A Restricted Cafeteria Diet ameliorates biometric and metabolic profile in a rat diet-induced obesity model

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Abstract

The administration of anti-obesity bioactive compounds and/or functional foods in rodents fed energy restriction diets based on chow food can be difficult to interpret. We propose an energy restricted cafeteria (CAF) diet as a dietetic intervention to be combined with other therapies. Postweaning male rats were fed standard chow, CAF diet or 30% energy restricted CAF diet (CAF-R) for 8 weeks. The CAF-R diet lowered energy intake and the increase of body weight and body mass index due to the CAF diet, lead to an intermediate feed efficiency, and dampened the CAF diet-induced alterations on body composition, serum levels of triacylglycerides and NEFAs, and insulin resistance. These effects were associated with diminished *Ucp1*, *Nrf1* and *Tfam1* gene expression in brown adipose tissue. In conclusion, the CAF-R diet ameliorated obesity and related metabolic disorders induced by a regular CAF diet, turning it in a useful tool to study anti-obesity compounds.

Keywords: body weight gain; energy restriction; cafeteria diet; metabolic syndrome; dietary treatment; adaptive thermogenesis

Introduction

Obesity and its comorbidities have been recognized as a worldwide health problem (Yumuk et al. 2015). It can lead to Metabolic Syndrome (MetS), a cluster of interconnected risk factors of cardiovascular disease including insulin resistance, obesity and dyslipidaemia (Saklayen 2018). Many factors, such as genetic predisposition, easy availability of energy-dense food and a sedentary lifestyle have contributed to the rise in obesity prevalence (Cui et al. 2017).

Several animal models have been developed to study human obesity. In some of them, animals develop obesity because of genetic or spontaneous mutations, while in others, obesity is induced by exposing animals to specific high-energy diets, such as the high-fat and/or high-sugar diets (Lutz 2018). The cafeteria diet (CAF) is a robust model of diet-induced obesity (DIO), consisting of exposing the rodents to high-energy highly palatable human foods promoting hyperphagia and a rapid weight gain (Sclafani and Springer 1976; La Fleur et al. 2010; Sampey et al. 2011; Lalanza et al. 2012, Lalanza et al. 2014; Leigh et al. 2019). It consists of supplementing rodents' standard chow diet with a variety of junk food fit for humans, like cakes, savoury snacks, cheese, and sugared milk. CAF diets induce the metabolic disorders associated with obesity and MetS in humans (hyperleptinemia, hypertension, hypertriglyceridemia, hyperglycaemia and insulin resistance) (Caimari et al. 2017; Mayneris-Perxachs et al. 2019).

Lifestyle modifications (diet and exercise), surgical interventions, pharmacology and, potentially, the supplementation of different bioactive compounds are widely used interventions to treat obesity and metabolic-related abnormalities in humans. The administration of hypocaloric diets (calorie-restricted diets) is effective in producing weight loss and health improvements but can induce a rebound body weight effect and increases anxiety and stress. The effects of functional foods and bioactive compounds are been investigated in rodent models. However, the effects of administering supplementary foods and/or bioactive compounds in animals fed with calorie restricted diets based on chow food can be difficult to interpret since the observed body weight reduction could be caused by the calorie restriction itself, or the supplementary item, or both.

Here, we have characterised an experimental cafeteria-restricted diet (CAF-R)

consisting of rodents' standard chow supplemented with small portions of palatable CAF items. Specifically, the CAF-R diet contains 30% calorie restricted CAF items (in relation to the calorie intake of the CAF-fed group) in addition to chow.

It is expected that the consumption of this CAF-R diet will decrease body weight and the metabolic disorders associated with the ad libitum consumption of the CAF diet but not as quickly or drastically as withdrawal with a normolipidic diet does. Therefore, this experimental strategy can emerge as a more realistic and precise tool to evaluate the anti-obesity effects of a caloric restriction-based intervention combined with other therapeutic strategies, such as bioactive compounds or functional foods.

Materials and methods

2.1 Animals and general procedures

Twenty-seven (23-25 day-old) male Sprague-Dawley rats were used (Harlan Laboratories, Barcelona, Spain). The animals were housed individually at 22 °C under a light/dark period of 12 h (lights on at 08:00 am) with chow and water availability ad libitum. After one week of habituation to the animal facility, the rats were assigned to three treatment groups (n = 9) with equivalent average of initial body weights: a group fed with a STD diet ad libitum; a group fed with a CAF diet ad libitum and a group fed with a CAF-R diet. Once a week, body weight and food consumption were recorded. At the end of the experiment, the animals were sacrificed by decapitation after 8 h of fasting. Total blood was collected and serum was obtained by centrifugation at 2000 g and 4 ºC for 15 min and stored at -80 ºC until further analysis. The liver; gastrocnemius and soleus muscles; white adipose tissue (WAT) depots (retroperitoneal -RWAT-, mesenteric -MWAT-, epididymal -EWAT- and inguinal -IWAT-); and interscapular brown adipose tissue (IBAT) were rapidly removed, weighed, frozen in liquid nitrogen and stored at -80 ºC. A macroscopic examination of all tissues removed were performed before storage.

All animals received human care under an institutionally approved experimental animal protocol, following the legislation applicable in Spain. The Animal Ethics Committee of the Technological Unit of Nutrition and Health of EURECAT (Reus, Spain) and the Generalitat de Catalunya approved all procedures (DAAM 9796). The experimental

protocol complied with the ARRIVE guidelines, followed the 'Principles of laboratory animal care' and was carried out in accordance to the EU Directive 2010/63/EU for animal experiments.

2.2 Diets

The animals were fed with the corresponding diets daily for 8 weeks until the end of the study. Food consumption was calculated as the difference between the food of each dietary component provided and the unconsumed that was remaining 24 h later. The CAF diet included (average quantity administered per rat/day): bacon (3 g); biscuit with pâté (4 g); biscuit with cheese (4 g); muffins (4 g); carrots (3 g); jellied sugared milk (44 g, 18% sucrose w/w); and standard chow (25 g). Mean total food and energy consumed per day were 55.6 g/day and 104 kcal/day, respectively. The caloric distribution of the CAF diet was 14% protein, 27% fat and 59% carbohydrates. The CAF-R diet was based on the same items and had a very similar qualitative composition than the CAF diet, but the amount of each food item administered was readjusted every week with a 30% calorie restriction relative to the energy intake consumed by the CAF group. Averaged over the 8 weeks, the CAF-R diet included (average quantity administered per rat/day): bacon (1 g); biscuit with pâté (2.5 g); muffins (1.5 g); carrots (2.5 g); jellied sugared milk (14.1 g, 18% sucrose w/w); and standard chow (13 g). Mean total food and energy consumed per day was 30.9 g/day and 70 kcal/day, respectively. The caloric distribution of this diet was 16% protein, 25% fat and 59% carbohydrates. The standard chow (Teklad Global 18% Protein Rodent Diet 2018, Harlan) was administered ad libitum to control animals. The caloric distribution of STD diet was 24% protein, 18% fat and 58% carbohydrates. Mean total standard chow consumed per day was 19 g/day (59 kcal/day).

