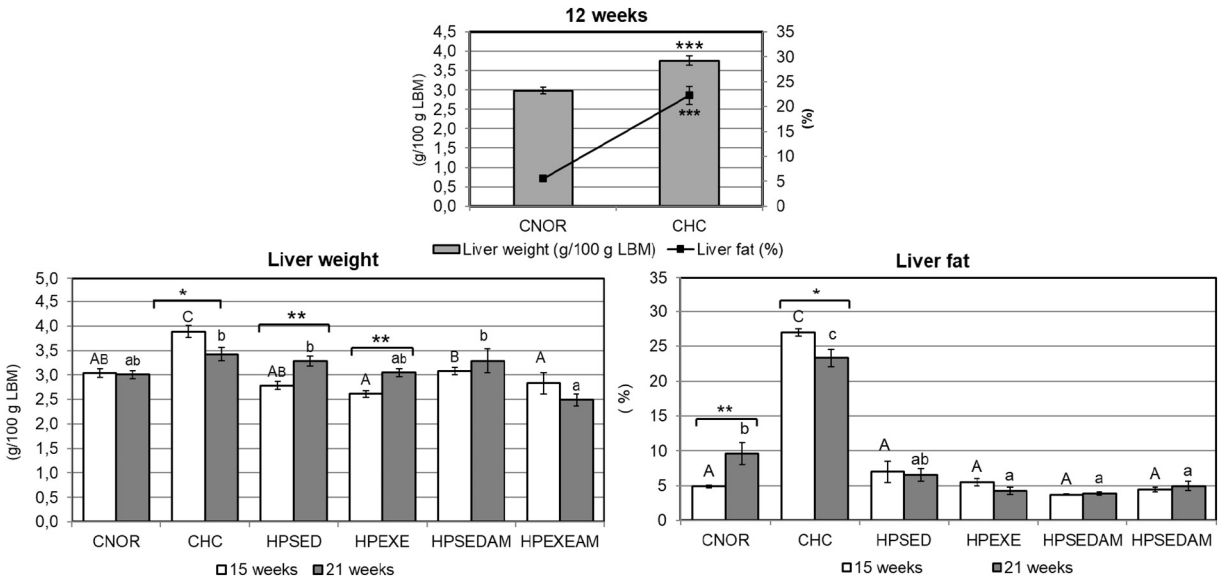

Fig. 3. Liver weight and total fat content of the different experimental groups at the end of each experimental stage (diet-induced obesity, weight-loss intervention, lost weight maintenance). LBM, Lean body mass. CNOR, control standard rat chow diet; CHC, control hypercaloric diet for dietary induction of obesity; HPSED, hyperprotein intervention diet for weight loss with sedentary lifestyle; HPEXE, hyperprotein intervention diet for weight loss with training protocol; HPSEDAM, hyperprotein intervention diet for weight loss with sedentary lifestyle and pharmacological treatment with AM251; HPEXEAM, hyperprotein intervention diet for weight loss with training protocol and pharmacological treatment with AM251. Results are means of eight rats ± SEM depicted by vertical bars or line points. ***P<.001 in the graph showing results from week 12; *P<.05 and **P<.01 in the graphs showing comparisons within the same experimental group between weeks 15 and 21; A,B,C, bars with different letters denote significant differences (P<.05) among experimental groups on week 15; a–c, bars with different letters denote significant differences (P<.05) among experimental groups on week 21.

pronounced in oleic acid and a concomitant significant increase was found in Oleoyl-CoA activity (oleic acid/stearic acid ratio).

At the end of the three-week intervention period (week 15) the modifications induced by obesity in total saturated, mono and polyunsaturated fatty acids as well as those in stearic, oleic, linoleic, arachidonic and DHA were overturned by caloric restriction. Palmitic acid was significantly lower both compared to CHC and even CNOR groups. In addition, changes due to caloric restriction were maintained by the training protocol. AM251 administration potentiated the effects of caloric restriction and physical exercise on mono and polyunsaturated fatty acids, specifically on oleic, linoleic, arachidonic and DHA, lessening palmitic acid content. Obesity-induced changes in Δelongase/desaturase ratio and Oleoyl-CoA activity were also reversed by caloric restriction and exercise and potentiated by AM251.

After the 6-week post-intervention maintenance period (week 21) (caloric restriction plus normocaloric maintenance diet), the following changes compared to the CHC group were induced by caloric restriction and exercise, and potentiated by AM251 administration: an increase in the percentage of total saturated and polyunsaturated fatty acids that was especially evident for stearic, arachidonic or DHA, a higher Δelongase/desaturase ratio, and a significant decrease in monounsaturated fatty acids that was especially pronounced in oleic acid and caused a concomitant decrease in Oleoyl-CoA activity.

3.5. Hepatic gene expression of lipogenic and lipolytic enzymes

DIO and different weight loss interventions critically affected hepatic gene expression of enzymes related to lipogenesis (*Fasn*, *G6pd*, *Scd-1*, *Ldlr*, *Gpat2*, *Hmgcr*), and lypolisis (*Cpt1a*, *Lpl*, *Lipc*, *Cyp7a1*, *Acox1*, *Ucp2*), although the effects on the transcription factors *Srebf1* and *Ppara*, known to control the expression of the former enzymes, were not significant and only trends to decrease the expression of *Srebf1* and increase that of *Ppara* by the HFD were observed (Fig 5 and Table 4). Regarding the individual lipogenic and lipolytic enzymes studied, there was a general inhibitory action of HFD consumption on the expression of lipogenic enzymes along the experimental period (12, 15 and 21 weeks). Such inhibitory action was partially or completely neutralized by the different weight-loss interventions with the exception of *G6pd* on week 15. Overall, intervention-derived effects were more pronounced at 21 weeks when compared to 15 weeks of experimental period.

Dietary induction of obesity consistently decreased the expression of *Lipc* along the entire experimental period, while it caused and increased expression of *Cyp7a1* in charge of bile acid synthesis. For the rest of lipolytic enzymes, obesity-induced changes varied within the different experimental periods, such as the decreased expression of *Lpl* on weeks 12 and 21 with no changes on week 15 or the increment in *Ucp2* on week 12. Although obesity-induction effects on the expression of lipolytic enzymes were complex an did not follow an uniform trend, the weight-loss interventions, especially the training protocol, showed a tendency to increase the expression of transcripts coding for lipolytic enzymes although such effect was more pronounced on week 21 (*Lpl*, *Lipc*, *Cyp7a1*, *Acox1*, *Ucp2*) compared to week 15 (*Lpl*, *Lipc*). Interestingly, the activating effects of exercise were considerably potentiated by AM251 administration at either one of the previously mentioned time points. Furthermore, there was a specific inducing effect of AM251 on the expression of *Lpl*, *Acox*, and *Ucp2* on week 15, and all the lipolytic enzymes on week 21. All these findings point out to the fact that the effects of the weight-loss interventions in hepatic gene expression of lipogenic and lipolytic enzymes were maintained and, in some cases, increased during the 6-week post-intervention stage.

The interactions among DIO and the three weight loss interventions tested in our rat model (caloric restriction, physical exercise, and AM251 administration) with regard to the expression of different transcripts related to lipid metabolism, are summarized in the heat map shown in Fig 6. Taking CNOR12 as control baseline group, time-dependent (12, 15 or 21 weeks) as well as intervention-related effects were observed in grouping of gene expression results. The three stronger factors affecting grouping were obesity induction, caloric restriction/physical exercise (that grouped together with the control CNOR experiments), and AM251administration. Most lipolytic genes

Table 2
Semi quantitative scoring system to assess the range of histological features of NAFLD in liver from Sprague–Dawley rats at different stages of the experimental period

