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Sustained exposure to diets with an unbalanced macronutrient proportion alters key genes involved in energy homeostasis and obesity-related metabolic parameters in rats

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Running title. Unbalanced intake of macronutrients in rats

Abstract

We have investigated the effects of long term intake of two unbalanced diets (rich in fat –HF– or protein –HP–) administered in isocaloric conditions to a control balanced diet (pair-feeding) to adult rats. Isocaloric intake of a HF diet did not affect body weight but increased adiposity, liver-fat deposition, and induced insulin resistance. Gene expression changes in liver and adipose tissue (increased lipolytic and decreased lipogenic gene expression) could try to compensate for increased adiposity. HP diet decreased caloric intake, body weight, size of subcutaneous adipocytes, and circulating cholesterol. Higher insulin levels apparently not related to insulin resistance were observed. Changes at gene expression level reflected an adaptation to lower diet carbohydrate content and to the use of amino acids as energy source. Kidney size was increased in HP-fed animals but serum creatinine was not affected. Circulating TNF-alpha levels were higher in both dietary models. Thus, a long-term increase in dietary fat proportion produces alterations related to metabolic syndrome even in the absence of increased body weight, whereas an increase in diet protein content reduces body weight but alters metabolic parameters and kidney size which could be linked to increased risk of suffering different pathologies.

Key words. High-fat diet, high-protein diet, isocaloric diets, energy homeostasis

Introduction

Energy homeostasis regulation and body weight maintenance is of high relevance, as increased adiposity is linked to obesity and related diseases^{1, 2}. Diet macronutrient composition is known to influence energy homeostasis, food intake and body weight in rats and humans³, and an unbalanced proportion of diet macronutrients has been related to several metabolic disorders⁴.

There are still discrepancies in the literature regarding the effects of dietary macronutrient composition on energy homeostasis and body weight control, as well as on the health effects of diets with an unbalanced proportion of macronutrients, mainly due to the duration of the experiments and the number of parameters measured and analyzed⁵. Fat intake has a deep impact on energy metabolism⁶. Particularly, the intake of animal fat is increasing in western diets⁷ and this type of fat is associated to increased adiposity, increased caloric intake, and to the development of disorders related to metabolic syndrome, such as insulin resistance, hypertriglyceridemia and hypercholesterolemia among others^{8, 9}. On the other hand, protein intake is also increasing, especially due to the popularity of high protein diets to decrease and control body weight¹⁰. An increasing body of evidence indicates that diets rich in proteins may improve biomarkers of metabolic health (insulin sensitivity, blood lipid profile)^{7, 11}. However, these diets have also been related to alterations of metabolic parameters in rat and human studies. For example, long-term protein intake has been suspected to promote insulin resistance, development and progression of renal disease and to increase urinary calcium excretion which could be linked to possible bone resorption^{12, 13, 14}.

The aim of this study was to estimate the metabolic consequences of a long-term intake of high fat or high protein diets in adult male Wistar rats by analyzing a wide range of

parameters related to body weight control and metabolic syndrome. We designed an iso-energetic pair-feeding experiment to ensure equal caloric intake between the control and experimental groups, enabling us to analyze the influence of high fat diet in absence of related overeating that would happen in *ad libitum* feeding conditions¹⁵. Adult animals were fed with the different diets for 4 months, which represents a quarter of their life span; it would be difficult to perform a controlled study on an equivalent period in humans.

Methods and Materials

Ethical approval. All experimental procedures followed in this study were reviewed and approved by the Ethical Committee of the University of the Balearic Islands and guidelines for the use and care of laboratory animals of the University were followed.

Animals. The experiments were conducted with 2-month-old male Wistar rats (Charles River Laboratories España, SA, Barcelona, Spain) pair-fed with different experimental diets for 4 months. We chose 2-month-old rats to begin the experiment because our objective was to analyze the effect of the intake of unbalanced diets during adulthood. Animals were single-housed at 22° C with a period of light/dark of 12 hours and were divided into three groups: a control group (n=7), a high-fat group (HF, n=7) and a high-protein group (HP, n=6). Control animals were fed a normolipidic diet (D12450B, Research Diets) containing 70% of energy (Kcal) from carbohydrate, 10% from fat and 20% from protein. HF animals were fed a high-fat diet (D12492, Research Diets) containing 20% of energy from carbohydrate, 60% from fat (40% saturated and 60% unsaturated fat) and 20% from protein. HP animals were fed a high-protein diet (Research Diets) containing 45% of energy from carbohydrates, 10% from fat and 45% from proteins (mainly casein). Diets were purchased from Brogaarden (Gentofte, Denmark). During the experimental trial, food was administered isocalorically. The HF and the HP groups received an equal amount of Kcal to the amount consumed by the control group the day before. We proceeded as follows: animals in the control group had free access to food and their food intake was daily recorded in order to calculate their exact energy (Kcal) consumption. The amount of food (grams) administered to the HF and the HP groups was calculated to contain the same Kcal as those ingested by the control group the day before. The energy density of the diets used for calculation was: control: 3.85; HF: 5.24 and HP: 3.85 Kcal per gram. When present, residual food in

each cage was weighed, discarded, and replaced with fresh diet every 24 h. Food was administered always at the same hour (13:00h) and was available to the animals over a 24-h period. Food intake of all groups was recorded daily to calculate the daily caloric intake and cumulative caloric intake throughout the experiment; body weight was recorded three times a week.

One week prior to sacrifice animals were submitted to nocturnal 14-h fasting to collect serum in fasted conditions to analyze the HOMA-IR score. Animals were sacrificed in the fed state by decapitation at the beginning of the light cycle (8:00-10:00h) and truncal blood was collected from the neck, stored at room temperature for 1 h and centrifuged at 1000 g for 10 min at 4°C to collect serum. Last meal was administered at 13:00h the day before the sacrifice (this pattern of food administration was conducted during the 4 months of the experiment); this last meal was isocaloric between the different groups (control: 69.8 ± 3.4 Kcal; HF: 65.7 ± 2.9 Kcal and HP 68.7 ± 2.7 Kcal). Liver, skeletal muscle, kidney, hypothalamus and different white adipose tissue (WAT) depots, both visceral (epididymal, mesenteric and retroperitoneal) and subcutaneous (inguinal), as well as the interscapular brown adipose tissue (BAT) were rapidly removed, weighed and frozen in liquid nitrogen and stored at -70°C until RNA, DNA, protein, lipid, triacylglycerol, and glycogen analysis or quantification. In addition, stomach content was quantified in the different animal groups to discard possible interferences in the results due to the lack of food (fasting) on the day of the sacrifice. All the animals presented food in the stomach in the moment of the sacrifice.

Adiposity. Adiposity was determined by an adiposity index computed for each rat as the sum of epididymal, inguinal, mesenteric and retroperitoneal white adipose tissue depot weights and expressed as a percentage of total body weight. In addition, body composition was measured 15 days before the sacrifice using an EchoMRI-700™ (Echo

Medical Systems, LLC., TX, USA) without anesthesia. Direct measurements of fat and lean mass (in grams) were taken from the analyzer, and expressed as a percentage of total body weight.

Quantification of protein lipid levels. Total protein and lipid levels were determined in liver and muscle by the methods of Bradford ¹⁶ and Folch ¹⁷, respectively.

Triacylglycerol determination. Triacylglycerol (TG) content was measured in liver and muscle lipid extracts as previously described ¹⁸ using the Serum Triglyceride Determination Kit (Sigma Aldrich, Madrid, Spain).

Quantification of DNA content. DNA content was analyzed in the different WAT depots studied (epididymal, inguinal, mesenteric and retroperitoneal). For quantification, 250–300 mg of the different adipose depots were homogenized in 3 volumes of PBS, using a polytron homogenizer, and then centrifuged at 500 g for 10 min; the supernatant was collected and used for DNA quantification by fluorescence with a multireader Mithras LB 940 (Berthold Technologies GmbH, Bad Wildbad, Germany) using the 3,5 diaminobenzoic acid method ¹⁹.

Histological analysis. Tissue samples (inguinal and retroperitoneal WAT, and liver) were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, overnight at 4°C, dehydrated in a graded series of ethanol, cleared, and embedded in paraffin blocks for light microscopy. Adipocyte area was measured in the inguinal and retroperitoneal WAT as selected depots. Five-micrometer-thick sections of tissues were cut with a microtome and mounted on slides. The area of adipocytes was measured in hematoxylin/eosin stained sections. Images from light microscopy were digitalized and an area of at least 100 cells of each section was determined using Axio Vision software (Carl Zeiss Imaging Solutions, Barcelona, Spain). Presence of steatosis was visually analyzed in stained liver sections.

Quantification of hepatic glycogen levels. For hepatic glycogen isolation, 0.3–0.8 g of liver from each sample was digested in 1 ml of 30% KOH at 100°C for 10 min, glycogen was precipitated overnight at –20°C with 2 ml of 100% ethanol, collected by centrifugation (at 3000 rpm and 4°C, as for the next centrifugations, for 30 min), dissolved in 1 ml of 8% trichloroacetic acid (TCA) and then centrifuged for 15 min. The supernatant was stored (4°C), whereas the pellet was dissolved in 1 ml of 8% TCA and centrifuged for 15 min, and the resulting supernatant was added to the stored one. Glycogen was again obtained from the supernatant by precipitation with 4 ml of 96% cold ethanol followed by centrifugation (15 min). The pellet was then dissolved in 1 ml of water. Glycogen concentration was measured with the anthrone reagent as previously described²⁰.