2.3 Body composition analyses

Lean, fat and water mass measurements (in grams) were performed without anaesthesia on weeks 0, 4 and 8 by quantitative nuclear magnetic resonance (qNMR) which provides a precise measurement of body composition (Taicher et al. 2003) using an EchoMRI™-700 device (Echo Medical Systems, L.L.C., Houston, USA). All the measurements were performed in triplicate. Data are expressed in relative values as a percentage of body weight (g/g).

2.4 Body mass index

At the final point, body length was measured as the nose:tail length in order to estimate the body mass index (BMI) using the following formula: weight (g)/body length $(cm)^2$.

2.5 Feed efficiency

Feed efficiency (FE) was calculated as the quotient between the final body weight gain (in grams) and the total energy (in kilocalories) consumed during the entire study.

2.6 Adiposity index

The adiposity index was determined as the sum of the EWAT, IWAT, MWAT and RWAT depot weights (in grams) and expressed as a percentage of body weight (g/kg). Visceral WAT referred to the sum of RWAT, MWAT and EWAT, while subcutaneous WAT was considered as the IWAT.

2.7 Serum analyses

Enzymatic colorimetric kits were used for the determination of serum total cholesterol, triglycerides and glucose (QCA, Barcelona, Spain), HDL-cholesterol and LDL/VLDL-cholesterol (Bioassay systems, CA, USA) and non-esterified free fatty acids (NEFAs) (WAKO, Neuss, Germany). Serum insulin, leptin and monocyte chemoattractant protein-1 (MCP-1) levels were measured using a mouse/rat insulin ELISA kit (Millipore, Barcelona, Spain), a rat leptin ELISA kit (Millipore), and a rat MCP-1 ELISA Kit (Thermo Scientific, Rockford, IL, USA), respectively. The homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated following the formula: HOMA-IR = Glucose X Insulin/22.5, according to (Matthews et al. 1985). Additionally, the insulin sensitivity was assessed by the revised quantitative insulin sensitivity check index (R-QUICKI) using the following formula: 1/[log insulin (μU/mL) + log glucose (mg/dL) + log free fatty acids (mmol/l)] (Perseghin et al. 2001).

2.8 ELISA analyses

Total and phosphorylated p70S6K protein levels from gastrocnemius muscle were determined by a commercially available ELISA kit (Abcam). Briefly, 100 mg of

gastrocnemius muscle samples were homogenized in 500 µL 1X Cell Extraction Buffer using a tissue homogenizer (Bullet Blender 24, Next Advancer, New York, USA). After remaining on ice for 20 min, samples were centrifuged at 18000 g for 20 min and the supernatant with total protein content was collected and stored at -80ºC. The protein concentration was determined by the Pierce BCA Assay kit (Thermo Fisher). A total of 50 µL of each sample were necessary to determine the total and phosphorylated protein levels.

2.9 Western blot analyses

Total MuRF1 (40 kD) and MAFbx32 (42 kD) protein levels in the gastrocnemius muscle were determined by western blot technique. Fifty mg of gastrocnemius muscle samples were homogenized in 1.5 mL lysis buffer (15 mM Tris-HCl pH 7.4, 0.5% sodium deoxycholate (NaD), 167 mM sodium chloride (NaCl), 1% Triton X- 100, 0.1% sodium dodecyl sulphate (SDS)) using a hand Ultra-Turrax® Homogenizer (IKA Company, China). After remaining on ice for 30 min, samples were centrifuged at 10000 g for 10 min and the supernatant with total protein content was collected. A total of 50 µg of protein per sample was solubilised and boiled at 95°C for 10 min in a 5X Loading buffer solution (0.5 M Tris–HCl pH 6.8, 30% glycerol, 2% SDS, 10% β-mercaptoethanol, 0.05% bromophenol blue), separated by SDS-PAGE on a 4-10% polyacrylamide gel and then transferred to a PVDF membrane (Millipore). The membranes were blocked in 5% BSA in TBS 1X (100 mM Tris-base pH 8.0, 1.5 M NaCl) for 1 h at room temperature, followed by an overnight incubation at 4°C with primary antibodies rabbit anti-MuRF1 and rabbit anti-MAFbx32 (1:5000; Abcam) diluted in 5% BSA in TBS-T (1X TBS, 0.1% Tween 20). Membranes were then washed and incubated for 1 h at room temperature with goat anti-rabbit secondary antibody (LI-COR, USA) and diluted 1/10000. Protein loading was monitored by immunodetection of β-Tubulin (49 kD). Thus, membranes were incubated with a rabbit anti-β-Tubulin primary antibody (1:1000, Abcam) and then with a goat anti-rabbit secondary antibody using the same dilution referred previously. Protein bands were detected and quantified by ODYSSEY CLx (LI-COR, USA). The signals for both MuRF1 and MAFbx32 proteins were relativized to the signal of the corresponding β-Tubulin protein, and the results were expressed as MuRF1/β-Tubulin and MAFbx32/β-Tubulin ratios.

2.10 Gene expression analyses

A total of 50 mg of IBAT samples were homogenized in 600 µL of Tripure Isolation Reagent (Sigma-Aldrich, Madrid, Spain) using a tissue homogenizer (Bullet Blender 24, Next Advancer). Quantification and quality (260/280 and 260/230 contamination ratios) of the RNA obtained were analysed in duplicate using a NanoPhotometer™ P-300 (BioNova Científica S.L., Madrid, Spain). RNA integrity was checked by agarose gel electrophoresis. The cDNA was synthesized from 0.5 µg/mL of total RNA using the MuLV reverse transcriptase (Applied Biosystems, Madrid, Spain) and the following RT-PCR program: 42 ºC for 60 minutes, 90 ºC for 5 minutes and stored at 4 ºC. The cDNA synthesized was disposed to the Q-PCR technique using the LightCycler 480 II System with SYBR Green I Master Mix (Roche Diagnostic Barcelona, Spain). The Q-PCR program was set as follows: 95 ºC for 5 min, 45 cycles of 94 ºC for 10 s, 60 ºC for 10 s, 72 ºC for 10 s. Each PCR was performed in duplicate. The relative expression of each mRNA was calculated using the 2-ΔΔCt method (Livak and Schmittgen 2001) with hypoxanthine guanine phosphoribosyl transferase (*Hprt*, for: 5'-TCCCAGCGTCGTGATTAGTGA-3', rev: 5'-CCTTCATGACATCTCGAGCAAG-3') selected as reference gene. Results were expressed as a percentage of the STD group. The primer sequences used to amplify the different genes analysed were: *Ucp1*, for: 5'-GGGCTGATTCCTTTTGGTCT-3', rev: 5'-GGTGGTGATGGTCCCTAAGA-3'; *Nrf1*, for: TTGTTTCCCACTCACCCATT, rev: 5'-GTCACTCCGTGTTCCTCCAT-3'; *Tfam1*, for: 5'-CAGGGGGCTAAGGATGAGTC-3', rev: 5'-ACACTGCGACGGATGAGAT-3' and *Pgc1*, for: 5'-AGGAGGGTCATCGTTTGTGG-3', rev: 5'-GGAGGCAGAAGAGCCGTC-3' (Biomers.net, Ulm, Germany).