	Item	Definition	Score	CNOR 12W	CNOR 15W	CNOR 21W	CHC 12W	CHC 15W	CHC 21W	Intervention 15W	Intervention 21W
STEATOSIS	**Grade**	<5%	0	**0**	**0**	**0**	**2**	**3**	**3**	**1**	**1**
	Low- to medium power of parenchymal involvement by steatosis	5%-33%	1								
		33%-66%	2								
		>66%	3								
	Location	Zone 3 (Centrilobular)	0	**-**	**-**	**-**	**3**	**3**	**3**	**2**	**2**
	Predominant distribution pattern	Zone 1 (Periportal)	1								
		Azonal	2								
		Panacinar	3								
	Microvesicular steatosis	Absent	0	**0**	**0**	**0**	**1**	**1**	**1**	**0**	**0**
	Contiguous patches	Present	1								
INFLAMMATION	**Lobular inflammation**	No foci	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
	Overall assessment of all inflammatory foci	<2 foci per 200× field	1								
		2–4 foci per 200× field	2								
		>4 foci per 200× field	3								
	Microgranulomas	Absent	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
	Small aggregates of macrophages	Present	1								
	Large Lipogranulomas	Absent	0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	Usually in portal areas or adjacent to central veins	Present	1								
	Portal Inflammation	None to minimal	0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	Assessed from low magnification	Greater than minimal	1								
LIVER CELL INJURY	**Ballooning or**	None	0	**0**	**0**	**0**	**1**	**1**	**1**	**0**	**0**
	Ballooning degeneration	Few balloon cells	1								
		Many cells/prominent ballooning	2								
	Acidophil bodies	None to rare	0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
		Many	1								
	Pigmented macrophages	None to rare	0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
		Many	1								
	Megamitochondria	None to rare	0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
		Many	1								
OTHER FINDINGS	**Mallory's hyaline or Mallory bodies**	None to rare	0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	Visible on routine stains	Many	1								
	Glycogenated nuclei or	None to rare	0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	Glycogen nuclei	Many	1								
	Contiguous patches										
FIBROSIS	**Stage**	None	0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
		Persinusoidal or periportal	1								
		Mild, zone 3, perisinusoidal	1A								
		Moderate, zone 3, persinusoidal	1B								
		Portal/periportal	1C								
		Persinusoidal and portal/periportal	2								
		Bridging fibrosis	3								
		Cirrhosis	4								
NAS Score (Steatosis Grade + Lobular inflammation + Ballooning) =				**1**	**1**	**1**	**4**	**5**	**5**	**2**	**2**

CNOR, Control standard rat chow diet; CHC, Control hypercaloric diet for dietary induction of obesity; Intervention: caloric restriction, physical exercise, AM251 administration.

were up-regulated by the different interventions, whereas such effects were much less pronounced in lipogenic ones.

3.6. Liver functionality and antioxidant activity

Plasma markers of liver damage such as ALT and AST activities were significantly increased by HFD treatment along the 12, 15 and 21 weeks of experimental period (Table 5), and decreased as a result of the weight loss interventions of caloric restriction and its combination with AM251 administration on week 15, but not by the training protocol. A similar trend towards intervention-related decrease in plasma ALT and AST activities was observed on week 21. Gene expression of *Nfe2l2* was decreased in obese animals on weeks 12 and 21, but not on week 15, whereas differential effects of the weight loss and post-intervention maintenance were observed, with a slight increase in expression caused by the combination of caloric restriction and the training protocol only in the animals that were not administered AM251. Catalase activity was reduced by nearly 50% after 15 or 21 weeks of HFD administration, and such decrease was reversed by caloric restriction associated or not to physical exercise. Nevertheless, the highest impact in catalase activity was exhibited by AM251 administration to sedentary animals, which caused a 60–63% increase compared to CNOR groups. Interestingly, implementation of a mixed training protocol in the AM251-administered animals led to a significant decrease in catalase activity *vs* their sedentary counterparts and resulted in values close to the control normocaloric groups.

Lipid peroxidation assessed by the development of thiobarbituric acid reactive substances tended to be increased by the dietary induction of obesity, but results were only significant at week 15 of the experimental period. All the weight loss interventions had a reducing effect on this parameter but the most efficient appeared to be the combination of caloric restriction, training protocol and AM251 administration.

4. Discussion

Obesity is an expanding disease related to metabolic alterations like hyperlipidemia, insulin resistance or the development of NAFLD.

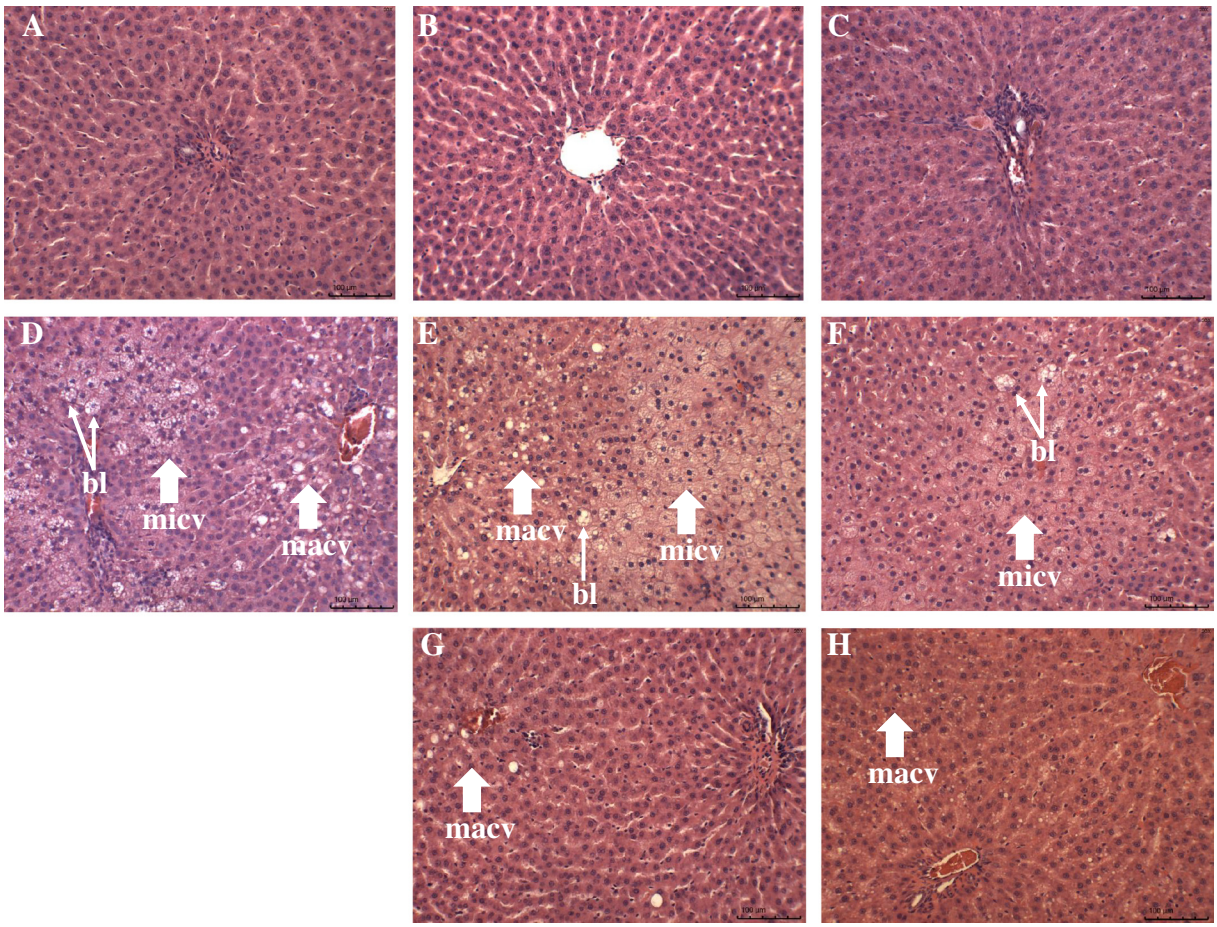

Fig. 4. Effect of dietary induction of obesity and weight loss interventions on liver histology (hematoxylin-eosin stain) of Sprague–Dawley rats (200×). A, B, C, CNOR, control standard rat chow diet (12, 15 and 21 weeks); D, E, F, CHC, control hypercaloric diet for dietary induction of obesity (12, 15 and 21 weeks); G, weight loss interventions (caloric restriction, physical exercise, AM251 administration, week 15); H, post-intervention maintenance period (normocaloric diet, physical exercise, AM251 administration, week 21). Photographs are representative of livers of 8 different rats for each experimental group. bl, cell ballooning, macv, macrovesicular steatosis, micv, microvesicular steatosis.