Quantification of serum parameters of interest. Blood glucose was measured using an Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). ELISA kits were used to measure serum levels of insulin (DRG Instruments, Marburg, Germany), leptin (R&D Systems, MN, USA) and TNF-alpha (R&D Systems Europe, Abingdon, UK). Circulating ghrelin was measured with an enzyme immunosorbent assay kit (Phoenix Europe GmbH, Karlsruhe, Germany). Commercial enzymatic colorimetric kits were used for the determination of triacylglycerols (Sigma Diagnostics, St Louis, MO, USA), free fatty acids (Wako Chemicals GmbH, Neuss, Germany), beta-hydroxybutyrate levels (β -HBA Procedure No. 310-UV, Sigma Diagnostics), total cholesterol (cholesterol-esterase and cholesterol-oxidase/peroxidase, BioSystems, Barcelona, Spain), urea (BUN-UV, Biosystems, Barcelona, Spain) and creatinine (alkaline picrate, BioSystems, Barcelona, Spain).

HOMA-IR analysis. Insulin resistance was assessed by the homeostatic model assessment for insulin resistance (HOMA-IR) in rats submitted to overnight (14 h)

fasting (n=6-7 for all groups). HOMA-IR score was calculated from fasting insulin and glucose concentrations using the formula of Matthews *et al.*²¹. $\text{HOMA-IR} = \text{fasting glucose (mmol/l)} \times \text{fasting insulin (mU/l)} / 22.5$.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Gene expression of key genes involved in energy homeostatic control was determined in different tissues (liver, muscle, hypothalamus and adipose tissue) by real time RT-PCR. Total RNA was extracted using Tripure reagent (Roche, Barcelona, Spain) according to the instructions provided by the supplier and was then purified using E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, Norcross, GA, USA) and treated with DNase I (Omega Bio-Tek, Norcross, GA, USA). Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies, Wilmington, DE, USA) and its integrity and purity confirmed using agarose gel electrophoresis. 0.25 μg of total RNA (in a final volume of 5 μl) was denatured at 65 °C for 10 min and then reverse transcribed to cDNA using murine leukaemia virus reverse transcriptase (from Applied Biosystem, Madrid, Spain) in a final concentration of 2 U/ μL at 20 °C for 15 min, 42 °C for 30 min, with a final step of 5 min at 95 °C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). PCRs were performed using diluted (1/20 for liver, muscle, WAT and BAT; and 1/5 for hypothalamus) cDNA template, forward and reverse primers (in a final concentration of 0.4 μM each), and 5 μL of 2x Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA) which includes AmpliTaq Gold® DNA Polymerase, in a total volume of 11 μL . PCR parameters were as followed: 10 min at 95°C, followed by a total of 40 temperature cycles (15 s at 95°C and 1 min at 60°C). Primers for the different genes analyzed were designed using Primer3Plus²² and are described in Table 1. All primers were obtained from Sigma Genosys (Sigma Aldrich Química SA, Madrid, Spain). In order to verify

the purity of the products and the specificity of the amplification, a melting curve was produced after each run according to the manufacturer's instructions. Specificity of the PCR amplification was also validated using agarose gel electrophoresis. The quantification cycle (Cq) was calculated by the instrument's software (StepOne Software v2.0) and the relative expression of each mRNA was calculated as a percentage of control rats, using the $2^{-\Delta\Delta C_t}$ method²³. PCR efficiency was always between 1.9-2.0. We tested four different reference genes (*β -actin*, *Lrp10*, *Gdil* and *18S rRNA*). At least two of these genes were analyzed in each tissue; we present data obtained after normalization using the reference gene with the lowest gene expression variation for each tissue. *β -actin*, a well-known reference gene was selected for muscle, hypothalamus and WAT. *β -actin* was also used in liver, except for the amino acid metabolism genes which were analyzed from a different RT product than the rest of the genes and for which *Gdil* which we have previously identified as a good constitutive gene based on microarray studies²⁴, resulted more adequate. *Lrp10* was used in BAT because it is a better constitutive in this tissue and it has been described as a good choice for expression studies in adipose tissue²⁵.

Western blot analysis of ATGL, ACC and phosphoACC. ATGL, ACC and phosphoACC protein levels were determined by western blot in selected inguinal and retroperitoneal WAT as selected depots. Tissue was homogenized at 4 °C in 1:3 (w:v) in RIPA buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher, Rockford, USA) using a Polytron homogenizer (VWR). Homogenate was centrifuged at 700 g for 10 min at room temperature and supernatant used for total protein ATGL, ACC and phospho-ACC analysis. Total protein content was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). Western blot was performed using 60 micrograms of total protein per lane in a 4–15% Criterion™

TGX™ Precast Gel (BioRad, Madrid, Spain), and then transferred to a nitrocellulose membrane. The primary antibodies used were the following: monoclonal rabbit anti-ATGL (Cayman Chemical, Ann Arbor, MI, USA), monoclonal rabbit anti-ACC (Cell Signaling, Inc., CA, USA) and monoclonal rabbit anti-phosphoACC (Cell Signaling, Inc., CA, USA) antibodies diluted 1:1000 in TBS-T; membranes were incubated during one hour for ATGL and phosphoACC and overnight for ACC. Membranes were also incubated with anti- β -actin antibody to ensure equal loading (from Cell Signaling, Inc., CA, USA). Specific infrared (IR)-dyed secondary anti-IgG antibodies (LI-COR Biosciences, Nebraska, USA) were used. For IR detection, membranes were scanned in Odyssey Imager (LI-COR); the bands were quantified using the software Odyssey V3.0 (LI-COR).

Statistical analysis. All data are expressed as the mean \pm SEM. Differences between groups were analyzed using Student's *t* test or one-way ANOVA. One-way ANOVA followed by least significant difference (LSD) post hoc comparison was used to compare the effect of the different diets (control, HF and HP), and Student's *t* test to directly compare the effect of the HF or HP diets *vs* the control diet. Effect of fasting was analyzed using Student's *t* test. All the analyses were performed with SPSS for windows (version 15.0, SPSS, Chicago, IL, USA). Threshold of significance was defined at $p < 0.05$ and is indicated when different.

Results

Body weight, adiposity and food intake

As shown in Table 2, high-fat pair-feeding did not affect body weight; animals of the HF group presented the same body weight as controls. However HF-fed animals had a greater fat mass compared to control animals; adiposity index, calculated from tissue weights after the sacrifice, was also higher in the rats fed with a HF diet. In these HF-fed animals there was an increase in the size of interscapular BAT and of two of the visceral WAT depots studied: epididymal and retroperitoneal, but not of the mesenteric depot. HF diet did not affect the size of the subcutaneous adipose depot (inguinal); however, there was a greater adipocyte area and a lower DNA content (used as an indicator of proliferative activity) in this depot in animals fed a HF diet, suggesting increased hypertrophy. No difference was observed in accumulated caloric intake during the experimental period (Table 2) between the HF and the control groups.

Concerning the HP group, animals presented a lower body weight in comparison to control animals (Table 2). However, there was no change in body fat mass or in the adiposity index in animals of the HP group in comparison to controls. No change was observed either in the size of the WAT depots; however, and contrary to what was observed in the HF group, a decrease was observed in the area of the adipocytes of the inguinal adipose depot (Table 2). Animals fed with the HP diet showed a lower accumulated energy intake during the whole experimental period (Table 2).

Liver and muscle parameters

Liver of the animals fed a HF diet had a lower size and lower glycogen content, but a higher content in total lipids, triacylglycerols and total protein in comparison to control-fed animals (Table 3). In accordance with the greater lipid content, histological analyses

revealed the presence of hepatic steatosis in the HF-fed animals (data not shown). In animals of the HP group, no change was evident for glycogen, lipid, triacylglycerol or protein content in liver or for liver size (Table 3). No evidence of hepatic steatosis was observed in HP animals. In muscle, an increased triacylglycerol content was observed in the animals fed with HF diet (Table 3).

Kidney parameters

Kidney weight was increased in the HP group; the greater size of this organ was even more evident when representing the results as a percentage of total body weight (Table 2). In accordance with higher protein content in the diet, urea levels in serum were increased in the HP, whereas there were decreased in the HF group (Table 4). Serum creatinine levels, used as possible indicator of kidney function, did not change as a result of the intake of HF or HP diets (Table 4).

Serum parameters related to energy/glucose homeostasis and adiposity

Measured serum parameters are represented in Table 4. HF diet did not affect circulating insulin levels but resulted in a tendency to increased levels of circulating glucose in fed conditions ($p=0.07$, Student's t test, *vs.* control group) and in lower insulin sensitivity as indicated by a higher HOMA-IR. On the other hand, HP diet did not affect circulating glucose levels in fed animals, but insulin levels were increased; in this case no change was observed in the HOMA index. Serum leptin levels were not affected by the intake of the HF or HP diets and decreased with fasting in all groups. HF diet intake produced a decrease in serum triacylglycerol levels, whereas free fatty acid levels were not affected by the intake of a HF or a HP diet. Interestingly, total cholesterol was decreased in HP-fed rats. We also analyzed beta-hydroxybutyrate levels, which was only present in detectable amounts in the HF group. Levels of the orexigenic signal ghrelin were higher in the HF and HP animals in the fed state. Serum

TNF-alpha levels, measured as a marker of inflammation were increased in both HF and HP groups.

Effect of HF and HP diet on mRNA and protein expression of key energy homeostasis-related genes in different tissues

Liver

In liver (Figure 1a), HF diet feeding produced a decrease in mRNA levels of genes involved in fatty acid synthesis: *Acc1*, *Fasn* and *Srebp1*; and an increase in mRNA levels of *Cpt1a* (liver isoform), a key gene involved in fatty acid oxidation. Interestingly, we also observed an increased expression of *Slc27a2* that codes for a fatty acid transporter. HP diet feeding did not affect the expression of genes related to fatty acid metabolism. In relation to the carbohydrate metabolism, the HF diet produced a decreased expression of the glycolytic gene *Pklr*. Additionally, both diets increased the expression of genes involved in amino acid metabolism: *Slc43a1* which codes for an amino acid transporter; *Got1* (increased only in the HP group) and *Gpt*, both coding for key transaminases; and the key urea cycle gene *Cps1*.