2.11 Statistical analyses

Statistical analyses were performed using SPSS Statistics 22 (SPSS, Inc., Chicago, IL, USA). The homoscedasticity among groups was measured using Levene's test. Grubbs' test was used to determine significant outlier values and consequently, discard for the analyses. One-way analysis of variance (ANOVA) followed by Duncan post hoc tests for comparisons between groups were performed to evaluate differences on biometric measures, nutrient consumption, energy intake and serum and molecular analyses. The evolution of body weight, total energy intake and body composition (lean, fat and water

mass) during the study were analysed by a repeated measures ANOVA with week as a within-subject factor and diet as between-subject factor. All the results were expressed as mean±SEM. The level of statistical significance was set at bilateral 5%.

Results

3.1 Body weight and BMI

The body weight increased over the weeks in all groups (ANOVA 'week': $F(8,192)=2519.535$, $P<0.001$). CAF-fed animals, but not CAF-R fed animals, showed a greater increase of body weight over weeks compared with STD-fed animals (ANOVA 'week x group': $F(16,192)=2.390$, $P=0.003$). The higher body weight of CAF animals was also corroborated by a significant diet effect ($F(2,24)=5.999$, $P=0.008$). Body weight of CAF animals was higher than STD animals in weeks 6, 7 and 8, and higher than CAF-R animals during the whole study (Figure 1A). At the end of the study, total body weight gain had increased by 467.3% in the CAF group, by 436.6% in the STD group and by 396.5% in the CAF-R group. Consistently, at the end of the experiment the CAF group showed increased BMI compared with the STD and CAF-R groups, whereas the CAF-R group showed intermediate values between STD and CAF groups (ANOVA, $F(2,24)=4.142$, $P=0.0288$) (Figure 1B).

[*Figure 1 near here*].

3.2 Food and energy intake

The relative food intake (food intake relativized to the body weight) decreased over weeks of the experiment (ANOVA 'week': $F(7,168)=177.191$, $P<0.001$). CAF-fed animals had a higher food intake than STD and CAF-R groups over weeks (ANOVA 'week x group': $F(14,168)=13.501$, $P<0.001$) (Figure 2A). CAF-R-fed rats exhibited intermediate values between STD and CAF groups (ANOVA, diet: $F(2,24)=345.960$, $P<0.001$). CAF-fed animals showed a lower chow intake than the STD-fed animals, whereas the CAF-R-fed animals displayed a higher chow intake (ANOVA, $F(2,24)=201.501$, $P<0.001$; Figure 2B) and a lower non-chow intake than the CAF group (Student's $t(16)=11.125$, $P<0.001$).

As in the case of food intake, the relative energy intake (relativized to body weight)

decreased over weeks (ANOVA 'week': $F(7,168)=217.987$, $P<0.001$). CAF-fed animals exhibited the highest energy intake over weeks, that was higher than both STD and CAF-R groups (ANOVA 'week x group': $F(14,168)=5.840$, $P<0.001$) (Figure 2C). Likewise, CAF-R animals showed intermediate energy intake values between CAF and STD groups during nearly the entire experiment (ANOVA, diet: $F(2,24)=146.473$, $P<0.001$), except at weeks 4 and 8. At the final time point, the CAF-fed animals exhibited a lower feed efficiency (FE) than both STD and CAF-R groups; the CAF-R-fed rats showing a higher FE compared to CAF group but lower than STD group (ANOVA, $F(2,24)=83.979$, $P<0.001$; Figure 2D). Therefore, STD-fed animals displayed the highest efficiency of energy conversion, CAF-fed animals displayed the lowest and CAF-R-fed animals displayed intermediate values.

[*Figure 2 near here*].

As expected, the CAF-fed animals showed the highest energy intake, followed by CAF-R in second place and then by STD who showed the lowest intake values (Table 1, first row) (ANOVA, $F(2,24)=146.473$, $P<0.001$). CAF-fed animals consumed a higher quantity of carbohydrates, fat and salt than STD and CAF-R groups. The protein intake was reduced in CAF and CAF-R groups compared with the STD. Except for proteins, the CAF-R group showed intermediate intake values between CAF and STD groups (ANOVA: carbohydrates: $F(2,24)=175.178$, $P<0.001$; fat: $F(2,24)=203.598$, $P<0.001$; salt: $F(2,24)=124.167$, $P<0.001$; proteins: $F(2,24)=44.698$, $P<0.001$). CAF-R-fed animals consumed half the amount of simple sugars compared with CAF-fed animals (Student's $t(16)=11.801$, $P<0.001$).

[*Table 1 near here*].

3.3 Body composition

In order to analyse whether dietary treatments induced changes in the body composition, nuclear magnetic resonances were performed at weeks 0 (baseline), 4 and 8 of the experiment. The relative fat mass changed over the experiment (ANOVA 'week': $F(2,48)=20.433$, $P<0.001$). The STD-fed animals maintained a nearly constant relative fat mass composition with a variation lower than 2%, whereas the other two dietary treatments induced changes in this parameter over weeks (ANOVA 'week x

group': F(4,48)=24.099, P<0.001). Specifically, the CAF group displayed an elevated relative fat mass which was increasing over time and was always higher compared to both STD and CAF-R animals (Figure 3A). The CAF-R group showed a soft increase of the relative fat mass, with intermediate values between CAF and STD groups (ANOVA, diet: F(2,24)=52.113, P<0.001) from week 4 to week 8. The relative lean mass also exhibited changes all through the experiment (ANOVA 'week': F(2,48)=44.584, P<0.001). As seen for the relative fat mass, STD group maintained a nearly constant relative lean mass composition (variation lower than 1%), whereas the CAF and CAF-R groups showed variations over weeks (ANOVA 'week x group': F(4,48)=16.011, P<0.001). In the CAF group, the relative lean mass decreased over weeks, with values lower than those of STD and CAF-R groups, whereas CAF-R animals showed intermediate values between STD and CAF-fed animals, although they also decreased all through the experiment (ANOVA, diet: F(2,24)=41.603, P<0.001) (Figure 3B). The relative body water composition decreased in all groups over the experiment (ANOVA 'week': F(2,48)=182.751, P<0.001), although there were differences among groups from week 1 to 8 (ANOVA 'week x group': F(4,48)=11.372, P<0.001). CAF group had a higher decrease than the other two groups and CAF-R-fed animals exhibited intermediate values between STD and CAF groups (ANOVA, diet: F(2,24)=29.251, P<0.001) (Figure 3C).

The differences in the relative lean mass between groups at week 8 were consistent with the diminution of the relative weight of gastrocnemius and soleus muscles in the CAF group compared with the STD group. No differences were observed in the weight of these muscles between CAF-R and STD groups (ANOVA: gastrocnemius: F(2,24)=5.531, P=0.011) (Figure 3D); soleus: F(2,24)=7.273, P=0.003) (Figure 3E).

[*Figure 3 near here*].

3.4 Adipose tissues depots

In regard of WAT depots and IBAT, CAF and CAF-R-fed animals displayed a higher relative white fat mass, both subcutaneous and visceral, and consequently, a higher adiposity index than STD group. CAF-R-fed animals decreased relative visceral WAT and adiposity index compared with CAF-fed animals (ANOVA: subcutaneous WAT: F(2,24)=20.257, P<0.001, Figure 4A; visceral WAT: F(2,23)=31.339, P<0.001, Figure

4B; adiposity index: F(2,23)=46.940, P<0.001, Figure 4C). The CAF group displayed the highest values of IBAT, showing a 50% higher relative IBAT weight than the STD group, which showed the lowest values, whereas the CAF-R group showed a 26% higher relative IBAT weight than the STD group and a 16% lower weight compared with the CAF group. (ANOVA, F(2,24)=13.937, P<0.001) (Figure 4D).