Table 3
Hepatic fatty acid profile (%) of Sprague–Dawley rats at different stages of the experimental period

	Palmitic (C16:0)	Palmitoleic (C16:1)	Stearic (C18:0)	Oleic (C18:1n9)	Linoleic (C18:2n6)	Linolenic (C18:3n3)	Arachidonic (C20:4n6)	DHA (C22:6n3)	Octadecenoic (C18:1n7)	Δelongase/ desaturase	Oleoyl-CoA	SAT	MON	POL
12 weeks														
CNOR	22.0	0.92 (0.17)	14.2	8.66	25.6	nd	21.9	2.60	3.76	0.87	0.62	36.3	13.4	53.9
CHC	23.3	nd	8.55***	29.88***	27.6	nd	7.72***	1.26**	1.73***	0.28***	4.06***	31.9*	31.6***	38.3***
SEM	1.45	-	1.18	1.18	1.29	-	2.04	0.41	0.19	0.089	0.76	1.92	1.26	2.46
15 weeks														
CNOR	20.9b	1.18	13.6b	7.07a	23.4b	0.04 (0.03)	26.1b	3.56b	4.09a	1.13b	0.53a	34.6b	13.0a	58.6c
CHC	21.1b	nd	5.42a	30.99d	32.2c	nd	6.65 a	1.31a	2.32b	0.20a	5.17c	26.5a	31.7c	41.1a
HPSED	16.9a	nd	17.3b	18.31bc	18.4ab	nd	23.6b	3.31b	2.86a	1.39b	1.16ab	34.2b	20.6ab	47.5ab
HPEXE	16.8a	nd	14.7b	21.09c	21.9b	nd	20.5b	3.45b	1.64b	1.07b	1.94b	31.5ab	22.7bc	49.2bc
HPSEDAM	14.3a	0.39a	18.3b	11.13ab	14.7a	nd	34.2c	5.08b	2.03a	2.36c	0.63a	32.5b	13.6a	55.9cd
HPEXEAM	14.7a	0.01a	15.8b	13.68abc	19.4ab	nd	29.6bc	4.66b	1.87a	1.64bc	0.91ab	30.5ab	15.6ab	55.5cd
SEM	1.27	0.186	1.64	3.18	1.93	-	3.11	0.74	0.26	0.26	0.47	1.61	3.08	2.43
21 weeks														
CNOR	22.9c	1.91bc	10.8ab	11.40ab	29.8c	0.40	17.0ab	2.18ab	3.55bc	0.58ab	1.13a	33.7b	16.9ab	53.0bc
CHC	19.9abc	nd	7.98a	30.15c	27.2bc	0.11	10.1a	2.08a	1.51a	0.37a	4.64b	27.9a	31.7c	41.0a
HPSED	21.3bc	2.04bc	11.0ab	17.52b	21.7ab	nd	19.3b	3.17abc	4.01c	0.99bc	1.70a	32.3ab	23.6b	48.1ab
HPEXE	18.9ab	2.51c	13.7bc	8.44a	19.9a	nd	29.4c	3.43abc	3.69c	1.49cd	0.64a	32.6ab	14.6a	56.5bc
HPSEDAM	17.9ab	0.92ab	15.0bc	7.70a	19.7a	nd	32.0c	3.93bc	2.88bc	1.66d	0.52a	32.9ab	11.5a	58.5c
HPEXEAM	16.7a	0.57a	15.6c	8.48a	21.9ab	nd	30.0c	4.38c	2.40ab	1.43cd	0.57a	32.3ab	11.5a	58.7c
SEM	1.47	0.42	1.43	2.24	1.83	0.103	2.86	0.57	0.40	0.19	0.58	1.70	2.41	2.89

CNOR, Control standard rat chow diet; CHC, Control hypercaloric diet for dietary induction of obesity; HPSED, hyperprotein intervention diet for weight loss with sedentary lifestyle; HPEXE, hyperprotein intervention diet for weight loss with training protocol; HPSEDAM, hyperprotein intervention diet for weight loss with sedentary lifestyle and pharmacological treatment with AM251; HPEXEAM, hyperprotein intervention diet for weight loss with training protocol and pharmacological treatment with AM251. DHA, Docosahexaenoic; Δelongase/desaturase ratio calculated on the basis of the Arachidonic acid/linoleic acid ratio; Oleoyl-coA activity (SCD) calculated using the oleic acid/stearic acid ratio as described by Martinez-Torres et al. [32]; SAT, total saturated fatty acids; MON, total monounsaturated fatty acids; POL, total polyunsaturated fatty acids. Results are means of 8 rats. SEM, standard error of the mean. $^{**}P<.01$, $^{***}P<.001$; nd: non-detected; a–d, means within the same column at each stage of the experimental period (12, 15 or 21 weeks) with different letters differ significantly ($P<.05$).

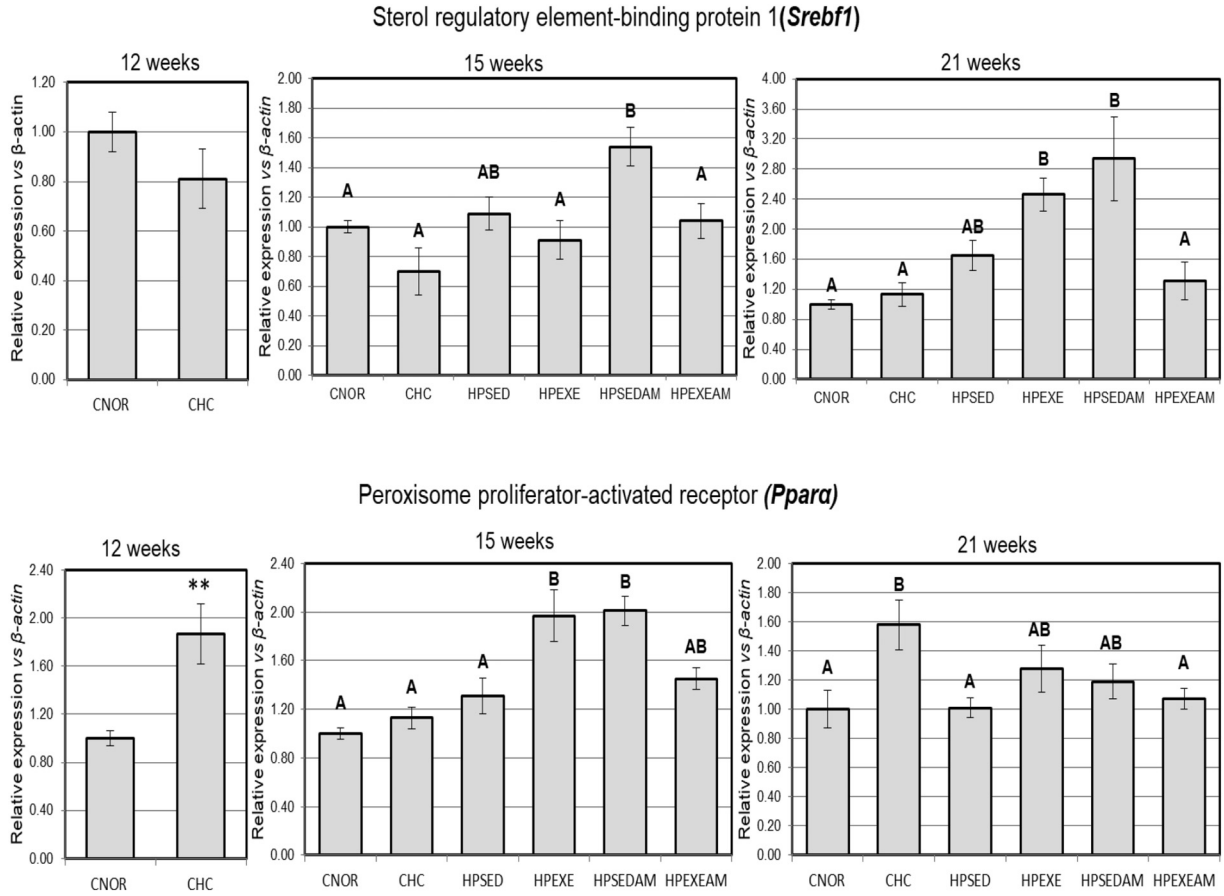

Fig. 5. Gene expression of lipogenic and lipolytic transcription factors SREBP1c and PPARα at the end of each experimental stage (diet-induced obesity, weight-loss intervention, lost weight maintenance). Results are expressed relatively *vs* β-actin. CNOR, control standard rat chow diet; CHC, control hypercaloric diet for dietary induction of obesity; HPSED, hyperprotein intervention diet for weight loss with sedentary lifestyle; HPEXE, hyperprotein intervention diet for weight loss with training protocol; HPSEDAM, hyperprotein intervention diet for weight loss with sedentary lifestyle and pharmacological treatment with AM251; HPEXEAM, hyperprotein intervention diet for weight loss with training protocol and pharmacological treatment with AM251. Results are means of eight rats ± SEM depicted by vertical bars. **P*<.05, ***P*<.01; A,B,C, bars with different letters denote significant differences (*P*<.05).