Muscle

As shown in Figure 1b, dietary treatment produced a decrease in gene expression levels of two key lipogenic genes studied, *Fasn* in the case of the HF diet and *Srebp1* in the case of the HP diet. Besides, a decreased expression was also observed for the genes involved in fatty acid oxidation: *Ucp3* in the HF group and *Cpt1b* (muscle isoform) and *Ucp3* in the HP group.

Hypothalamus

In hypothalamus (Figure 1c), HF-feeding produced an increased expression of the orexigenic gene *Agrp* and a decreased expression of the anorexigenic gene *Cart*. HP-feeding did not significantly affect mRNA expression of the peptides studied.

Adipose tissue

Depot-specific differences were evident for the studied genes and proteins in the different adipose tissues. As shown in Figures 2 and 3, HF diet feeding produced a decrease in the expression of key genes involved in fatty acid synthesis: *Acc1* and *Fasn*, both in BAT (Figure 2) and in the different WAT (Figure 3) depots studied. ACC protein levels, analyzed in the inguinal and retroperitoneal WAT depots, were also decreased while the phosphorylated ACC (inactive form)/total ACC ratio was increased, but only in the inguinal depot (Figure 4). On the other hand, the HF diet produced an increase in the expression of genes related to fatty acid oxidation: *Cpt1b* in BAT, *Atgl* in the inguinal and mesenteric WAT, and *Cpt1b* in the retroperitoneal WAT. *Cpt1b* was measured in adipose tissue because it is the predominant isoform expressed in this tissue²⁶. At protein level we analyzed ATGL expression in two adipose depots and it followed the same regulatory pattern than mRNA: increased ATGL levels were observed in the inguinal adipose depot while no change was evident in the retroperitoneal one (Figure 4). We observed an increase in mRNA levels of the main effector of BAT thermogenesis, UCP1, in the HF group, although it did not reach statistical significance due to variability between animals ($p=0.1$, Student's *t* test). No change was observed in BAT in the expression of *Adrb3* or *Pgc1a*, involved in thermogenic response and UCP1 expression respectively. HF diet produced an increase in the expression of the adipogenic gene *Pparg* in the inguinal WAT.

HP diet also decreased the expression of genes involved in fatty acid synthesis, *Acc1* and *Fasn*, in BAT, and *Fasn* in the inguinal and retroperitoneal WAT, but increased the expression of *Acc1* in the WAT depots studied. However, this increased *Acc1* expression was translated into higher protein levels in none of the two adipose depots analyzed, inguinal or retroperitoneal, and a tendency ($p=0.08$, Student's *t* test) to a higher phosphorylated ACC/total ACC ratio was observed in retroperitoneal adipose depot (Figure 4). Concerning genes involved in fatty acid oxidation, an apparently controversial effect was also found, as HP diet increased *Atgl* expression in the inguinal (also at protein levels) and retroperitoneal WAT but decreased *Cpt1b* mRNA levels in the different WAT depots. Gene expression of the adipogenic *Pparg* was increased in the inguinal and retroperitoneal depots, but decreased in the mesenteric WAT of HP-fed rats. In BAT, HP diet produced a decrease in expression levels of *Ucp3*, a gene involved in fatty acid oxidation.

Discussion

Macronutrient composition has a deep impact in energy metabolism with coherent adaptive physiological response in the selected organs and tissues. However there are still discrepancies regarding the metabolic effects, especially in the long term, of diets with an unbalanced macronutrient proportion⁵. We performed this study to explore whether or not the intake of unbalanced diets rich in fat or proteins offered in isocaloric amounts in comparison to a control diet might be detrimental to healthy animals when administered over a long period of time.

In our study, the intake of an isocaloric HF-diet with 60% energy from fat, administered for 4 months, did not affect body weight. However, HF-fed animals showed around 25% increased fat mass content and adiposity index. The lack of overweight but increased adiposity is consistent with other experiments administering HF diets in isocaloric conditions^{27,28}. However, Lomba *et al.*²⁹ have described overweight using a pair-feeding experiment with a similar experimental design to ours. The difference can be due to the fact that they used a HF diet which contained mainly saturated fatty acids provided by hydrogenated coconut oil, while we used a diet with fat provided by lard (with approximately 40% saturated and 60% unsaturated fat), and it is well known that the type of fat diet consumed can affect lipid metabolism in a different manner³⁰. In spite of increased adiposity, we did not observe any difference in serum leptin levels in comparison to control animals, in agreement with other authors using a HF diet in isocaloric conditions^{31,32}.

It is well documented that the obesity derived from high intake of fat in *ad libitum* conditions originates overweight which is linked to insulin resistance and related metabolic disorders in different animal model and human studies^{8,33}. Our data show that the intake of an isocaloric pair-fed HF diet, despite causing no overweight, is also

inducing the development of insulin resistance and other metabolic alterations related to metabolic syndrome as discussed next. HF-fed animals presented a higher HOMA index and higher glucose levels in feeding conditions, even when the amount of carbohydrates provided by the diet was much lower than the control diet (20% vs 70% total energy); suggesting an alteration of glucose metabolism and insulin signaling due to fat intake, in agreement with what has recently been described by other authors with a similar diet and conditions ²⁷. HF-fed animals also presented increased lipid and triacylglycerol content in liver and increased serum levels of the inflammatory marker TNF-alpha, all of them parameters related to metabolic syndrome.

Metabolically, HF-fed animals were in a fasting-like situation because of sustained decreased carbohydrate intake, which is evidenced by the lower levels of hepatic glycogen and by the presence of circulating beta-hydroxybutyrate. The HF diet produced a high impact on mRNA expression of regulatory genes involved in energy homeostasis maintenance which would be intended to maintain glycaemia, to use fatty acids from diet as energy source, as well as to try to compensate increased adiposity. In accordance with a low input of diet carbohydrates, we observed decreased expression of the key glycolytic gene *Pklr* in liver, in agreement with what has previously been reported for HF diets administered *ad libitum* ³⁴. We also found increased liver expression of genes involved in amino acid uptake (*Slc43a1*) and handling (*Gpt* and *Cps1*) which could reflect increased amino acid metabolism to maintain glucose homeostasis by gluconeogenesis; however, circulating levels of urea, indicator or protein catabolism, were not increased in the HF rats. Important changes were also observed in lipid metabolism. In general terms, in the different tissues analyzed (liver, skeletal muscle and adipose tissue) HF feeding induced a reduced mRNA expression of key genes involved in lipid synthesis pathways (*Fasn*, *Srebp* and *Acc1*). The decreased

lipogenic capacity was confirmed in the adipose tissue by a decrease in ACC protein levels and also by an increase in the phosphorylated (inactive) ACC/total ACC ratio observed in the inguinal WAT depot. *De novo* lipogenesis has been repeatedly demonstrated to be reduced during *ad libitum* high fat feeding³⁵, but data regarding isocaloric feeding are very scarce. Decreased lipogenesis in liver would be related to a reduced very low-density lipoprotein production rate which would explain the decrease in circulating triacylglycerols detected in HF-fed animals. Moreover, animals of the HF group showed an increased expression of fatty acid oxidation genes in liver and in brown (BAT) and white adipose tissue (WAT), as described for HF diets administered *ad libitum*³⁶. Lipolytic genes with increased expression were: *Cpt1* (*Cpt1a* in liver and *Cpt1b* in BAT and retroperitoneal WAT) and *Atgl* (in inguinal and mesenteric WAT depots). Increased mRNA levels correlated with increased protein levels for ATGL, studied as representative of lipolysis; as happened with mRNA levels, we observed increased ATGL expression in the inguinal but not in the retroperitoneal depot. WAT depot-specific differences observed in gene expression regulation, mainly in lipolysis, could be due to hormonal and/or signaling differences (e.g. amount of beta-adrenergic receptors) between fat depots^{37, 38}. We also report increased liver expression of *Slc27a2*, which codes for FATP2, a fatty acid transporter that has been related to hepatic steatosis³⁹. This increased *Slc27a2* expression in liver observed in HF-fed animals could be related to increased fatty acid uptake, which correlates with the greater liver lipid and triacylglycerol content and with the signals of hepatic steatosis observed by histological analysis in our animals. In muscle, a tissue with an important contribution to energy waste, we do not observe an increased expression of *Cpt1b* and we even observe a decrease in the expression of *Ucp3*, also related to fatty acid oxidation. This could suggest a decreased lipolytic capacity in this tissue under a HF diet, as suggested

by other authors⁴⁰ and thus a recirculation of the flux of fat excess to adipose tissue depots. In addition, triacylglycerol levels in muscle were increased. Decreased lipolysis and *Ucp3* gene expression in muscle under conditions of HF diets has also been related to an increase in insulin resistance^{41, 42}. One powerful mechanism suggested to compensate diet-induced obesity in rodents consists of increasing energy expenditure through adaptive thermogenesis⁴³. *Ad libitum* intake of HF diets has been related to BAT hypertrophy and to an increased expression in this tissue of uncoupling protein 1 (UCP1), the main effector of adaptive thermogenesis^{44, 43}, however, the effect of isocaloric HF diets on BAT thermogenic potential is less studied. In our HF pair-fed animals, we observed an increased size of BAT, and a non-significant increase in *Ucp1* mRNA expression. Other group has previously reported lack of effect of isocaloric HF feeding on UCP1 expression and thermogenic capacity²⁸. High caloric intake and HF diet intake independently of caloric intake are known to stimulate sympathetic nervous system activity which controls UCP1-mediated thermogenesis²⁸. Thus, probably, the fact that the HF diet was administered in isocaloric conditions without increased caloric intake produced a not so potent UCP1 induction. However, we cannot discard an effect on thermogenesis, as it has been reported that isocaloric HF feeding in rats induces BAT thermogenesis by augmenting the activation of UCP1 rather than its expression⁴⁵.