[*Figure 4 near here*].

3.5 Serum metabolic parameters

Several biochemical parameters associated with obesity were measured in serum at the end of the experiment (Table 2). CAF diet increased the circulating levels of insulin (ANOVA, F(2,23)=9.940, P=0.001) and insulin resistance, as was indicated by a greater HOMA-IR values (ANOVA, F(2,22)=10.183, P=0.001) and decreased R-QUICKI values (ANOVA, F(2,22)=7.924, P=0.003), compared to STD and CAF-R-fed animals. Nevertheless, no significant changes were observed in serum glucose levels among groups (ANOVA, F(2,24)=2.167, P=0.136). In addition, CAF diet increased circulating levels of triacylglycerides (ANOVA, F(2,24)=7.435, P=0.003) compared to the other groups, and NEFAs levels were also higher in CAF group (ANOVA, F(2,24)=3.764, P=0.038). No changes in either total or HDL/LDL cholesterol were detected among groups (ANOVA: total cholesterol: F(2,24)=1.040, P=0.369; HDL: F(2,24)=0.628, P=0.542; LDL/VLDL: F(2,24)=1.910, P=0.170). CAF diet also induced an increase in serum levels of leptin (ANOVA, F(2,24)=19.560, P<0.001), and tended to increase the inflammatory marker MCP-1 (student's t(16)=-2.088, P=0.053). Interestingly, CAF-R diet prevented hyperinsulinemia, the loss of insulin sensitivity and the hypertriglyceridemia associated to CAF diet, showing levels of these parameters similar to those of the STD group and lower than those of the CAF group. Likewise, the restriction of CAF foods induced a smaller increase in leptin levels, being those of the CAF-R group lower than the ones of the CAF group, as well as in MCP-1, whose levels in CAF-R group did not differ from those of CAF and STD groups.

[*Table 2 near here*].

3.6 Protein expression in skeletal muscle

In order to determine whether the lean mass loss observed in CAF-fed animals and to a lesser extent in CAF-R-fed animals (Figure 3B) was associated with changes in the muscle protein synthesis and/or degradation, the protein levels of p70S6K, Murf1 and MAFbx32 in the gastrocnemius muscle were analysed. However, results showed no significant differences in the levels of these proteins (ANOVA: p70S6K: $F(2,24)=0.872$, $P=0.431$; MuRF-1: $F(2,23)=1.176$, $P=0.326$; MAFbx32: $F(2,24)=2.784$, $P=0.082$) (Figure 5).

[*Figure 5 near here*].

3.7 mRNA expression in IBAT

To explore whether changes in FE and relative IBAT mass could be associated with changes in the expression of genes involved in thermogenesis and mitochondrial biogenesis in this tissue, we analysed the mRNA levels of *Ucp1*, and those of *Pgc1*, *Nrf1* and *Tfam1*. The results showed that CAF diet did not modify the expression of any of these genes (Figure 6), whereas CAF-R diet decreased the *Nrf1* and *Tfam1* mRNA levels (ANOVA, *Nrf1*: $F(2,22)=11.795$, $P<0.001$, Figure 6C; *Tfam1*: $F(2,22)=7.288$, $P=0.004$, Figure 6D). The same trend was observed in the case of *Ucp1*, whose mRNA levels diminished in the CAF-R group compared to the CAF group (Student's $t(14)=1.993$, $P=0.066$) (Figure 6A). No differences were found between groups in the mRNA levels of *Pgc1* (ANOVA $F(2,20)=0.114$, $P=0.893$) (Figure 6B).

[*Figure 6 near here*].

Discussion

In this study, the administration of the CAF diet to young male rats for 8 weeks increased body weight, BMI, food and energy intake, fat mass and visceral adiposity compared with STD diet. Furthermore, the CAF diet produced different metabolic alterations associated with obesity and MetS, such as hyperinsulinemia, insulin resistance, dyslipidaemia with elevated serum triglycerides and NEFAs levels, hyperleptinemia and mild inflammation. These results are in accordance with others indicating that CAF diet is a valid pre-clinical animal model of human obesity and its

co-morbidities (Gomez-Smith et al. 2016).

In addition, CAF diets induce hyperphagia and increase energy intake (Shafat et al. 2009), in part due to gut sensors that stimulate the preference for carbohydrate and fat-rich foods via an appetite process that activates the brain reward system (Sclafani 2018). Moreover, exposure to CAF diet can change feeding patterns in rats (Martire et al. 2013), alters gene expression in brain regions implicated in reward, and withdrawal from this diet alters gene expression in brain regions associated with stress (Martire et al. 2014). These properties and effects make CAF diet similar to the human Western obesogenic dietary pattern (Sampey et al. 2011).

We aimed to propose a dietary intervention based on cafeteria restriction, which would allow us to test the efficacy and the effects of other therapeutic strategies such as the use of bioactive natural compounds or functional foods. The problem of using standard chow diets to experimentally reverse obesity and the MetS features is that chow calorie restricted diets are very effective and produce their effects too much quickly. For example, a single month of switching CAF diet-fed rats to a standard chow diet has been shown to completely reverse these conditions (Gomez-Smith et al. 2016). Moreover, the effects of administering supplementary foods and/or bioactive compounds in animals fed with calorie restricted diets based on chow food can be difficult to interpret since the observed changes in the parameters analysed could be caused by the calorie restriction itself, or the supplementary item, or both.

Our proposal consists of exposing the animals to a 30% calorie restricted CAF items (in relation to the calorie intake of the CAF-fed group) in addition to a limited amount of chow (13 g/rat/day). As a result, the CAF-R-fed rats ate more chow (daily average) than the CAF-fed rats (11 g/rat/day vs 7 g/rat/day), decreased body weight, prevented BMI increase and ameliorated the fat mass accretion associated with the ad libitum consumption of the CAF diet, but not too drastically or quickly, as we previously mentioned.

Moreover, CAF-R-fed animals showed a healthier metabolic profile compared with the CAF-fed animals, the leptin levels being the only elevated serum parameter compared with the STD-fed animals. Unlike the CAF-fed animals, CAF-R-fed animals exhibited standard serum values of circulating insulin, insulin resistance, insulin sensitivity,

triacylglycerides, NEFAs and of the inflammation marker MCP-1. No changes in weight nor evident macroscopic lesions such as signs of steatosis or fibrosis in the liver were observed among CAF, CAF-R and STD groups. Since the liver plays a central role in all metabolic process including fat, carbohydrate and protein metabolism, a deeper analysis of its metabolic status (i.e. serum levels of alanine aminotransferase -ALT-, alkaline phosphatase -ALP-, aspartate transaminase -AST-) in CAF and CAF-R animals compared with STD animals is pending of another study.

Therefore, CAF-R-diet attenuated the alterations observed in the CAF group in obesity-related biochemical parameters, but without completely reversing the situation induced by the CAF diet. These results make us to hypothesise that the beneficial metabolic effects observed in our young adult rats, could also be reproduced in other developmental stages. In fact, similar effects of the CAF-R diet have been obtained in other studies from our group developed in older rats with different experimental designs. Altogether, the CAF-R diet appears to be a good model for studying the anti-obesity effects of bioactive compounds.