Different lifestyle and pharmacological interventions can be combined to achieve a more efficient weight loss and avoid the rebound effects on body weight that are usual after many weight control treatments. The aim of this study was to test how different body weight loss strategies (i.e. caloric restriction, physical exercise, and a pharmacological treatment with a food-intake inhibitor), could reverse different obesity-induced alterations in body weight and hepatic lipid metabolism that lead to the development of NAFLD, paying especial attention to the potential molecular mechanisms involved. In addition to the weight-loss intervention period, we assayed a post-intervention phase of body weight maintenance aimed to avoid the post-treatment rebound effects. After consuming the HFD for 12 weeks, obesity was observed in line to marked changes in hepatic morphology and lipid metabolism associated to an altered hepatic acid profile and decreased gene expression of lipogenic enzymes, together with the appearance of plasma markers of hepatic injury, increased liver lipid peroxidation, and modifications in antioxidant enzyme activity. Body weight, alterations in liver morphology, fatty acid profile, gene expression of lipogenic enzymes, plasma markers of hepatic injury and hepatic lipid peroxidation were partial or totally reversed by caloric restriction for three weeks in combination with a mixed training protocol and/or AM251 administration, whereas multiple and complex interactions were observed in antioxidant enzyme activity. Body weight as well as the beneficial effects attained by the weight-loss interventions in the above mentioned parameters were maintained along the 6-week post-intervention treatment with normocaloric diet, physical exercise and once-a-week administration of CB1 receptor blocker.

Under our experimental conditions, HFD consumption was effective to induce obesity in an experimental model of SD rat in agreement to what has been previously reported [38]. The induction of obesity was especially effective during the first weeks of experimental period, after which a marked decrease in food:gain ratio was observed. On the other hand, weight loss was associated to a lower caloric intake, particularly during the first week of intervention period. It is well stablished in humans that a sustained reduction in energy intake is necessary for significant weight loss and such caloric restriction is often facilitated by a reduced fat intake and increased fiber in the diet [23]. Under our experimental conditions, the observed caloric restriction can be attributed to the lower fat content combined with the satiating effect of high levels of soluble dietary fiber present in the intervention diet (10%), the anorexigenic action of physical exercise, and the inhibitory action on food intake exhibited by AM251. In addition, rodent DIO models have been described to exhibit leptin resistance associated with higher circulating levels of leptin derived from excess adipose tissue activity [17,39,40], and such resistance could be lessened upon termination of HFD treatment, thus potentiating the leptin anorexigenic action. Furthermore, Tam et al. [41] have reported that blockade of CB1 receptors in obese mice restored sensitivity to endogenous leptin, which in turn triggered hypophagia via re-activation of melanocortin signaling in hypothalamus arcuate nucleus. Nevertheless, the inhibitory action of the different interventions on food intake, and, specifically, of AM251 administration, was very intense during the first week of weight-loss intervention but lessened its effects during the two-following ones. In this regard,

Table 4
Hepatic gene expression of lipogenic and lipolytic enzymes.

		Fasn	Ldlr	G6pd	Scd1	Gpat2	Hmgcr
12 W	**CNOR**	1.00 (0.05)	1.00 (0.05)	1.00 (0.01)	1.00 (0.28)	1.00 (0.01)	1.00 (0.04)
	CHC	0.29 (0.06)***	0.65 (0.09)*	0.39 (0.04)***	0.41 (0.11)	0.49 (0.09)*	0.36 (0.08)***
15 W	**CNOR**	1.00 (0.09) b	1.00 (0.09) d	1.00 (0.02) c	1.00 (9.88) a	1.00 (0.01) abc	1.00 (0.05) ab
	CHC	0.12 (0.01) a	0.20 (0.02) a	0.13 (0.02) ab	0.07 (0.02) a	0.67 (0.08) a	0.12 (0.02) a
	HPSED	1.05 (0.17) b	0.48 (0.02) bc	0.12 (0.01) ab	0.29 (0.10) a	0.75 (0.08) ab	2.92 (0.54) c
	HPEXE	0.60 (0.10) b	0.42 (0.07) abc	0.08 (0.01) a	0.07 (0.02) a	0.78 (0.09) ab	2.14 (0.26) bc
	HPSEDAM	0.95 (0.14) b	0.65 (0.07) c	0.17 (0.02) b	5.92 (2.33) a	1.28 (0.27) bc	1.68 (0.47) abc
	HPEXEAM	0.66 (0.09) b	0.36 (0.03) ab	0.11 (0.01) ab	2.27 (0.88) a	1.32 (0.27) c	2.63 (0.63) c
21 W	**CNOR**	1.00(0.08) a	1.00 (0.09) b	1.00 (0.02) ab	1.00 (0.75) ab	1.00 (0.01) ab	1.00 (0.04) ab
	CHC	0.20 (0.04) a	0.30 (0.05) a	0.51 (0.02) a	0.06 (0.01) a	0.33 (0.05) a	0.33 (0.06) a
	HPSED	1.61 (0.20) bc	1.21 (0.18) b	1.18 (0.31) ab	4.74 (0.82) b	1.18 (0.09) ab	2.77 (0.36) bc
	HPEXE	6.06 (0.62) d	1.07 (0.12) b	1.32 (0.14) b	11.61 (1.79) c	1.41 (0.19) b	4.90 (0.42) cd
	HPSEDAM	2.62 (0.05) c	0.94 (0.11) b	0.77 (0.08) ab	3.71 (1.26) ab	1.39 (0.32) b	4.11 (0.43) cd
	HPEXEAM	1.14 (0.22) ab	0.77 (0.08) b	1.00 (0.14) ab	2.19 (0.37) ab	3.00 (0.44) c	5.56 (1.04) d
		Cpt1a	**Lpl**	**Lipc**	**Cyp7a1**	**Acox**	**Ucp2**
12W	**CNOR**	1.00 (0.09)	1.00 (0.01)	1.00 (0.11)	1.00 (0.04)	1.00 (0.32)	1.00 (0.02)
	CHC	0.93 (0.08)	0.42 (0.10)***	0.48 (0.06)*	2.75 (0.40)***	1.57 (0.22)	2.42 (0.25)**
15W	**CNOR**	1.00 (0.15) a	1.00 (0.02) a	1.00 (0.13) ab	1.00 (0.17) a	1.00 (0.15) a	1.00 (0.02) a
	CHC	0.98 (0.08) a	1.00 (0.11) a	0.79 (0.05) ab	2.51 (0.30) a	1.17 (0.12) a	0.56 (0.06) a
	HPSED	1.21 (0.14) a	0.95 (0.14) a	0.80 (0.08) ab	6.47 (0.85) c	1.06 (0.11) a	1.03 (0.15) a
	HPEXE	1.00 (0.14) a	1.11 (0.12) a	0.90 (0.12) ab	2.86 (0.44) ab	1.05 (0.09) a	0.86 (0.08) a
	HPSEDAM	0.92 (0.22) a	2.84 (0.69) a	0.52 (0.12) a	2.48 (0.46) a	1.55 (0.60) a	0.91 (0.17) a
	HPEXEAM	1.66 (0.38) a	4.61 (1.05) b	1.35 (0.25) b	6.30 (1.73) bc	3.25 (0.53) b	1.91 (0.37) b
21W	**CNOR**	1.00 (0.10) a	1.00 (0.02) ab	1.00 (0.04) a	1.00 (0.06) a	1.00 (0.14) a	1.00 (0.01) a
	CHC	0.63 (0.07) a	0.53 (0.08) a	0.59 (0.05) a	2.95 (0.36) ab	0.67 (0.08) a	0.62 (0.09) a
	HPSED	0.84 (0.13) a	0.60 (0.10) ab	1.27 (0.12) a	4.87 (0.99) ab	0.58 (0.03) a	0.80 (0.09) a
	HPEXE	0.79 (0.05) a	0.66 (0.08) ab	1.53 (0.16) a	8.74 (1.35) ab	0.95 (0.04) a	1.28 (0.11) a
	HPSEDAM	1.55 (0.21) a	1.66 (0.37) b	1.50 (0.18) a	12.43 (4.56) b	2.23 (0.36) a	3.05 (0.33) b
	HPEXEAM	4.62 (1.09) b	3.20 (0.41) c	2.82 (0.62) b	12.79 (3.63) b	5.13 (1.14) b	6.29 (0.89) c