Isocaloric HF-feeding also affected the expression of hypothalamic neuropeptides involved in the control of food intake. Although daily caloric intake was not affected, HF diet produced an increase in mRNA expression of the orexigenic *Agrp* and a decrease in the expression of the anorexigenic *Cart* gene; no change was observed in the expression of the orexigenic *Npy* or in the anorexigenic *Pomc* gene. Moreover, serum levels of the orexigenic signal ghrelin were also increased in HF-fed rats. The higher AgRP/CART ratio and higher basal circulating ghrelin levels could contribute to

overfeeding and obesity if animals were given free access to food. Interestingly, long-term intake of a HF diet in *ad libitum* conditions in rats has been described to have an opposite effect, producing a decrease in plasma ghrelin levels⁴⁶ probably to counteract the positive energy balance.

We also analyzed the effects of a long-term HP diet intake. Animals fed with this diet presented 9% lower body weight in comparison to control animals. However, we did not observe any change in adiposity, although there was a decrease in adipocyte area in the subcutaneous WAT depot, which was not translated into a lower size of this depot. No change was observed either in the circulating levels of the adiposity signal leptin measured in *ad libitum* conditions. Other authors have previously reported a lower body weight and adiposity in animals fed a HP diet which has been attributed, in part, to a lower caloric intake⁴⁷. In fact, our HP-fed animals showed a lower, voluntary cumulative caloric intake after 4 months of experiment, which could be explained by the greater sensory-specific satiety of proteins compared with carbohydrates⁴⁸. Circulating levels of the orexigenic hormone ghrelin were increased in the animals of the HP group. Ghrelin is responsive to short but also to long-term nutritional status, and its levels increase with fasting and weight loss⁴⁹. It has been described that individuals who have lost weight by means of chronic caloric restriction have elevated levels of this hormone⁵⁰. Thus, high ghrelin levels in HP-fed animals could be reflecting the long-term caloric restriction with a lower cumulative intake during the 4 months of the experiments. One of the suggested and controversial mechanisms to take part in weight reduction due to the intake of HP diets is the increased energy expenditure through adaptive thermogenesis⁵¹. It has been reported that BAT thermogenesis activation is dependent on the protein content in the diet⁵²; we do not observe any effect in mRNA expression

of UCP1 or of its transcriptional coactivator PGC1 α using a HP diet with a 45% energy from protein.

Weight loss is normally associated with an improvement in insulin resistance and serum parameters related to metabolic syndrome, irrespectively of the type of diet ⁵³. We observe a decrease in circulating cholesterol levels in our HP-fed animals that could be related to the weight loss, while triacylglycerol levels were not affected. Regarding glucose metabolism, although HOMA-IR and glucose levels remained unchanged, higher insulin levels were required to maintain glycaemia in HP-fed animals. It has been previously shown that long-term protein intake is associated with a higher insulin release and has also been suspected to promote insulin resistance ¹². When considering the effect of HP diets on insulin resistance it may be important to take into consideration the source of protein. The protein in our diet comes mainly from casein, which has been demonstrated to have a more negative impact on insulin sensitivity when compared to proteins from other sources such as cod and soy proteins ⁵⁴. Remarkably, we observed an increase in serum TNF- α levels in the HP group which could indicate a proinflammatory effect of the HP diet used in our study and, thus, a higher risk of developing metabolic complications. In fact, TNF- α serum levels have been related to insulin resistance and metabolic syndrome ⁵⁵. However, also in this case, protein source could be relevant, as peptides derived from casein digest are known to enhance immune system ⁵⁶.

As expected, we observed increased liver expression of genes involved in amino acid uptake and handling (*Cps1*, *Got1*, *Gpt* and *Slc43a1*) which correlated with higher serum levels of urea in the HP-fed group, reflecting an increased oxidation of dietary amino acids. Metabolic adaptation to HP intake is characterized by a down regulation of lipogenesis from glucose ⁴⁷. Our results show a decreased mRNA expression of

lipogenic genes in muscle (*Srebp1*), WAT (*Fasn*) and BAT (*Fasn* and *Acc1*), possibly as a result of decreased carbohydrate intake due to increased protein proportion in diet⁵⁷. However, apparently contradictory, the expression of lipogenic gene *Acc1*, coding for the enzyme involved in malonyl-CoA synthesis from acetyl-CoA, rose in WAT in animals of the HP group. This increased *Acc1* expression could be originated by the excess acetyl-CoA generated by the catabolism of the excess amino acids⁵⁸, and it is not necessarily translated into increased protein levels or activity. In fact, ACC protein levels measured in WAT were not increased and even a tendency to higher ratio between the phosphorylated (inactive) form and total protein was observed in the retroperitoneal adipose depot which agrees with the expected inhibition of lipogenesis in the HP fed animals. Surprisingly, we did not observe any change in gene expression of lipogenic or lipolytic enzymes in liver, although other authors have reported a reduction in hepatic lipogenesis in animals fed with HP diets measuring parameters including fatty acid synthase activity⁴⁷. With respect to genes involved in lipolysis, we observed increased ATGL expression in the inguinal (mRNA and protein) and retroperitoneal (only mRNA) WAT depots, correlating with the smaller adipocyte diameter in the former. In contrast, the key gene related to fatty acid oxidation, *Cpt1b*, decreased its expression in muscle and in the different WAT depots analyzed in the animals of the HP group, probably due to the fact that these animals obtain energy mainly by oxidation of dietary amino acids. In accordance with the lower carbohydrate levels in the HP diet, we also observe a decreased expression of the glycolytic gene *Pfk*. Finally, HP diets have been related to renal alterations¹³. In this sense the intake of a HP diet produced an increase in the weight of kidney in our animals, which has been previously described in humans⁵⁹. It has been demonstrated that high-protein diets produce greater renal complications in diabetic than in non-diabetic animals⁶⁰. Here in

healthy animals, in spite of a greater kidney size we did not observe any difference in serum creatinine, commonly used as an indicator of renal function. However, an increased kidney size is present in different renal alterations like diabetic renal hypertrophy and tubulointerstitial injury, among others^{61,62}, and for that reason it could be a detrimental effect of the HP diet, particularly in the face of compromised kidney function in diabetic patients.

In conclusion, long-term intake of unbalanced diets with an excess of fat or protein have an impact on metabolic and molecular parameters related to body weight control and adiposity (see Figure 5). Thus, care has to be taken when altering the recommended macronutrient proportions in diet. A reduction in diet carbohydrates or bad habits consisting of eating fat-rich foods, can lead to a disproportion in fat intake which, even if not associated to overweight when ingested calories are controlled, can be accompanied by metabolic syndrome-related alterations. Although there are some recent research describing the development of insulin resistance in animals fed an isocaloric HF diet²⁷, our study has a more global approach. The intake of calorie-controlled high-fat diets can increase the incidence of non-obese individuals with an increased fat content, the so called “thin-outside-fat-inside” or “metabolically-obese normal-weight”. Thus, it would be important to establish analytical measures to identify these individuals as a health preventive strategy, as they can be at increased risk of metabolic disease. On the other hand, unbalanced diets with an increased proportion of proteins, although reduce body weight and decrease circulating cholesterol, increase serum insulin and TNF-alpha levels, and produce increased kidney size which could indicate a higher risk of developing insulin resistance, pathologies related to increased inflammation, or renal complications. For this reason we do not discard that diets rich in proteins could have

adverse effects in non-healthy and more susceptible individuals, as individuals with increased body weight, those who usually follow these diets.

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Conflict of interest. None

Authorship. Author's responsibilities were as follows: AP, PO, RDR and AC developed the experimental design; RDR and EGR did the major part of the experimental work; PO and AC collaborated in the animal studies; PO, AP, RDR analyzed and discussed the results; RDR and PO wrote the manuscript and AP, AC and EGR participated in critical revising of the manuscript. All the authors read and approved the final manuscript.

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Table legends

Table 1. Primers used for real time RT-PCR amplification. Nucleotide sequences, product size -P- (bp) and gene accession numbers are indicated. The position of the primers relative to the coding sequence (indicated as subscripts), their location by exon (indicated between parentheses) as well as annealing temperature -AT- (°C) are also provided. For genes related to fatty acid synthesis: *Acc1*: acetyl-coenzyme a carboxylase alpha; *Fasn*: fatty acid synthase; *Pparg*: peroxisome proliferator-activated receptor gamma and *Srebp-1*: sterol regulatory element-binding protein 1. For genes related to fatty acid oxidation: *Adrb3*: beta-3 adrenergic receptor; *Atgl*: adipose triglyceride lipase; *Cpt1a*: carnitine palmitoyltransferase-1a; *Cpt1b*: carnitine palmitoyltransferase-1b; *Pgc1a*: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *Ucp1*: uncoupling protein 1 and *Ucp3*: uncoupling protein 3. For fatty acid transport: *Slc27a2*: solute carrier family 27 (fatty acid transporter), member 2. For genes related to carbohydrate metabolism: *Pepck*: phosphoenolpyruvate carboxykinase; *Pfkl*: phosphofructokinase; *Pklr*: pyruvate kinase. For genes related to amino acid metabolism: *Cps1*: carbamoyl phosphate synthetase 1; *Got1*: glutamic-oxaloacetic transaminase 1 and *Gpt*: glutamic-pyruvate transaminase and *Slc43a1* solute carrier family 43. For genes related to food intake: *Agrp*: agouti related protein; *Cart*: cocaine and amphetamine regulated transcript; *Ghsr*: ghrelin receptor; *Npy*: neuropeptide y; *Lepr*: leptin receptor; *Pomc*: proopiomelanocortin and *Socs3*: suppressor of cytokine signaling 3. Reference genes: *Actb*: actin beta; *Gdi1*: guanosine diphosphate dissociation inhibitor 1 and *Lrp10*: low-density lipoprotein receptor-related protein 10.