The analysis of the response to the CAF-R- diet administration revealed that animals fed with this diet weighted even slightly lower than the STD-fed animals during the first two weeks of the study. However, from the third week onwards, the body weights of the two groups were very similar, and the final BMI did not differ between them. Moreover, CAF-R-fed animals exhibited intermediate values of both absolute (data not shown) and relative food and energy intake between STD and CAF groups. Specifically, they consumed more standard chow than the CAF-fed animals, and both groups consumed less chow than the STD-fed animals (58% and 39%, respectively). Regarding the CAF food, i.e. the non-chow intake, the CAF-R-fed animals consumed nearly half the quantity that the CAF-fed animals did.

The greater consumption of calories coming from carbohydrate and fat of CAF-R-fed animals compared to the STD ones led to an increase in the relative fat mass and body fat stores, as was indicated by the resonance data, which was consistent with the increased weights of subcutaneous and visceral WAT depots and adiposity index at the final point of the study. These results were also in accordance with the increased serum levels of leptin observed in CAF-R-fed animals, since this hormone is produced primarily by adipose cells, and its levels are considered a marker of the adipose tissue

mass (Jéquier 2002). Both the CAF diet and, to a lesser extent, the CAF-R diet produced greater overall adiposity, which was associated with a lower body water content in these animals compared with the STD ones, in accordance with the low water content of adipose tissues (Yang et al. 2013).

Interestingly, the relative weight of subcutaneous WAT did not differ between the CAF-fed and CAF-R-fed animals, whereas the relative weight of the visceral WAT was different between the two groups, it being the highest in the CAF group and intermediate between CAF and STD in the CAF-R-fed group. This is important because recently, increasing evidence has shown that the subcutaneous WAT has an important role in accommodating the excess of energy intake, being protective and preventing the ectopic fat deposition in other tissues involved in metabolic homeostasis (Iacobini et al. 2019; Vishvanath and Gupta 2019). Therefore, the increase of subcutaneous WAT induced by the CAF-R diet could be indicating a reactive response induced by the increase of food and energy intake vs the STD diet. That increase of subcutaneous WAT would have prevented a greater increase in visceral WAT, which has been shown to be a better predictor of obesity-related metabolic abnormalities and cardiovascular disease (CVD) risk than total fat mass per se (Iacobini et al. 2019; Vishvanath and Gupta 2019). The analysis of the adipocytes' morphology and the quantification of several inflammatory markers on that tissue should confirm this hypothesis.

The fact that CAF-R-fed animals increased food and energy intake compared with STD-fed animals while there were no differences in the body weight between these two groups from week 3 onwards indicated a lower FE due to the CAF-R diet compared to the STD diet. This difference could be explained by the different body composition of animals fed each diet, already commented. Thus, the increased fat mass of the CAF-R fed animals might derive from their greater intake of fat and sugar, while their decreased lean mass might derive from their lower absolute (data not shown) and relative protein intake. As it is well known, protein intake is necessary for lean mass development while excessive intake of sugar and fat is accumulated in the form of adipose tissue (Carbone et al. 2012; Engin 2017). However, the lower FE shown by the CAF-R group compared to the STD group could also be explained, at least in part, by the activation of compensatory mechanisms in the former group, such as adaptive thermogenesis and perhaps the mitochondrial biogenesis process. The relative mass of

IBAT in the CAF-R-fed group was intermediate between STD-fed and CAF-fed animals. The CAF-fed animals had increased body weight and white fat depots and displayed the lowest FE and the highest weight of IBAT among the three dietary interventions, thus indicating that these animals could also lose usable energy as heat by diet-induced non-shivering thermogenesis (DIT). It has been well described that CAF diets increase DIT and cause BAT hypertrophy (Rothwell et al. 1982; Rothwell 1997). BAT, which is rich in mitochondria, plays an important role in DIT and energy expenditure. This process allows the dissipation of energy through the production of heat by uncoupling respiration from ATP synthesis (Cannon and Nedergaard 2004). UCP1 was the first uncoupling protein identified in BAT. Later, other UCPs were identified based on sequence homology (Boss et al. 1997; Fleury et al. 1997). In addition to thermogenesis, the activation of mitochondrial biogenesis processes can also increase the energy expenditure of the animals. This process is regulated by the transcriptional coactivator PGC1, which is responsible for the increase in expression of transcription factors such as NRF1, which in turn stimulates the expression of the mitochondrial transcription factor TFAM1 (Scarpulla 1997; Wu et al. 1999). Moreover, PGC1 regulates UCP1 expression by acting on PPARγ activation in BAT mitochondria (Puigserver et al. 1998).

Interestingly, the relative daily food and energy intake decreased progressively over the study in all experimental groups, the decrease was high during weeks 1-3, and slowed down from weeks 3-4 onwards. This intake pattern would be related to the growth rate of the animals during the period analysed, where adolescent to early-adulthood transition took place and energy requirements changed. However, although CAF-R and STD groups had a similar energy intake at the end of the study, the consumption of protein, fat and carbohydrate was very different between them, the protein intake being always lower and fat and sugar intakes higher in the CAF-fed and CAF-R-fed animals than in the STD ones. These results would be in accordance with those of (Rothwell and Stock 1987) who showed that changes in energy balance, thermogenesis and BAT activity that result from dietary protein deficiency cannot be ascribed to changes in the level of energy intake or to a specific increase in the amount or proportion of either carbohydrate or fat intake, but rather to the protein-to-energy ratio (Rothwell and Stock 1987).

When we analysed the expression levels of several genes involved in thermogenesis and mitochondrial biogenesis, we found that CAF-R-fed animals showed the lowest values of IBAT *Ucp1*, *Nrf1* and *Tfam1* expression among the three dietary groups. Thus, the decrease in the energy intake of CAF-R-fed animals at the end of the experiment (last weeks, by reaching similar values to the STD group at the last one) could have induced a downregulation of these genes in the increased IBAT (compared with the relative IBAT of the STD group) due to the imbalance in macronutrient intake. This downregulated gene expression may be a compensatory effect in order to decrease energy expenditure by UCP1-dependent thermogenesis as well as NRF1 and TFAM1-regulated mitochondrial biogenesis. These results are consistent with (Palou et al. 2015), who observed that BAT thermogenic capacity was diminished in calorie restricted animals, and that the effect included a decreased expression of BAT *Ucp1*.

By contrast, in the present study, no change in the IBAT expression of *Ucp1* or of mitochondrial replication-related genes such as *Pgc1*, *Tfam1* and *Nrf1* appeared in the CAF-fed animals, thus suggesting that other mechanisms associated with energy expenditure and FE were probably functioning in these animals. The presence of UCP1-independent thermogenic regulators, even in other tissues than IBAT, such as creatine (Bertholet et al. 2017), N-acyl amino acids (Long et al. 2016) or leptin (Mottillo et al. 2014) can also increase the energy expenditure and decreases FE. Anyway, changes in the levels of IBAT UCP1 protein as well as in the expression in *Ucp1*-containing cells in WAT cannot be discarded. IBAT *Ucp1* mRNA and protein expression increase in rats fed high-fat diet (Fromme and Klingenspor 2011), and mRNA expression levels of *Ucp2* (Roca et al. 1999) or *Ucp3* (Margareto et al. 2001) increase in CAF diet-fed rats. Therefore, further analyses of other UCP family members may be useful to gain insight into the regulation of thermogenesis and metabolic efficiency by CAF diet.