Gene expression data are expressed relatively *vs* β-actin for *Fasn*, fatty acid synthase; *Ldlr*, low density lipoprotein receptor; *G6pd*, glucose 6 phosphate dehydrogenase; *Scd1*, stearoyl-CoAdesaturase-1; *Gpat2*, glycerol-3-phosphate acyl transferase 2; *Hmgcr*, 3-hydroxy-3-methyl-glutaryl-CoA reductase; *Cpt1a*, carnitine palmitoyl transferase 1A; *Lpl*, lipoprotein lipase; *Lipc*, hepatic triacylglycerol lipase; *Cyp7a1*, cholesterol 7 alpha-hydroxylase; *Acox1*, peroxisomal acyl-CoA oxidase; *Ucp2*, uncoupling protein 2; CNOR, Control standard rat chow diet; CHC, Control hypercaloric diet for dietary induction of obesity; HPSED, hyperprotein intervention diet for weight loss with sedentary lifestyle; HPEXE, hyperprotein intervention diet for weight loss with training protocol; HPSEDAM, hyperprotein intervention diet for weight loss with sedentary lifestyle and pharmacological treatment with AM251; HPEXEAM, hyperprotein intervention diet for weight loss with training protocol and pharmacological treatment with AM251; W, week. Results are means of 8 rats, data in parenthesis correspond to the standard error of the mean. *P<.05, **P<.01, ***P<.001; a–d, means within the same column at each stage of the experimental period (12, 15 or 21 weeks) with different letters differ significantly (P<.05).

the anorexigenic action of the drug has been shown to decrease after 2–3 weeks of administration [13,20,21] and can be attributed to the development of tolerance. In addition, other factors such as the initial stress induced by daily handling of the animals until they became used to drug administration or toxicity of the drug or vehicle, might also be involved and would constitute a limitation to our study.

Interestingly, body weight lost during the 3-week intervention was not regained during the 6-week post-intervention period. At this later stage of the experiment, a normocaloric dietary pattern was implemented, slightly decreasing the amount of food consumed in order to avoid a rebound effect. During this maintenance period, the physical exercise was continued to implement a healthy lifestyle habit, and the pharmacological intervention was decreased to a minimum of once a week administration. The implementation of the three interventions (two of them at a lower scale compared to the 3-week weight-loss stage) was efficient at maintaining the benefits of our weight-loss program for a long-term post-intervention period, either related to body weight or metabolic and liver functionality.

HFD intake induced obvious signs of NAFLD characterized by steatosis and liver histological alterations that run in parallel to considerable changes in hepatic fatty acid profile. The NASH grading score gave a NAS score value of 4–5 based on the semiquantitative evaluation of steatosis, lobular inflammation, hepatocellular ballooning and fibrosis, which are exhibited by other experimental models of NAFLD such as the Zucker rat [42]. HFD-derived steatosis and histological lesions have been reported by Chen et al. [30], who found that liver morphology of SD rats fed for 16 weeks a HFD showed more yellow color and larger size together with azonal steatosis described as a mix of macro and microvesicular steatosis and few ballooning damage compared to the control normocaloric diet-fed rats. An interesting finding of our research work is that caloric restriction implemented for a 3-week intervention period was efficient at reversing not only liver weight and total fat accumulation, but also most of hepatic histological alterations, specifically macro and microvesicular steatosis as well as ballooning, decreasing the NAS score to a value of 1–2. In addition, the benefits of intervention were maintained during the post-intervention lost-weight maintenance period in which the NAS score index to measure the development of NAFLD remained the same as that obtained right after the weight-loss intervention, and consistently lower than the CHC control.

Steatotic lesion induced by HFD can be strongly associated to the toxicity of certain fatty acids. Palmitic acid has shown strong lipotoxic action in either *in vitro* models of HepG2 or primary hepatocyte culture or *in vivo* high-fat DIO, causing not only the standard hepatomegaly and alterations in hepatic histology, but also the development of inflammation, endoplasmic reticulum stress and apoptosis [30]. The hypercaloric diet used in our study for dietary induction of obesity almost doubled the percentage of palmitic acid compared to its normocaloric control and therefore presented greater lipotoxic potential. However, when the liver fatty acid profile is compared, differences between normo- or hypercaloric groups were hardly noticeable, whereas the percentages of stearic and oleic acid were significantly altered. These changes can be attributed to the activation of elongase desaturase pathways that convert palmitic into stearic acid followed by desaturation to oleic acid as seen by the marked increase in the ratio oleic/stearic paradigmatic of Scd1 activity. Since oleic acid exhibits a much lower lipotoxicity [30], the activation of its synthesis could have a protective action on hepatic viability, although it can also be associated to an easier storage and metabolism compared to palmitic or stearic acids.

Several mechanisms are most likely involved in the effects of the different interventions on hepatic fatty acid profile and may show interesting synergies to strengthen the overall action. On one hand, caloric restriction can modify the hepatic expression of genes involved

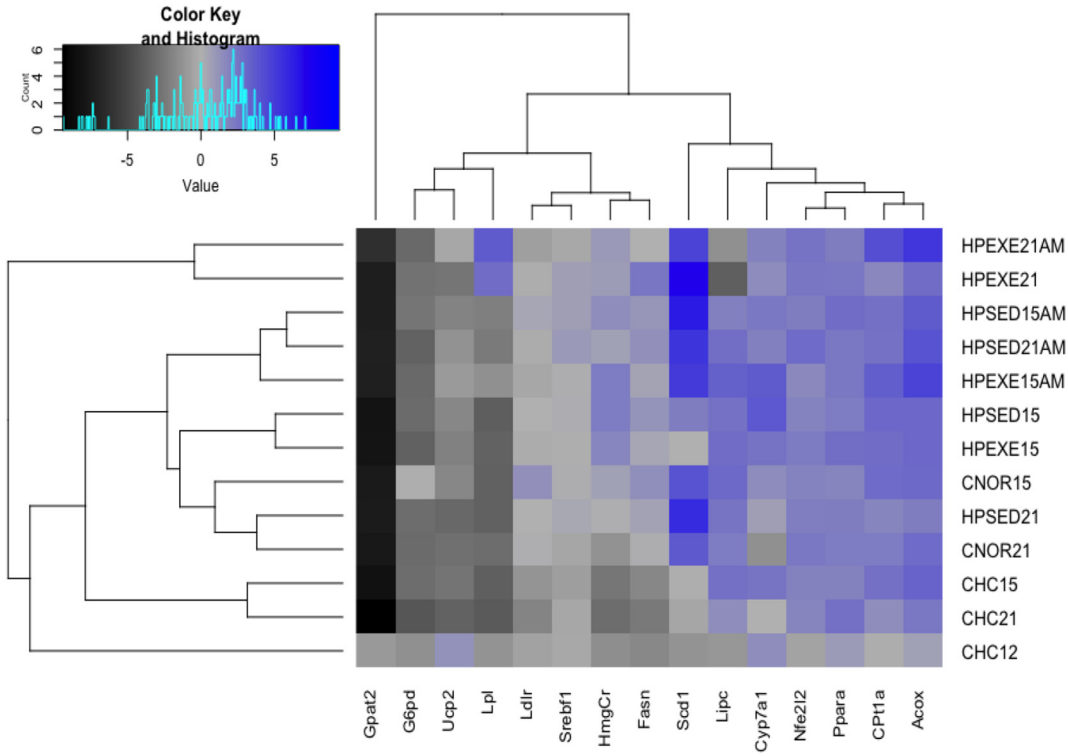

Fig. 6. Heatmap depicting gene expression of hepatic lipogenic and lipolytic enzymes in the different experimental groups at the end of each experimental stage (diet-induced obesity, weight-loss intervention, lost weight maintenance). The graph was generated using the relative mRNA expression values compared to the CNOR12 control group shown as log2 of the values obtained by the 2-(ΔΔct) method using β-actin as housekeeping gene. CNOR, control standard rat chow diet; CHC, control hypercaloric diet for dietary induction of obesity; HPSED, hyperprotein intervention diet for weight loss with sedentary lifestyle; HPEXE, hyperprotein intervention diet for weight loss with training protocol; HPSEDAM, hyperprotein intervention diet for weight loss with sedentary lifestyle and pharmacological treatment with AM251; HPEXEAM, hyperprotein intervention diet for weight loss with training protocol and pharmacological treatment with AM251.