Table 2. Body weight, adiposity-related parameters, food intake, DNA content, kidney weight and kidney/body weight ratio of male Wistar rats fed with a control, a high fat (HF) or a high protein (HP) diet from the age of 2 until the age of 6 months. Food in HF and HP groups was offered in isocaloric amounts to the control group and water was offered *ad libitum*. The adiposity index was computed as the sum of epididymal, inguinal, mesenteric and retroperitoneal white adipose tissue depot weights and expressed as a percentage of total body weight. Fat and lean mass were measured using an EchoMRI and are expressed as percentage of total body weight. Adipocyte area was measured using specific software. DNA content in different adipose tissues was measured by fluorescence. One kidney per animal was considered. Kidney/body weight ratio represents the weight of the kidney expressed as a percentage of total body weight. BAT: interscapular brown adipose tissue; EWAT: epididymal white adipose tissue; IWAT: inguinal WAT; MWAT: mesenteric WAT and RWAT: retroperitoneal WAT. Results represent mean \pm SEM (n=7 in control and HF groups and n=6 in HP group). Statistics: D, effect of the type of diet (one-way ANOVA, $p < 0.05$, and indicated when different). Values not sharing a common letter (a, b, c) are significantly different (one-way ANOVA, $p < 0.05$). Letter “a” is used to indicate the higher values. No letters = no statistical difference. #, different vs control group (Student’s t test, $p < 0.05$).

Table 3. Liver and muscle parameters analyzed in the same animals and conditions described in Table 2. Glycogen, lipid and protein content were measured by using the anthrone, Folch and Bradford methods respectively. Triacylglycerol (TG) content was measured using a colorimetric kit. Results represent mean \pm SEM (n=7 in control and HF groups and n=6 in HP group). Statistics: D, effect of the type of diet (one-way ANOVA, $p < 0.05$). Values not sharing a common letter (a, b) are significantly different

(one-way ANOVA, $p < 0.05$). Letter “a” is used to indicate the higher values. No letters = no statistical difference. # different *vs* control group (Student’s *t* test, $p < 0.05$).

Table 4. Serum parameters analyzed in the same animals described in Table 2.

Some of the parameters were also analyzed in fasting conditions (nocturnal 14-h fasting). Fed and fasted data correspond to serum from the same set of animals which was collected with one week of difference. Insulin, leptin and TNF-alpha levels were measured by ELISA kits. Ghrelin levels were measured an enzyme immunosorbent assay kit. Circulating levels of tricylglycerols, free fatty acids, beta-hydroxybutyrate, total cholesterol, urea and creatinine were determined using commercial enzymatic colorimetric kits. HOMA-IR was computed using the formula of Matthews *et al.*²¹. Glucose was measured using a glucometer. Results represent mean \pm SEM (n=7 in control and HF groups and n=6 in HP group). Statistics: D, effect of the type of diet (one-way ANOVA, $p < 0.05$). Values not sharing a common letter (a, b, c) are significantly different (one-way ANOVA, $p < 0.05$). Letter “a” is used to indicate the higher values. No letters = no statistical difference. #, different *vs* control group (Student’s *t* test, $p < 0.05$). * Effect of fasting (fasted *vs* fed animals) (Student’s *t* test, $p < 0.05$).

Figure legends

Figure 1. mRNA levels of key energy homeostasis-related genes in liver (a), muscle (b) and hypothalamus (c) analyzed in male Wistar rats fed with a control, a high fat (HF) or a high protein (HP) diet from the age of 2 until the age of 6 months. Food in HF and HP groups was offered in isocaloric amounts to the control group and water was offered *ad libitum*. mRNA expression levels in the different tissues were measured by real time RT-PCR. Results represent mean \pm SEM (n=7 in control and HF groups and n=6 in HP group) of ratios of specific mRNA levels to β -actin (used as reference gene), except for the amino acid metabolism genes whose data were normalized using *Gdi1*. Data of the control group was set to 100% and the rest of the values are referred to this. Statistics: Values not sharing a common letter (a, b) are significantly different (one-way ANOVA, $p < 0.05$). Letter “a” is used to indicate the higher values. No letters = no statistical difference. #, different vs control group (Student’s *t* test, $p < 0.05$).

Figure 2. mRNA levels of key energy homeostasis-related genes in brown adipose tissue (BAT) depots analyzed in the same animals and conditions described in Figure 1. mRNA expression levels in BAT was measured by real time RT-PCR. Results represent mean \pm SEM (n=7 in control and HF groups and n=6 in HP group) of ratios of specific mRNA levels to *Lrp10* (used as reference gene). Data of control group was set to 100% and the rest of the values are referred to this. Statistics: Values not sharing a common letter (a, b) are significantly different (one-way ANOVA, $p < 0.05$). Letter “a” is used to indicate the higher values. No letters = no statistical difference. #, different vs control group (Student’s *t* test, $p < 0.05$).

Figure 3. mRNA levels of key energy homeostasis-related genes in different white adipose tissue, iWAT (a), mWAT (b), rWAT (c) depots analyzed in the same animals and conditions described in Figure 1. mRNA expression levels in the different white

adipose tissues were measured by real time RT-PCR. iWAT: inguinal WAT; mWAT: mesenteric WAT and rWAT: retroperitoneal WAT. Results represent mean \pm SEM (n=7 in control and HF groups and n=6 in HP group) of ratios of specific mRNA levels to β -actin (used as reference gene). Data of control group was set to 100% and the rest of the values are referred to this. Statistics: Values not sharing a common letter (a, b) are significantly different (one-way ANOVA, $p < 0.05$). Letter “a” is used to indicate the higher values. No letters = no statistical difference. #, different vs control group (Student’s *t* test, $p < 0.05$).

Figure 4. ATGL, ACC, phosphoACC protein levels and phosphoACC/ACC ratio in inguinal and retroperitoneal WAT in the same animals and conditions described in Figure 1. Protein levels were measured by western blot. Representative bands obtained in the western blot are shown; sixty micrograms of protein was loaded per lane. Results represent mean \pm SEM (n=7 in control and HF groups and n=6 in HP group) of ratios of specific protein levels to β -actin, (loading control). For protein levels, data of control group was set to 100% (for protein levels) or one (for phosphoACC/ACC ratio) and the rest of the values are referred to this. Statistics: Values not sharing a common letter (a, b) are significantly different (one-way ANOVA, $p < 0.05$). Letter “a” is used to indicate the higher values. No letters = no statistical difference. #, different vs control group (Student’s *t* test, $p < 0.05$ or indicated when different).

Figure 5. Scheme of the metabolic effects of the intake of unbalanced high fat or high protein diets administered in isocaloric conditions to adult Wistar rats from the age of 2 until the age of 6 months.

Table 1. Primers used for real time RT-PCR amplification

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	P (bp)	AT (°C)	Gene accession number
Fatty acid synthesis					
<i>Acc1</i>	753-772 TGCAGGTATCCCCACTCTTC (e8)	964-944 TTCTGATTCCCTTCCCTCCT (e9)	212	59.2	NM_174224.2
<i>Fasn</i>	799-818 CGGCGAGTCTATGCCACTAT (e6)	1020-1003 ACACAGGGACCGAGTAAT (e8)	222	59.1	NM_017332.1
<i>Pparg</i>	470-489 AGAGCCTTCAAACCTCCCTCA (e3)	699-681 GAGACATCCCCACAGCAAG (e4)	230	59.1	NM_013124.3
<i>Srebf1</i>	663-680 CCCACCCCTTACACACC (e3)	860-843 GCCTGCGGTCTTATTGT (e4)	198	59.2	NM_001276707.1
Fatty acid oxidation					
<i>Adbl3</i>	1230-1250 CCTTCAACCCGCTCATCTAC (e1)	1418-1401 TGGGAAATGGACGCTCAC (e2)	189	59.2	NM_013108.2
<i>Atgl</i>	573-592 TGTGGCCTCATTCTCCTAC (e4)	843-824 AGCCCTGTTTGACATCTCT (e5)	271	59.2	NM_001108509.2
<i>Cpt1a</i>	1773-1793 GCTCGCACATTACAAGGACAT (e14-15)	2041-2022 TGACACCACATAGAGGCAG (e16)	250	59.1	NM_031559.2
<i>Cpt1b</i>	896-915 GCAAACCTGGACCGAGAAGAG (e8)	1075-1056 CCTTGAAGAAGCGACCTTTG (e10)	180	59.1	NM_013200.1
<i>Pgc1a</i>	358-380 CATTTGATGCACTGACAGATGGA (e3)	427-410 CCGTCAGGCATGGAGGAA (e3)	69	59.1	NM_031347.1
<i>Ucp1</i>	144-163 GGGCTGATTCCTTTTGGTCT (e1)	373-354 GGTGGTGATGGTCCCTAAGA (e2)	229	59.2	NM_012682.2
<i>Ucp3</i>	576-598 GGAGGAGAGAGGAAATACAGAGG (e4)	793-773 CCAAAGGCAGAGACAAAGTGA (e6)	218	59.1	NM_013167.2
Fatty acid transport					
<i>Slc27a2</i>	1308-1326 CTGAAAAAGGAGGGCGTGT (e1)	1463-1444 TATGTAGACTGCGGGTGTGG (e2)	156	59.1	NM_031736.1
Carbohydrate metabolism					
<i>Pepck</i>	517-534 CATTCAACGCCAGGTTC (e3)	680-663 AGAGGTCCCCATCCGTGT (e4)	164	59.1	NM_198780.3
<i>Pfkfb</i>	169-187 AGGGCTGTGACTCGTATGG (e2)	273-254 GTTGGCTGGCTTGATGTTCT (e3)	105	59.1	NM_013190.4
<i>Pklr</i>	956-975 CTGCGGAGAAGGTTTTCTTG (e7)	1127-1208 ATACAGTCAGCCCCATCCAG (e8)	172	59.1	NM_012624.3
Amino acid metabolism					
<i>Cps1</i>	1595-1614 AGGTCTTGGGAACATCGGTC (e15)	1739-1720 ATGGTGCTGCTGCCTTCAA (e16)	145	59.1	NM_017072.1
<i>Got1</i>	275-294 GACCACAGTCTCAACCACGA (e2)	396-378 ACACCCCAACCCGATTCT (e2-3)	122	59.1	NM_012571.2
<i>Gpt</i>	36-55 ACTTTCCCATTCAGCCCT(e1)	172-153 ACCTTTCCCTTCAGCCATT (e1)	137	59.1	NM_031039.1
<i>Slc43a1</i>	1119-1139 GAGGGGACTGACGGCTTTATT (e6-7)	1234-1215 AGGCTCCACAGGAAAATGGG (e8)	116	59.1	NM_001107742.1
Food intake					
<i>Agrp</i>	327-347 AGAGTCTCAGGTCTAAGTCT (e3-4)	536-513 CTTGAAGAAGCGGCAGTAGCACGT (e5)	210	59.1	NM_033650.1
<i>Cart</i>	276-296 AGAAGAAGTACGGCCAAGTCC (e2)	359-342 CACACAGCTTCCCGATCC (e3)	84	59.1	NM_017110.1
<i>Ghr</i>	893-912 TCAGCCAGTACTGCAACCTG (e2)	1114-1005 GGAGAGATGGGATGTGCTGT (e2)	222	59.1	NM_032075.3
<i>Npy</i>	126-145 TGGACTGACCCTCGCTCTAT (e2)	313-294 GTGTCTCAGGGCTGGATCTC (e3)	188	59.2	NM_012614.2
<i>Lepr</i>	846-865 AGCCAAACAAAAGCACCATT (e6)	1019-1000 TCCTGAGCCATCCAGTCTCT (e7)	174	59.2	NM_012596.1
<i>Pomc</i>	545-568 CCTGTGAAGGTGTACCCCAATGTC (e3)	809-790 CACGTTCTTGATGATGGCGTTC (e3)	266	59.2	NM_139326.2
<i>Socs3</i>	548-567 ACTGAGCCGACCTCTCTCT (e1)	719-700 CCCCTCTGACCTTTCTTTG (e1)	172	59.2	NM_053565.1
Reference genes					
<i>Actb</i>	670-689 TACAGCTTACCACCACAGC (e4)	790-771 TCTCCAGGGAGGAAGAGGAT (e4)	120	59.1	NM_031144.3
<i>Gdil</i>	1155-1174 GACTCTCTGAACCGTCATCAA (e9)	1313-1293 CCGCACAAGGCAAATACATC (e10)	159	59.1	NM_017088.2
<i>Lrp10</i>	411-430 TCCCCTTCTTCTCCTCCTC (e1)	608-589 TTACCGTCTGTTCCTTGCTG (e3)	198	59.1	NM_001037777.1