Regarding the lack of differences in the *Pgc1* mRNA expression among groups, it must be considered that this transcription factor is a central modulator of cell metabolism, acting both on controlling mitochondrial biogenesis and on oxidative phosphorylation, under tight regulatory mechanisms. Alterations in its activity and expression have been associated with many diseases in different tissues (Wu et al. 1999; Liang and Ward 2006).

The diminution of the lean mass in both CAF and CAF-R groups and the diminution of

relative weight of gastrocnemius and soleus muscles in the CAF group compared to STD group, lead us to study whether CAF diets had negatively affected the proper development of skeletal muscles. The lack of differences in the weight of gastrocnemius or soleus muscles between CAF-R and STD groups suggests that other skeletal muscles would be responsible for the lower lean mass observed. We analysed the protein expression levels of p70S6K, a ribosomal kinase involved in the mTORC1 (mammalian target of rapamycin complex-1) signalling pathway, and of the muscle-specific ubiquitin ligases MuRF-1 and MAFbx which participate in protein breakdown. The mTORC1 complex regulation plays a key role in the skeletal muscle protein synthesis and it can be activated by several inputs such as the presence of growth factors, energy status, amino acids and mechanical stimuli such as physical activity (Adegoke et al. 2012). Nevertheless, our results showed no differences among groups in the gastrocnemius protein levels of p70S6K, MuRF-1 or MAFbx, suggesting that effects of CAF diets on these proteins could be exerted at other levels, such as mRNA expression, and/or regulation of other protein turnover-related mechanisms in skeletal muscle. Furthermore, it would be interesting to investigate whether the diminution of the relative lean and skeletal muscle mass due to CAF diets that we observed in young adult rats is also present in latter developmental stages.

In conclusion, in young rats, the intake of a 30% energy restricted CAF diet partially ameliorated obesity and related metabolic diseases induced by a regular CAF diet, but not as quickly or drastically as normolipidic diets based on chow food, suggesting that this approach could be used as a realistic and precise tool to evaluate the anti-obesity effect of a caloric restriction-based intervention combined with other therapeutic strategies, such the intake of bioactive compounds, functional foods or/and physical activity. Further studies aimed at evaluating this CAF-R diet combined with these other complementary treatments to tackle obesity are needed to support this hypothesis.

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Disclosure of interest

The authors report no conflict of interest.

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Figure legends

Figure 1. Effects of the dietary treatments on body weight (A) and final BMI (B). Data are expressed as the mean±SEM. * P<0.05 vs. STD group; + P<0.05, ++ P<0.01, +++ P<0.001 between CAF and CAF-R groups (Duncan's post-hoc test).

Figure 2. Effects of the dietary treatments on food and energy intake and FE. (A) Daily food intake during the 8 weeks of the dietary treatments. (B) Average food intake expressed as chow and non-chow foods. (C) Daily energy intake during the 8 weeks of dietary treatments. (D) FE at week 8 of the experiment. All the parameters were relativized to the body weight (BW, kg). Data are expressed as the mean±SEM. * P<0.05, ** P<0.01, *** P<0.001 vs. STD group; + P<0.05, ++ P<0.01, +++ P<0.001 between CAF and CAF-R groups (Duncan's post-hoc test). $ P<0.05 between CAF and CAF-R groups (Student's t test).

Figure 3. Effects of the dietary treatments on body composition and weight of skeletal muscles. Relative fat mass (A), lean mass (B) and water (C) composition were documented at weeks 0, 4 and 8 of the experiment and weight of gastrocnemius (D) and soleus (E) muscles at week 8. Relative fat and lean masses and water composition were calculated according to the formula: 100*body component/body weight and expressed as percentage. Muscle weights were relativized to total body weight (BW, kg). Data are expressed as the mean±SEM. * P<0.05, ** P<0.01, *** P<0.001 vs. STD group; + P<0.05, ++ P<0.01, +++ P<0.001 between CAF and CAF-R groups (Duncan's post-hoc test).

Figure 4. Effects of the dietary treatments on subcutaneous WAT (A), visceral WAT (B), adiposity index (C) and IBAT (D) at the end of the experiment. Tissue weights were relativized to total body weight (BW, kg). Data are expressed as the mean±SEM. * P<0.05, ** P<0.01, *** P<0.001 vs. STD group; + P<0.05, ++ P<0.01, +++ P<0.001 between CAF and CAF-R groups (Duncan's post-hoc test).

Figure 5. Effects of the dietary treatments on protein expression levels of genes associated with muscle protein synthesis (A) and degradation (B, C) in the gastrocnemius muscle. p70S6K, p70 ribosomal protein S6 kinase; MuRF-1, muscle

RING finger 1; MAFbx32, muscle atrophy F-box 32. Data are expressed as the mean±SEM.

Figure 6. Effects of the dietary treatments on the mRNA expression levels of *Ucp1* (A), *Pgc1* (B), *Nrf1* (C) and *Tfam1* (D) in the IBAT. *Ucp1*, mitochondrial brown fat uncoupling protein 1; *Pgc1*, proliferator-activated receptor-γ coactivator-1α; *Nrf1*, nuclear respiratory factor 1; *Tfam1*, mitochondrial transcription factor A 1. Data are expressed as the mean±SEM and relativized to the STD values (%). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. STD group; + $P<0.05$, ++ $P<0.01$, +++ $P<0.001$ between CAF and CAF-R groups (Duncan's post-hoc test). $ $P=0.066$ between CAF and CAF-R groups (Student's t test).

Graphical abstract (**schematic diagram of the experimental design)**

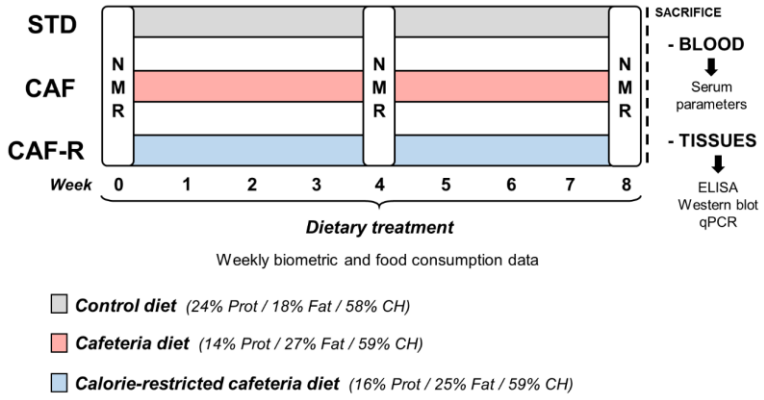

Figure 1.