in lipid metabolism to promote lipid utilization through hepatic transcriptional alteration, thus preventing hepatic steatosis [43]. On the other hand, the mixed training protocol during 8 weeks can exert marked beneficial effects in altered hepatic levels of stearic, palmitic, oleic and arachidonic acid through decreased Scd-1 and increased 5-elongase activities in obese animals as reported by Martinez et al. [16]. Here, the combination of caloric restriction and the mixed training protocol tended to decrease Scd-1 activity and the percentage of

Table 5
Markers of liver damage and activity of antioxidant enzymes

	AST (U/L)	ALT (U/L)	γ-GT (U/L)	ALP (U/L)	Mn-SOD (UAA/mg protein)	Cu/Zn-SOD (UAA/mg protein)	CAT (U/mg protein)	GPX (nmol NADPH/min/mg protein)	TBARS (nmol MDA/mg protein)	NFe2l2 expression vs b-actin
12 weeks										
CNOR	69.0	20.2	2.72	109.0	29.5	555.7	16.8	12.7	1.31	1.00 (0.10)
CHC	92.1*	31.6*	0.58**	133.1	33.0	501.7	15.7	10.3	1.85	0.67 (0.07)**
SEM	9.60	4.58	0.68	13.1	7.04	90.5	1.67	2.15	0.33	-
15 weeks										
CNOR	58.3a	30.5a	5.08d	88.4b	35.7ab	548.8bc	17.2b	5.78a	1.88a	1.00 (0.07) ab
CHC	178.9b	74.6b	0.15a	78.8ab	39.5bc	416.8a	10.6a	11.2c	8.40b	1.05 (0.07) ab
HPSED	86.1a	48.5ab	2.46ab	78.0ab	29.2a	624.6c	21.5b	6.15a	1.26a	1.01 (0.07) ab
HPEXE	94.9a	41.6a	2.62bc	51.6a	41.7bc	524.0b	19.9b	10.3bc	1.67a	1.33 (0.08) a
HPSEDAM	63.9a	35.6a	4.83cd	90.0b	47.8c	475.8ab	28.4c	7.91ab	1.00a	1.20 (0.16) ab
HPEXEAM	66.6a	34.7a	4.96d	87.7b	40.2bc	452.0ab	20.9b	10.1bc	0.78a	0.85 (0.02) b
SEM	15.4	9.27	0.77	12.5	3.23	33.2	2.03	0.93	1.10	-
21 weeks										
CNOR	146.2ab	38.9a	0.43a	117.2ab	27.5a	367.4ab	15.9ab	6.94a	2.01bc	1.00 (0.10) a
CHC	181.9b	50.5b	0.74a	130.3b	26.4a	386.4ab	8.82a	3.36a	2.42c	0.73 (0.08) a
HPSED	64.7a	31.9a	2.59b	96.6ab	47.3bc	592.1c	21.6b	17.8c	1.99bc	0.89 (0.07) a
HPEXE	89.6ab	27.8a	4.32bc	77.1a	39.7b	626.7c	22.9b	16.8c	1.90bc	1.20 (0.08) a
HPSEDAM	99.8ab	31.8a	4.67c	82.2a	52.0cd	456.6b	33.4c	13.0b	1.13ab	1.53 (0.41) a
HPEXEAM	56.4a	27.6a	4.87c	79.1[a]	58.0d	342.0a	17.9b	11.3b	0.70a	1.26 (0.25) a
SEM	33.0	3.90	0.62	15.0	2.75	30.5	2.63	1.16	0.36	-

AST, aspartate amino transferase; ALT, alanine amino transferase; γ-GT, γ-glutamyl transferase; ALP, alkaline phosphatase; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; TBARs, thiobarbituric acid reactive substances; CNOR, Control standard rat chow diet; CHC, Control hypercaloric diet for dietary induction of obesity; HPSED, hyperprotein intervention diet for weight loss with sedentary lifestyle; HPEXE, hyperprotein intervention diet for weight loss with training protocol; HPSEDAM, hyperprotein intervention diet for weight loss with sedentary lifestyle and pharmacological treatment with AM251; HPEXEAM, hyperprotein intervention diet for weight loss with training protocol and pharmacological treatment with AM251. Results are means of eight rats. SEM, standard error of the mean. *P<.05, **P<.01; a,b,c,d, means within the same column at each stage of the experimental period (12, 15 or 21 weeks) with different letters differ significantly (P<.05).

palmitic, oleic, and linoleic acids, while increasing those of stearic and arachidonic both during the weight-loss and the maintenance period. Another interesting finding is the effect of the interventions on the recovery and increment of DHA levels, given that this fatty acid is the precursor of different mediators that counter-regulate the persistent inflammatory status which is usually associated to the development of obesity [44].

Knowledge about the effect of CB1 receptor blockers on liver fatty acid profile and gene expression of lipogenic/lypolitic enzymes is scarce. Previously, we detected that daily AM251 administration at a dose of 3 mg/kg for a period of 21 days decreased hepatomegalia, steatosis and plasma levels of transaminases, bilirubin, total and LDL cholesterol in obese Zucker rats [20]. In the present study, its strong action on oleoyl-CoA and Δelongase/desaturase activities, which led to significant changes in mono and polyunsaturated fatty acids, appeared of especial relevance. The influence of endocannabinoid system in the lipogenesis pathway has been reported by different authors [45,46] who emphasized that endocannabinoid activation of CB1 receptor induced glucose intolerance, hepatic steatosis and novel CB1-responsive genes. In contrast, CB1 receptor blockade has been shown to cause an inhibitory action on lipogenesis by hepatocyte and peripheric adipose tissue [47,48]. Furthermore, Vida et al [48] described a significant inhibition of SREBP1c and CHREBP expression in animals fed a HFD as well as significant CB1-mediated signaling of lipogenic genes that were down-regulated in normocaloric but not HFD-fed rats after AM251 administration. On the other hand, Chen et al. [49] have recently identified the main proteomic changes in the liver of mice fed a HFD after daily administration of AM251 for 7 days at a dose of 5 mg/kg. The authors described alterations in the expression of proteins involved in glucose, lipid, and xenobiotic metabolism, as well as on mitochondrial process of ATP synthesis, and concluded that blockade of CB1 receptor may contribute to the improvement of mitochondrial function in hepatic steatosis. In our experiment, the blockade of CB1 contributed to the normalization of hepatic lipid content and metabolism through normalization in the expression of lipogenic enzymes and significant up-regulation of the lipolytic transcripts. In this regard, the association of physical exercise and AM251 administration was especially effective, specifically on the expression of *Cyp7a1*, an enzyme involved in bile acid formation that reached a 12-fold increase, or the sixfold increase in the expression of *Ucp2* at 21 weeks.

DIO models have been usually associated to increased hepatic lipid peroxidation, decreased antioxidant enzyme activities, and compromised oxidative stress status [50,51]. Under our present experimental conditions, obesity caused a general decrease in the expression of nuclear transcription factor *Nrf2* and the activity of antioxidant enzymes, especially Cu/Zn-SOD, CAT and GPX, with a concomitant increase in the lipid peroxidation marker TBARs. The combined interventions appeared to differentially counter the obesity-related alterations in liver antioxidant activity, although several interactions depending on the experimental period (12, 15 or 21 weeks) should be pointed out. Among the most striking, we found exercise-AM251 interaction on Mn-SOD or catalase activity. Mendes et al. [52] have reported enhancements in hepatic antioxidant status caused by caloric restriction-derived weight loss in HFD-induced NAFLD model in mouse. Specifically, the gene and protein expression of *Nrf2*, catalase, SOD, GPX and GR were improved by the intervention.