Table 2. Body weight, adiposity-related parameters, food intake, DNA content, kidney weight and kidney/body weight ratio of male Wistar rats fed with a control, a high fat (HF) or a high protein (HP) diet from the age of 2 until the age of 6 months

	control group	HF group	HP group	ANOVA
Body weight (g)	500 ± 13 ^b	495 ± 10 ^b	455 ± 12 ^a #	D
Adiposity index (% of fat)	8.43 ± 0.70 ^b	11.1 ± 0.9 ^a #	8.31 ± 1.07 ^b	
Fat mass (%)	17.8 ± 1.6 ^b	22.8 ± 2.1 ^a #	18.3 ± 1.7 ^b	D
Lean mass (%)	68.2 ± 1.3 ^a	62.2 ± 1.9 ^b #	67.6 ± 2.1 ^a	D
Accumulated caloric intake (Kcal)	10227 ± 182 ^a	9789 ± 203 ^a	8971 ± 495 ^b #	D
Daily food intake (Kcal)	73.2 ± 3.4	69.8 ± 3.1	69.0 ± 3.4	
Weight of adipose tissues (g)				
BAT	0.54 ± 0.03 ^b	0.67 ± 0.03 ^a #	0.44 ± 0.03 ^c #	D
EWAT	12.8 ± 1.0 ^b	18.4 ± 1.5 ^a #	12.0 ± 1.7 ^b	D
IWAT	9.78 ± 0.92	11.4 ± 1.2	8.30 ± 0.96	
MWAT	6.44 ± 0.76	8.37 ± 1.10	6.08 ± 1.80	
RWAT	12.2 ± 1.1 ^a	16.3 ± 1.2 ^b #	11.5 ± 2.1 ^a	D
Adipocyte area (µm ²)				
IWAT	4473 ± 359 ^b	6716 ± 300 ^a #	3262 ± 290 ^c #	D
RWAT	5881 ± 834	6770 ± 1013	5405 ± 706	
DNA content (µg/g tissue)				
EWAT	5.29 ± 0.46	6.47 ± 1.09	6.65 ± 0.76	
IWAT	42.9 ± 6.0 ^a	26.7 ± 1.6 ^b #	36.4 ± 5.9 ^{a,b}	D
MWAT	9.97 ± 1.1	10.2 ± 1.2	13.0 ± 1.5	
RWAT	7.35 ± 0.91	5.41 ± 0.52	7.70 ± 0.80	
Weight of kidney (g)	1.32 ± 0.04 ^b	1.23 ± 0.05 ^b	1.45 ± 0.04 ^a #	D
Kidney/body weight ratio (%)	0.27 ± 0.01 ^b	0.25 ± 0.01 ^b	0.32 ± 0.01 ^a #	D

Table 3. Liver and muscle parameters analyzed in the same animals and conditions described in Table 2

	control group	HF group	HP group	ANOVA
Liver weight (g)	13.9 ± 0.4 ^a	11.9 ± 0.3 ^b #	13.9 ± 0.3 ^a	D
Liver glycogen content (mg glycogen/ g tissue)	25.3 ± 1.5 ^a	16.8 ± 1.4 ^b #	22.3 ± 3.9 ^{a,b}	
Lipid content (mg lipid/g tissue)				
Liver	42.6 ± 3.3 ^b	68.7 ± 6.6 ^a #	38.8 ± 1.3 ^b	D
Muscle	14.8 ± 1.3	15.3 ± 1.3	13.0 ± 0.7	
TG content (mg TG/ g tissue)				
Liver	22.7 ± 3.1 ^b	48.9 ± 6.7 ^a #	39.9 ± 6.8 ^b	D
Muscle	54.5 ± 14.6 ^b	121 ± 21 ^a #	46.8 ± 6.8 ^b	
Protein content (mg protein/g tissue)				
Liver	16.3 ± 0.8 ^b	19.5 ± 0.6 ^a #	15.7 ± 0.4 ^b	D
Muscle	43.1 ± 2.4	38.0 ± 1.7	44.2 ± 1.9	

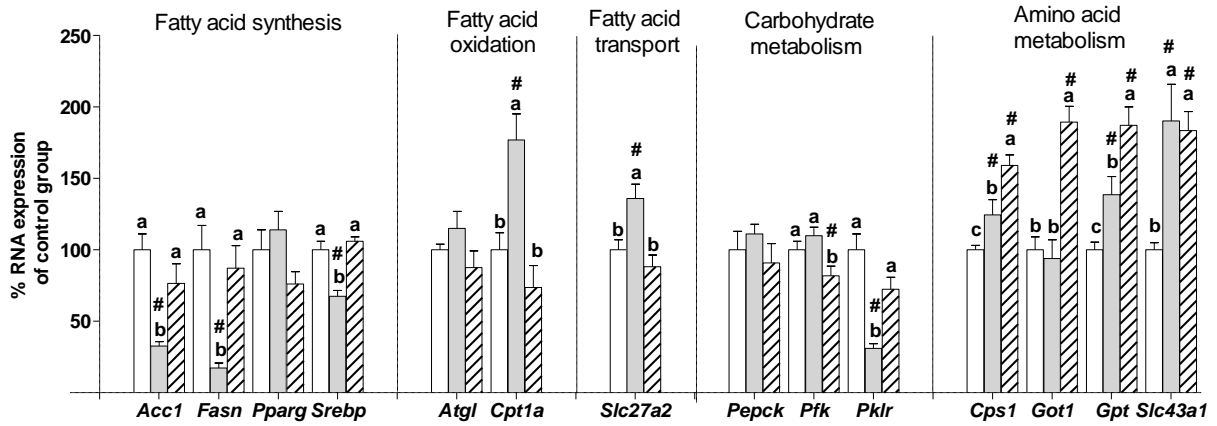
Table 4. Serum parameters analyzed in the same animals described in Table 2

	control group	HF group	HP group	ANOVA
Glucose (mg/dl)				
Feeding	97.1 ± 4.4 ^b	107 ± 2.4 ^a	91.5 ± 3.0 ^b	D
Fasting	80.5 ± 4.3 [*]	86.4 ± 1.4 [*]	87.8 ± 4.0	
Insulin (µg/l)				
Feeding	1.23 ± 0.22 ^b	0.88 ± 0.21 ^b	2.12 ± 0.27 ^a #	D
Fasting	0.36 ± 0.08 [*]	0.82 ± 0.08 [#]	0.37 ± 0.16 [*]	D
HOMA index	2.17 ± 0.49 ^b	4.18 ± 0.49 ^a #	1.85 ± 0.65 ^b	D
Leptin (pg/µl)				
Feeding	9.27 ± 0.72	9.68 ± 0.87	8.58 ± 0.86	
Fasting	5.78 ± 0.61 [*]	6.66 ± 0.51 [*]	4.43 ± 0.54 [*]	
TNF-alpha (pg/ml)	2.64 ± 0.55 ^b	5.90 ± 0.75 ^b #	13.3 ± 5.7 ^a #	
Ghrelin (ng/ml)	1.75 ± 0.12 ^c	3.40 ± 0.30 ^b #	5.12 ± 0.48 ^a #	
Triacylglycerols (mg/ml)	7.08 ± 0.63 ^a	4.31 ± 0.43 ^b #	6.93 ± 1.02 ^a	
Free fatty acids (µM)	0.62 ± 0.07	0.63 ± 0.09	0.52 ± 0.05	
Beta hydroxybutyrate (µmol/ml)	-	0.21 ± 0.13	-	D
Total Cholesterol (mg/dl)	1.54 ± 0.21 ^a	1.75 ± 0.32 ^a	0.92 ± 0.16 ^b #	
Urea (mg/dl)	5.08 ± 0.26 ^b	4.22 ± 0.27 ^c #	8.10 ± 0.68 ^a #	
Creatinine (mg/dl)	0.81 ± 0.09 ^{a,b}	1.12 ± 0.21 ^a	0.55 ± 0.07 ^b	D