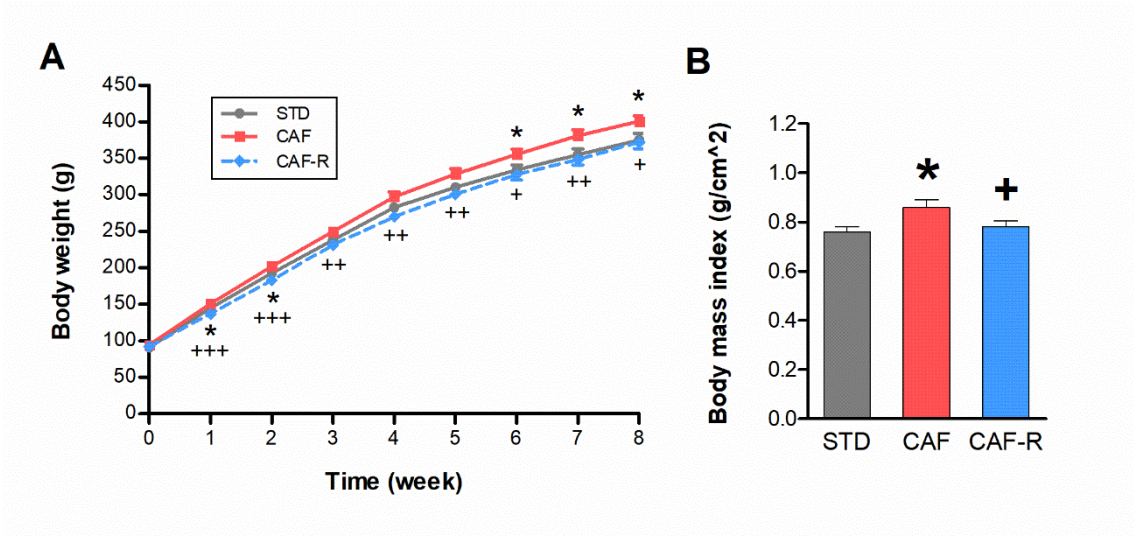

Figure 2.

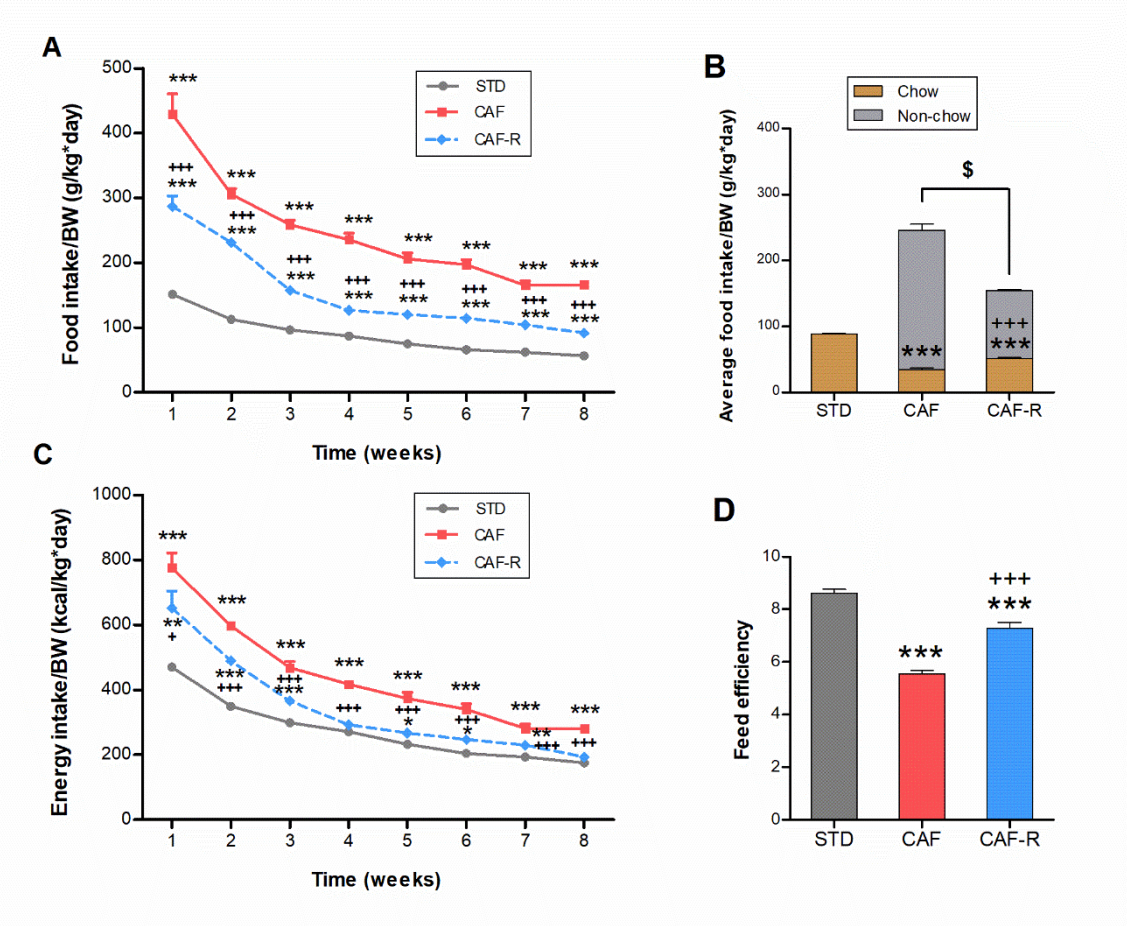
A
Food intake/BW (g/kg*day)
Time (weeks)
STD
CAF
CAF-R
B
Average food intake/BW (g/kg*day)
Chow
Non-chow
$
STD
CAF
CAF-R
C
Energy intake/BW (kcal/kg*day)
Time (weeks)
STD
CAF
CAF-R
D
Feed efficiency
STD
CAF
CAF-R

Figure 3.

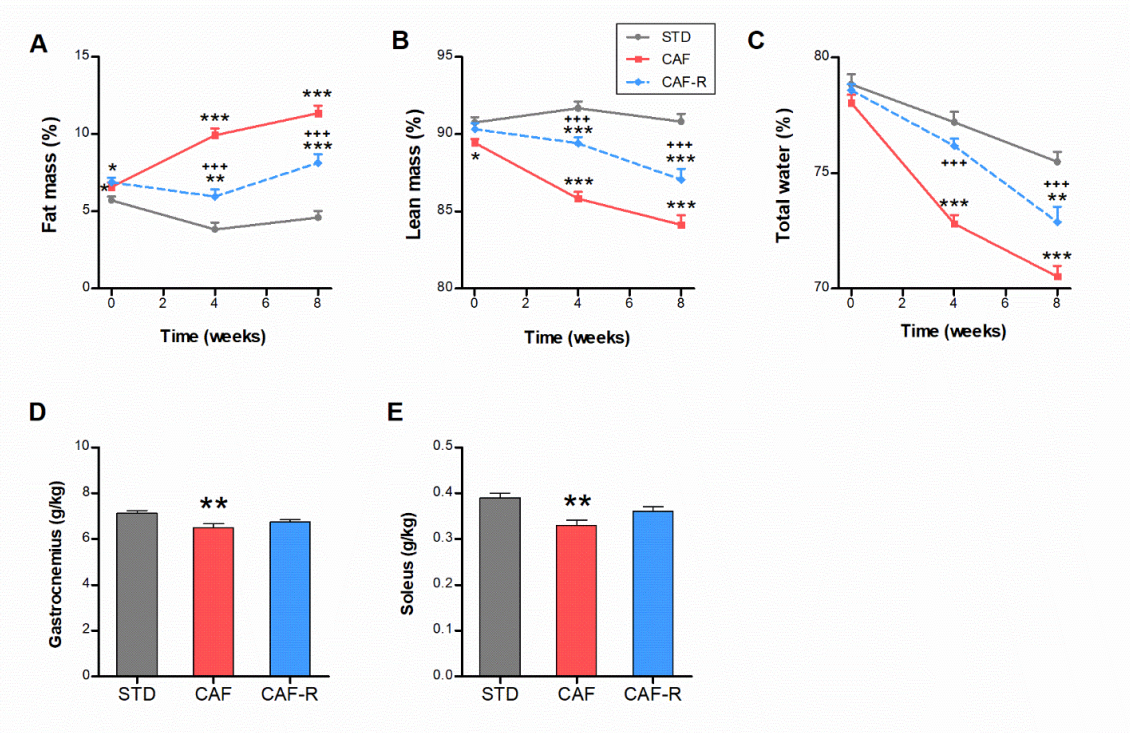
A
Fat mass (%)
Time (weeks)
B
Lean mass (%)
Time (weeks)
STD
CAF
CAF-R
C
Total water (%)
Time (weeks)
D
Gastrocnemius (g/kg)
STD
CAF
CAF-R
E
Soleus (g/kg)
STD
CAF
CAF-R