In conclusion, the body weight control strategies assayed achieved the goal not only of decreasing body weight during the intervention period, but also to limit body weight regain during the post-intervention stage, avoiding the rebound effect common to weight control treatments. In this phase of lost weight maintenance, the training protocol played a crucial role. As for the weight-loss treatments, the three interventions assayed proved to be efficient, although caloric restriction exhibited a stronger effect to which the training protocol combined or not with CB1 receptor blockade had a synergistic action. The different interventions were also efficient to reverse the obesity-associated alterations in hepatic lipid metabolism through the improvement of steatosis and balancing of hepatic fatty acid profile mediated by the up-regulation in expression of lipogenic and lipolytic enzymes, the latter mainly during the post-intervention maintenance period. During this later stage, maintenance of body weight within adequate limits was reflected in sustained or even increased benefits on hepatic morphology and functionality. The strong influence shown by the endocannabinoid system on hepatic lipid metabolism was clearly modulated by the administration of the CB1 receptor blocker either by itself or combined with the training protocol, showing potential for the design and development of new therapeutic strategies for the treatment of NAFLD.

Author contributions

R. M., M. L.-J., P. A., J. M. P and M. G. designed the experimental model; G. K., R. M., L. L. T., J. M. P., and M. G conducted research; R. M., G. K., L. L. T., F. A., and M. G. analyzed data; J. M. P., M. G., R. M., and M. L. J. drafted and revised the paper, and all authors read and approved the final manuscript.

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Declaration of competing interest

No conflicts of interest, financial or otherwise, are declared by the authors.

References

[1] Huang TD, Behary J, Zekry A. Non-alcoholic fatty liver disease (NAFLD): a review of epidemiology, risk factors, diagnosis and management. Intern Med J. 2019. https://doi.org/10.1111/imj.14709.
[2] Hruby A, Manson JE, Qi L, Malik VS, Rimm EB, Sun Q, et al. Determinants and consequences of obesity. Am J Public Health. 2016;106(9):1656–62. https://doi.org/10.2105/AJPH.2016.303326.
[3] Bluher M. Obesity: global epidemiology and pathogenesis. Nat Rev Endocrinol. 2019;15(5):288–98. https://doi.org/10.1038/s41574-019-0176-8.
[4] Gaggini M, Morelli M, Buzzigoli E, De Fronzo RA, Bugianesi E, Gastaldelli A. Non-alcoholic fatty liver disease (NAFLD) and its connection with insulin resistance, dyslipidemia, atherosclerosis and coronary heart disease. Nutrients. 2013;5(5):1544–60. https://doi.org/10.3390/nu5051544.
[5] Jarukamjorn K, Jearapong N, Pimson C, Chatuphonprasert W. A high-fat, high-fructose diet induces antioxidant imbalance and increases the risk and progression of nonalcoholic fatty liver disease in mice. Scientifica. 2016;5029414. https://doi.org/10.1155/2016/5029414.
[6] Takakura K, Oikawa T, Nakano M, Saeki C, Torisu Y, Kajihara M, et al. Recent insights into the multiple pathways driving non-alcoholic steatohepatitis-derived hepatocellular carcinoma. Front Oncol. 2019;9:762. https://doi.org/10.3389/fonc.2019.00762.
[7] Foright RM, Presby DM, Sherk VD, Kahn D, Checkley LA, Giles ED, et al. Is regular exercise an effective strategy for weight loss maintenance? Physiol Behav. 2018;188:86–93. https://doi.org/10.1016/j.physbeh. 2018.01.025.
[8] Giles ED, Steig AJ, Jackman MR, Higgins JA, Johnson GC, Lindstrom RC, et al. Exercise decreases lipogenic gene expression in adipose tissue and alters adipocyte cellularity during weight regain after weight loss. Front Physiol. 2016;7:32. https://doi.org/10.3389/fphys.2016.00032.
[9] Elfhag K, Rossner S. Who succeeds in maintaining weight loss? A conceptual review of factors associated with weight loss maintenance and weight regain. Obes Rev. 2005;6(1):67–85. https://doi.org/10.1111/j.1467-789X.2005.00170.x.
[10] de Oliveira Otto MC, Anderson CAM, Dearborn JL, Ferranti EP, Mozaffarian D, Rao G, et al. Dietary diversity: implications for obesity prevention in adult populations: a science advisory from the American Heart Association. Circulation. 2018;138(11):e160–8. https://doi.org/10.1161/CIR.0000000000000595.