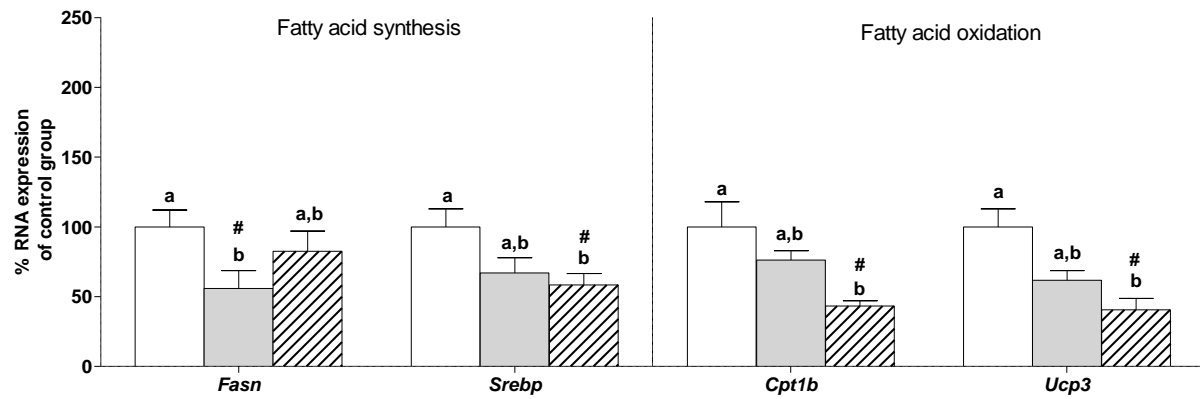
Figure 1

□ Control
 ■ High fat
 ▨ High protein

a) Liver



b) Muscle



c) Hypothalamus

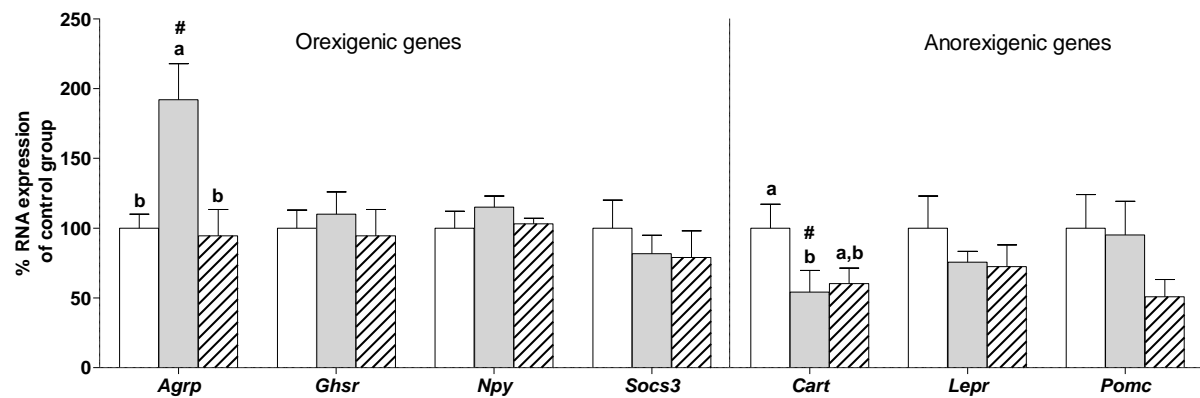


Figure 2

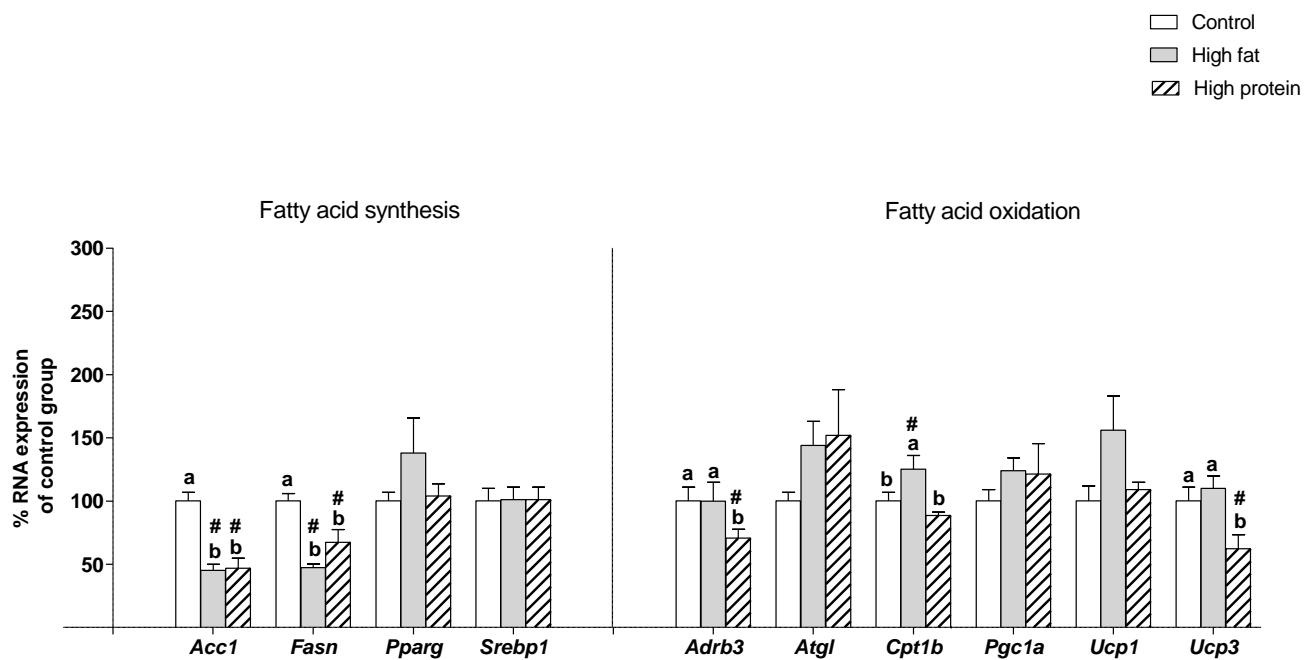


Figure 3

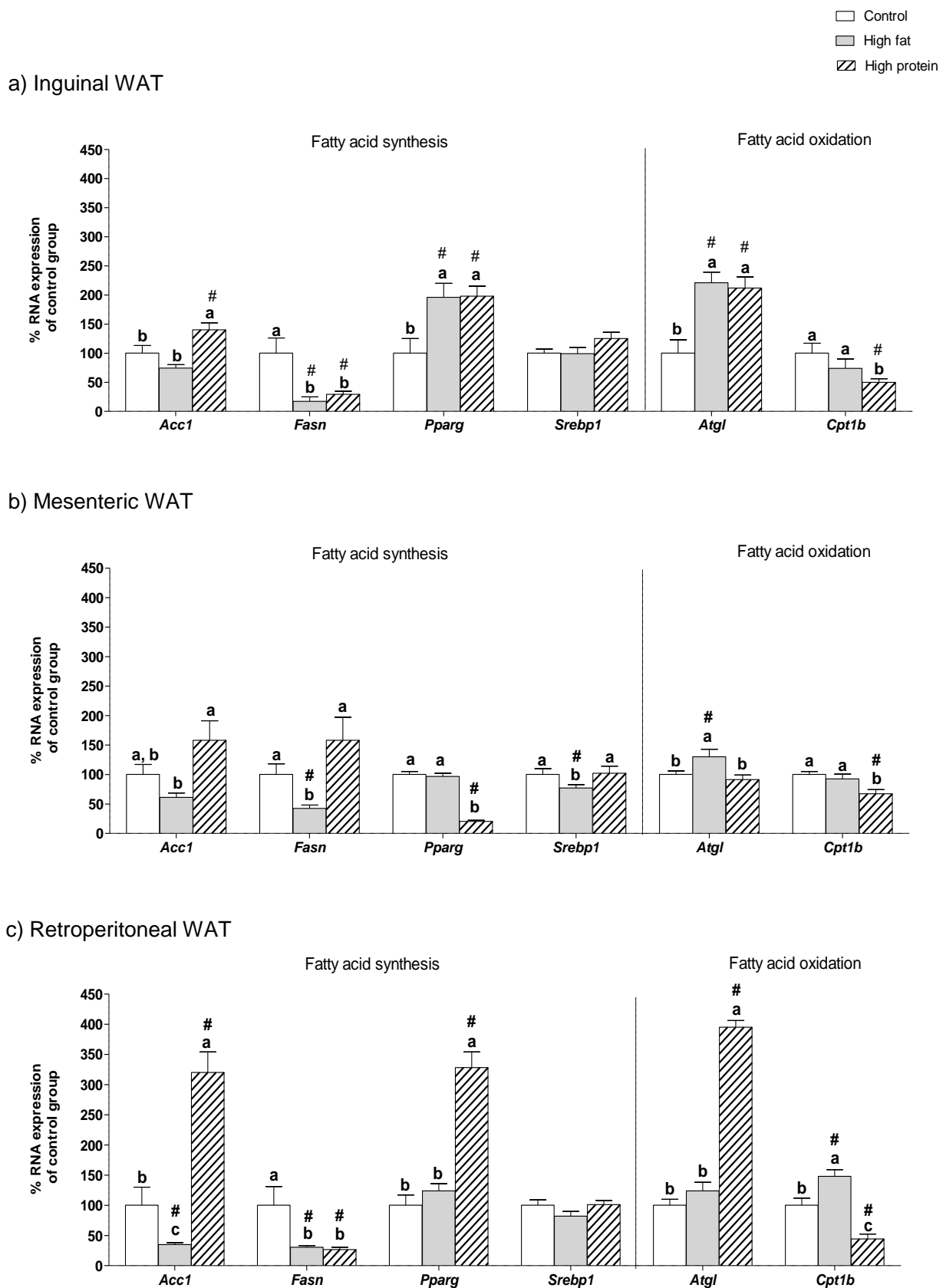


Figure 4

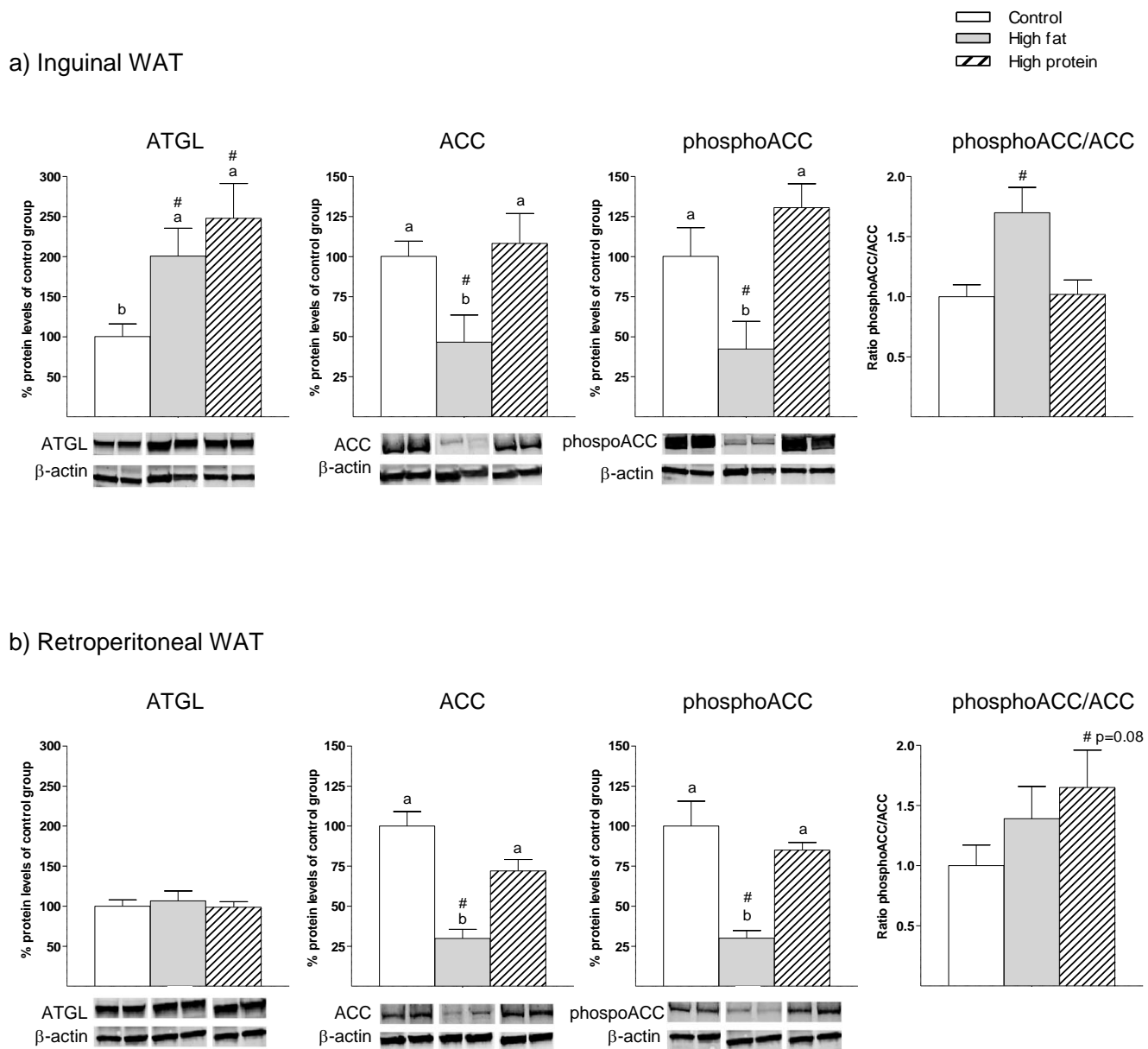