Figure 4.

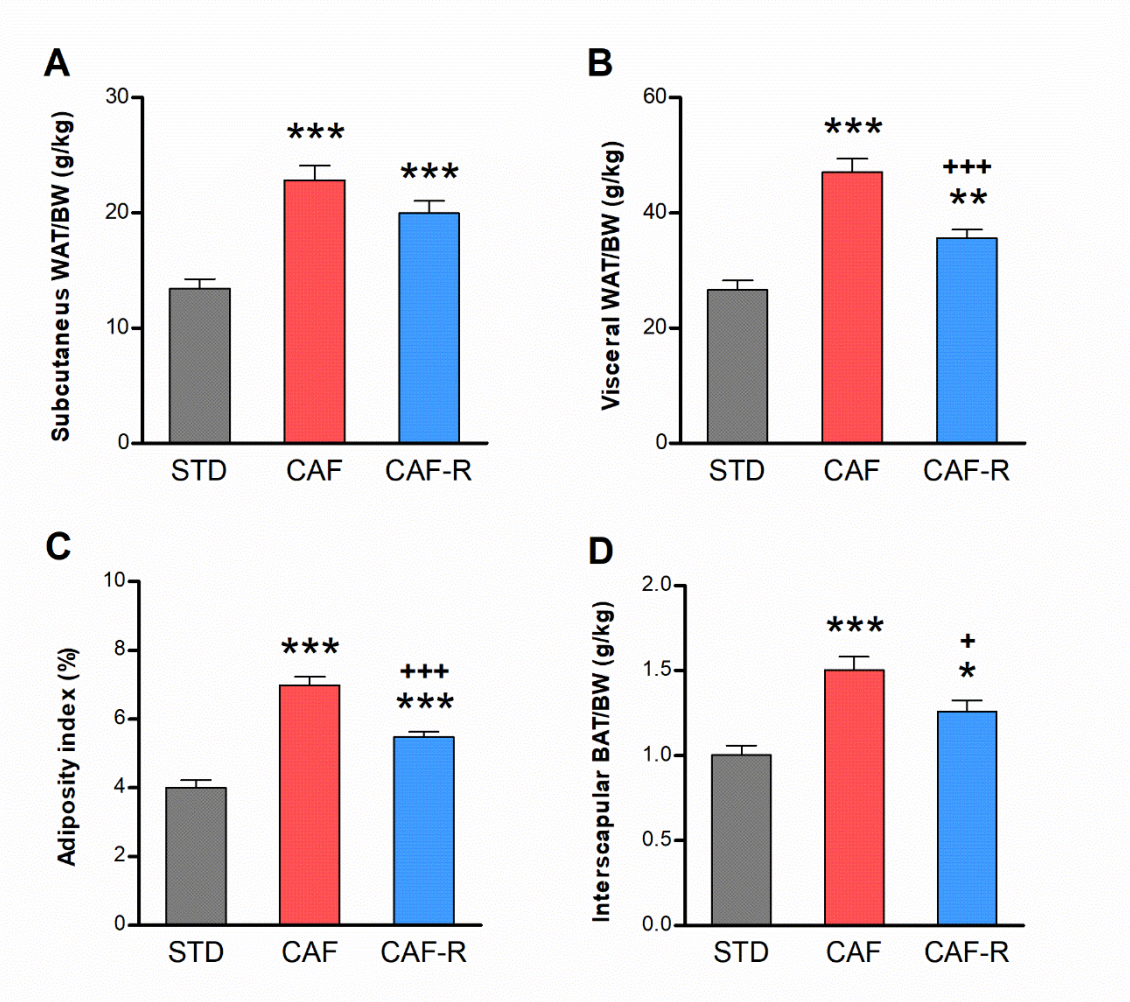
A
Subcutaneus WAT/BW (g/kg)
30
20
10
0
***
***
STD
CAF
CAF-R
B
Visceral WAT/BW (g/kg)
60
40
20
0
***
+++
**
STD
CAF
CAF-R
C
Adiposity index (%)
10
8
6
4
2
0
***
+++
***
STD
CAF
CAF-R
D
Interscapular BAT/BW (g/kg)
2.0
1.5
1.0
0.5
0.0
***
+
*
STD
CAF
CAF-R

Figure 5.

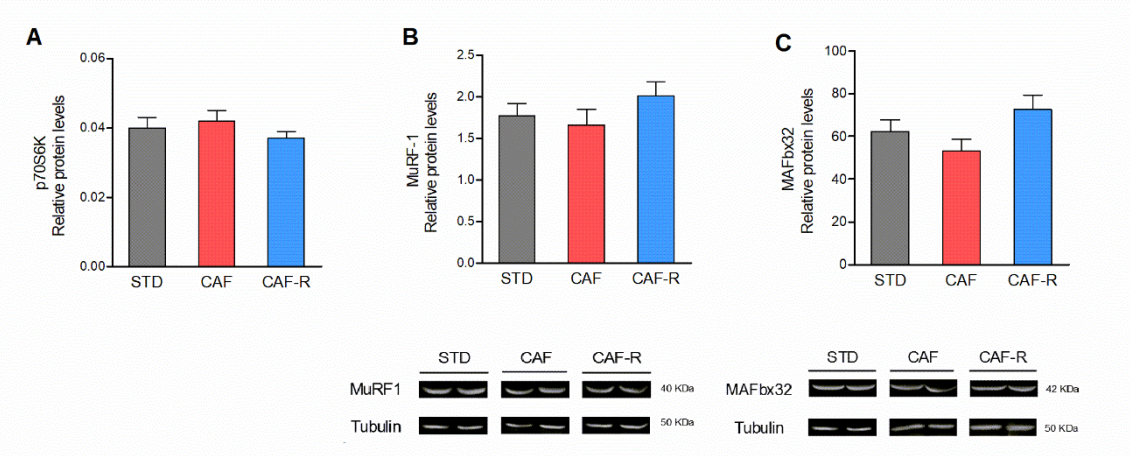

Figure 6.