[11] Thorpe MP, Jacobson EH, Layman DK, He X, Kris-Etherton PM, Evans EM. A diet high in protein, dairy, and calcium attenuates bone loss over twelve months of weight loss and maintenance relative to a conventional high-carbohydrate diet in adults. J Nutr. 2008;138(6):1096–10018492840.
[12] Galisteo M, Morón R, Rivera L, Romero R, Anguera A, Zarzuelo A. *Plantago ovata* husks-supplemented diet ameliorates metabolic alterations in obese Zucker rats through activation of AMP-activated protein kinase. Comparative study with other dietary fibers. Clin Nutr. 2010;29:261–7.
[13] Galisteo M, Sánchez M, Vera R, González M, Anguera A, Duarte J, et al. A diet supplemented with husks of *Plantago ovata* reduces the development of endothelial dysfunction, hypertension, and obesity by affecting adiponectin and TNF-alpha in obese Zucker rats. J Nutr. 2005;135(10):2399–404. https://doi.org/10.1093/jn/135.10.2399.
[14] Verheggen RJHM, Maessen MFH, Green DJ, Hermus ARMM, Hopman MPE, Thijssen DHT. A systematic review and meta-analysis on the effects ofexercise training versus hypocaloric diet: distinct effects on body weight and visceral adipose tissue. Obes Rev. 2016;17:664–90. https://doi.org/10.1111/obr.12406.
[15] Coll-Risco I, Aparicio VA, Nebot E, Camiletti-Moirón D, Martínez R, Kapravelou G, et al. Effects of interval aerobic training combined with strength exercise on body composition, glycaemic and lipid profile and aerobic capacity of obese rats. J Sports Sci. 2016;34:1452–60. https://doi.org/10.1080/02640414. 2015.1119296.
[16] Martínez R, Kapravelou G, Donaire A, Lopez-Chaves C, Arrebola F, Galisteo M, et al. Effects of a combined intervention with a lentil protein hydrolysate and a mixed training protocol on the lipid metabolism and hepatic markers of NAFLD in Zucker rats. Food Funct. 2018;9:830–50. https://doi.org/10.1039/c7fo01790a.
[17] Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med. 1995;1:1311–4.
[18] Muller T, Demizieux L, Troy-Fioramonti S, Gresti J. Pais de Barros JP, Berger H, et al. Overactivation of the endocannabinoid system alters the antilipolytic action of insulin in mouse adipose tissue. Am J Physiol Endocrinol Metab. 2017;313(1): E26–36. https://doi.org/10.1152/ajpendo.00374.2016.
[19] Merroun I, Errami M, Hoddah H, Urbano G, Porres JM, Aranda P, et al. Influence of intracerebroventricular or intraperitoneal administration of cannabinoid receptor agonist (WIN 55,212-2) and inverse agonist (AM251) on the regulation of food intake and hypothalamic serotonin levels. Br J Nutr. 2009;101(10):1569–78. https://doi.org/10.1017/S0007114508083530.
[20] Merroun I, Sánchez-González C, Martínez R, López-Chaves C, Porres JM, Aranda P, et al. Novel effects of the cannabinoid inverse agonist AM 251 on parameters related to metabolic syndrome in obese Zucker rats. Metabolism. 2013;62(11): 1641–50. https://doi.org/10.1016/j.metabol.2013.06.011.
[21] Chambers AP, Koopmans HS, Pittman QJ, Sharkey KA. AM251 produces sustained reductions in food intake and body weight that are resistant to tolerance and conditioned taste aversion. Br J Pharmacol. 2006;147(1):109–16.
[22] Sengupta P. A Scientific review of age determination for a laboratory rat: How old is it in comparison with human age? Biomed Int. 2012;2:81–9.
[23] Ramage S, Farmer A, Eccles KA, McCargar L. Healthy strategies for successful weight loss and weight maintenance: a systematic review. Appl Physiol Nutr Metab. 2014;39(1):1–20.
[24] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, Off J Eur Union. 2010:L276/33–79.
[25] Wisløff U, Helgerud J, Kemi OJ, Ellingsen Ø. Intensity-controlled treadmill running in rats: V02max and cardiac hypertrophy. Am J Physiol-Heart Circ Physiol. 2001; 280:H1301–10. https://doi.org/10.1152/ajpheart. 2001.280.3.H1301.
[26] Clemente VJC, Martín S, Porres J, Fuentes S, Ramírez PA. Efecto de la suplementación de vitamina en el rendimiento de una prueba incremental de consumo máximo de oxígeno en ratas Wistar. Arch Med Dep. 2011;XXVIII:168–73.
[27] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem. 1957;226:497–509.
[28] Kapravelou G, Martínez R, Andrade AM, López-Chaves C, López-Jurado M, Aranda P, et al. Improvement of the antioxidant and hypolipidaemic effects of cowpea flours (*Vigna unguiculata*) by fermentation: results of *in vitro* and *in vivo* experiments. J Sci Food Agric. 2015;95:1207–16. https://doi.org/10.1002/jsfa.6809.
[29] Kleiner DE, Brunt EM, Natta Van. M, Behling C, Contos MJ, Cummings OW, et al. Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41(6):1313–21. https://doi.org/10.1002/hep.20701.
[30] Chen X, Li L, Liu X, Luo R, Liao G, Li L, et al. Oleic acid protects saturated fatty acid mediated lipotoxicity in hepatocytes and rat of non-alcoholic steatohepatitis. Life Sci. 2018;203:291–304. https://doi.org/10.1016/j.lfs.2018.04.022.
[31] Lepage G, Roy CC. Direct transesterification of all classes of lipids in a one-step reaction. J Lipid Res. 1986;27(1):114–203958609.
[32] González-Torres L, Matos C, Vázquez-Velasco M, Santos-López JA, Sánchez-Martínez I, García-Fernández C, et al. Glucomannan- and glucomannan plus spirulina-enriched pork affect liver fatty acid profile, LDL receptor expression and antioxidant status in Zucker fa/fa rats fed atherogenic diets. Food Nutr Res. 2016;61(1):1264710. https://doi.org/10.1080/16546628.2017.1264710.
[33] Cohen G, Kim M. Ogwu V.A modified catalase assay suitable for a plate reader and for the analysis of brain cell cultures. J Neurosci Methods. 1996;67:53–6.
[34] Lawrence RA, Sunde RA, Schwartz GL, Hoekstra WG. Glutathione peroxidase activity in rat lens and other tissues in relation to dietary selenium intake. *Exp Eye* Res. 1974;18:563–9.
[35] Ukeda H, Maeda S, Ishii T, Sawamura M. Spectrophotometric assay for superoxide dismutase based on tetrazolium Salt 3′-{1-[(Phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate reduction by xanthine–xanthine oxidase. Anal Biochem. 1997;251:206–9. https://doi.org/10.1006/abio.1997.2273.
[36] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
[37] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95:351–8.
[38] Touati S, Meziri F, Devaux S, Berthelot A, Touyz RM, Laurant P. Exercise reverses metabolic syndrome in high-fat diet-induced obese rats. Med Sci Sports Exerc. 2011;43(3):398–407. https://doi.org/10.1249/MSS.0b013e3181eeb12d.
[39] Shawky NM, Segar L. Sulforaphane improves leptin responsiveness in high-fat high-sucrose diet-fed obese mice. Eur J Pharmacol. 2018;835:108–14. https://doi.org/10.1016/j.ejphar.2018.07.050.
[40] Shin AC, Mohankumar SMJ, Balasubramanian P, Sirivelu MP, Linning K, Woolcock A, et al. Responsiveness of hypothalamo-pituitary-adrenal axis to leptin is impaired in diet-induced obese rats. Nutr Diabetes. 2019;9(1):10. https://doi.org/10.1038/s41387-019-0076-y.
[41] Tam J, Szanda G, Drori A, Liu Z, Cinar R, Kashiwaya Y, et al. Peripheral cannabinoid-1 receptor blockade restores hypothalamic leptin signaling. Mol Metab. 2017;6(10): 1113–25. https://doi.org/10.1016/j.molmet.2017.06.010.
[42] Kapravelou G, Martínez R, Nebot E, López-Jurado M, Aranda P, Arrebola F, et al. The combined intervention with germinated *Vigna radiata* and aerobic interval training protocol is an effective strategy for the treatment of non-alcoholic fatty liver disease (NAFLD) and other alterations related to the metabolic syndrome in Zucker rats. Nutrients. 2017;9(7). https://doi.org/10.3390/nu9070774 pii: E774.
[43] Higami Y, Tsuchiya T, Chiba T, Yamaza H, Muraoka I, Hirose M, et al. Hepatic gene expression profile of lipid metabolism in rats: Impact of caloric restriction and growth hormone/insulin-like growth factor-1 suppression. J Gerontol A Biol Sci Med Sci. 2006;61(11):1099–110. https://doi.org/10.1093/gerona/ 61.11.1099.
[44] López-Vicario C, Rius B, Alcaraz-Quiles J, García-Alonso V, Lopategi A, Titos E, et al. Pro-resolving mediators produced from EPA and DHA: overview of the pathways involved and their mechanisms in metabolic syndrome and related liver diseases. Eur J Pharmacol. 2016;785:133–43. https://doi.org/10.1016/j.ejphar.2015.03.092.
[45] Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Bátkai S, et al. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. J Clin Invest. 2005;115(5): 1298–305. https://doi.org/10.1172/JCI23057.
[46] Ruby MA, Nomura DK, Hudak CS, Barber A, Casida JE, Krauss RM. Acute overactive endocannabinoid signaling induces glucose intolerance, hepatic steatosis, and novel cannabinoid receptor 1 responsive genes. PLoS One. 2011;6(11):e26415. https://doi.org/10.1371/journal.pone.0026415.
[47] Wu HM, Yang YM, Kim SG. Rimonabant, a cannabinoid receptor type 1 inverse agonist, inhibits hepatocyte lipogenesis by activating liver kinase B1 and AMP-activated protein kinase axis downstream of Gα i/o inhibition. Mol Pharmacol. 2011;80(5):859–69. https://doi.org/10.1124/mol.111.072769.
[48] Vida M, Rivera P, Gavito AL, Suárez J, Pavón FJ, Arrabal S, et al. CB1 blockade potentiates down-regulation of lipogenic gene expression in perirenal adipose tissue in high carbohydrate diet-induced obesity. PLoS One. 2014;9(2):e90016. https://doi.org/10.1371/journal.pone.0090016.
[49] Chen C, Lee TY, Kwok C, Hsu Y, Shih K, Lin YJ, et al. Using proteomics to discover novel biomarkers for fatty liver development and response to CB1R antagonist treatment in an obese mouse model. Proteomics. 2017;17(1-2):1600292. https://doi.org/10.1002/pmic.201600292.
[50] Mukthamba P, Srinivasan K. Hypolipidemic and antioxidant effects of dietary fenugreek (*Trigonella foenum-graecum*) seeds and garlic (*Allium sativum*) in high-fat fed rats. Food Biosc. 2016;14:1–9.
[51] Zhu Z, Lin Z, Jiang H, Jiang Y, Zhao M, Liu X. Hypolipidemic effect of Youcha in hyperlipidemia rats induced by high-fat diet. Food Funct. 2017;8(4):1680–7. https://doi.org/10.1039/c7fo00089h.
[52] Mendes IKS, Matsuura C, Aguila MB, Daleprane JB, Martins MA, Mury WV, et al. Weight loss enhances hepatic antioxidant status in a NAFLD model induced by high-fat diet. Appl Physiol Nutr Metab. 2018;43(1):23–9. https://doi.org/10.1139/apnm-2017-0317.