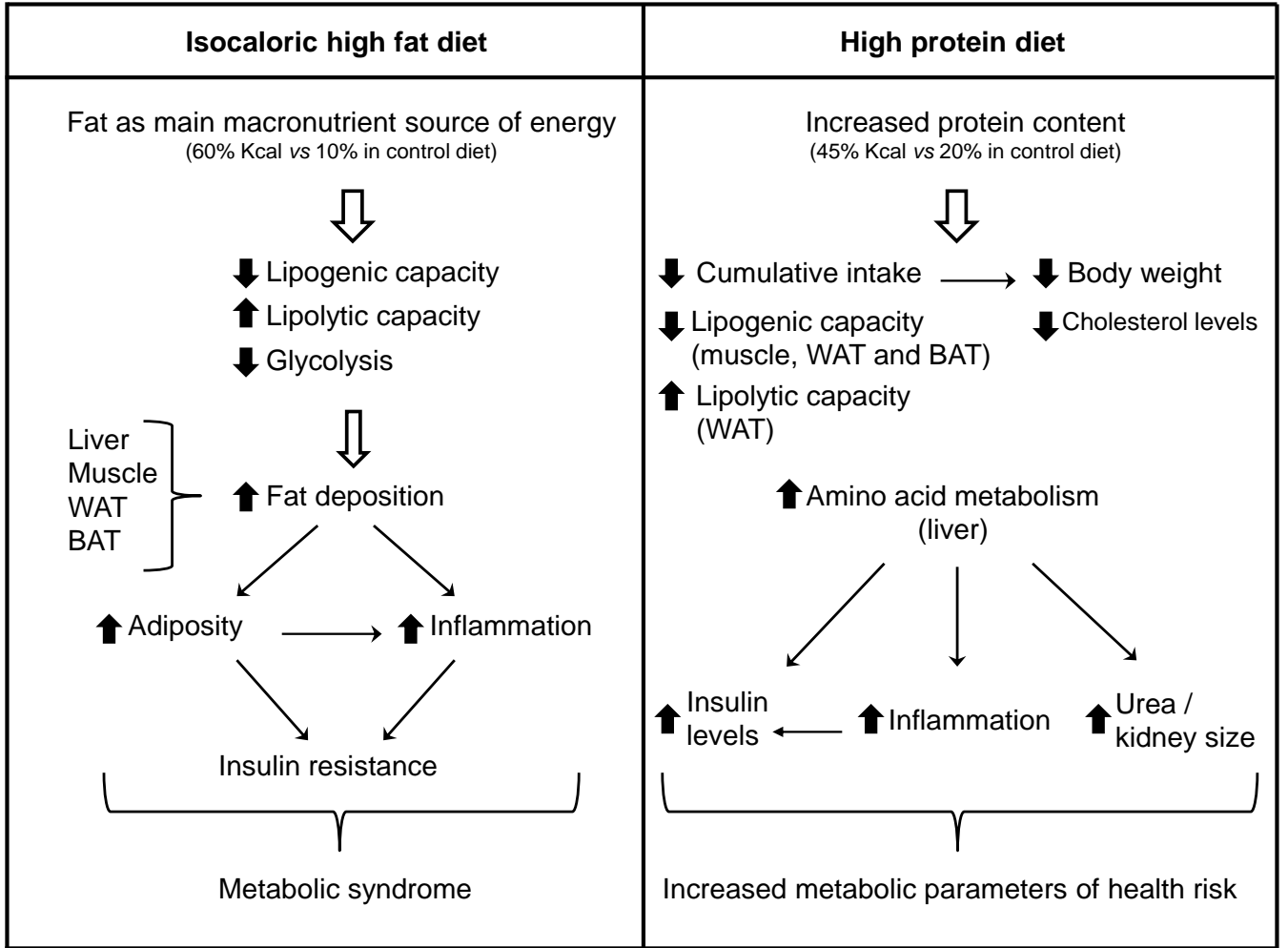
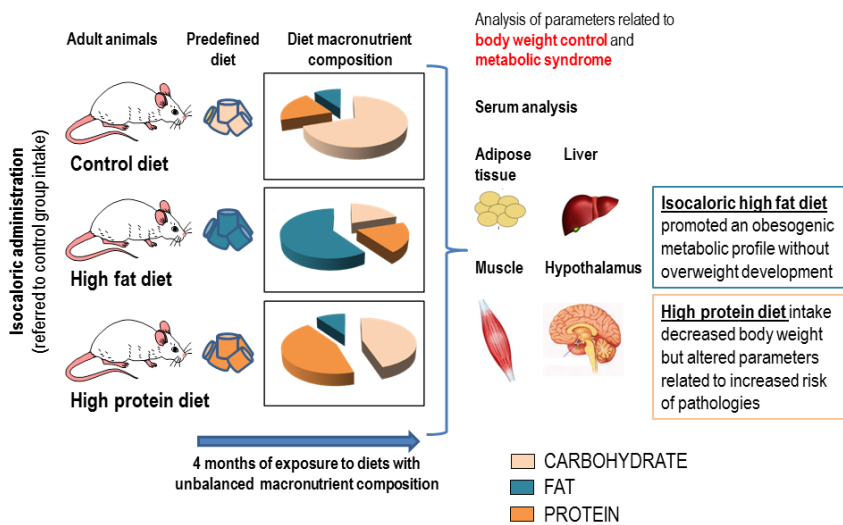


Figure 5



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Highlight

Chronic intake of diets with high proportion of fat or protein administered in isocaloric conditions to a control balanced diet is associated to changes in metabolic parameters related to adiposity and health.