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**Long-term follow-up of muscle lipid accumulation, mitochondrial activity
and oxidative stress and their relationship with impaired glucose
homeostasis in high fat high fructose diet-fed rats**

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Running title: Muscle lipid accumulation and glucose homeostasis in HFHFr diet-fed rats

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28 **Abstract**

29 Metabolic syndrome components, including obesity, dyslipidemia and impaired glucose
30 homeostasis, become a major public health issue. Muscles play a predominant role in insulin-mediated
31 glucose uptake, and high fat diets may negatively affect muscle function and homeostasis. This work
32 aimed to study the time-course of muscle lipid accumulation, oxidative stress and mitochondrial
33 dysfunction and their association to impaired glucose homeostasis in rats fed an obesogenic diet. Male
34 Wistar rats were fed with a standard or a high fat/high fructose (HFHFr) diet and sacrificed on 4, 8, 12,
35 16, 20 weeks. Rats fed the HFHFr diet developed mild overweight, increased liver and adipose tissue
36 weights and glucose intolerance. The impaired glucose homeostasis increased gradually with the
37 HFHFr diet to become significant on the 12th and 16th weeks of diet. In parallel, the muscle lipid
38 composition showed an increase in the saturated fatty acids and the monounsaturated fatty acids with a
39 marked decrease in the polyunsaturated fatty acids. The HFHFr diet also increased muscle contents of
40 both diacylglycerols and Ceramides. Surprisingly, HFHFr diet did not induce major muscle
41 mitochondrial dysfunction or oxidative stress. These results indicate that muscle lipid alterations, as
42 well as impaired glucose homeostasis occur as early as the 8th week of HFHFr diet, increase to reach a
43 plateau around the 12th-16th weeks of diet, and then attenuate towards the end of study. At these diet
44 treatment durations, muscle mitochondrial activity and oxidative stress remained unchanged and do
45 not seem to have a major role in the observed impaired glucose homeostasis.

46

47 *Keywords*

48 Free radicals, High fat/high fructose diet; Impaired glucose homeostasis; Mitochondrial function;
49 Muscle lipid composition; Muscle metabolism; Oxidative stress.

50

1. Introduction

Metabolic syndrome components, including obesity, dyslipidemia and impaired glucose homeostasis, are increasing and become a major public health issue. Indeed, increased body weight becomes a major public health challenge in developed countries and increasingly in developing countries, where food supplies are abundant and physical activity is reduced [1]. Obesity generally results from imbalanced energy intake and expenditure of the body from a metabolic point of view. Skeletal muscle is an essential tissue for whole-body energy metabolism and is the predominant site of insulin-mediated glucose uptake in the post prandial state [2]. Skeletal muscle fatty acid metabolism is subject to extensive *in vivo* regulation, in particular by control of fatty acid entry into the cell, their transfer into the mitochondria and the capacity of the β -oxidation cycle [3]. The balance between uptake and utilization of fatty acids will ultimately determine the magnitude of lipid accumulation in muscle cells. Consequently, high fat intake may lead to ectopic lipid deposition in muscle and metabolically active lipid intermediates that accumulate, such as diacylglycerols (DAG) and sphingolipids, in particular ceramides and sphingomyelin. These active/toxic lipids have been demonstrated to play essential roles in the establishment of impaired glucose homeostasis muscle and insulin resistance [4, 5]. Indeed, the high fat diet-stimulated sphingolipid formation contributes to dysregulated lipid accumulation, systemic insulin resistance, and cytokine expression and secretion from skeletal muscle and adipose tissues, exacerbating obesity-related physiopathological conditions. In addition, many reports have indicated that some skeletal muscle phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and their fatty acyl composition, have also a role in insulin sensitivity regulation [6-10].

Reactive oxygen species (ROS) are overproduced in obesity and the resulting oxidative damage could play a key role in the pathogenesis of impaired glucose homeostasis, diabetes and its complications [11]. Mitochondria are one of the main cellular ROS generation sites and mitochondrial ROS production is closely associated with activity of the respiratory chain complexes [12, 13]. The relationship between mitochondria and insulin action is highly complex [14]. Several studies in various models of metabolic diseases or using genetically manipulated mice have reported a decrease

in mitochondrial activity, while others have reported either no change or even an increase in mitochondrial oxidative metabolism in obese and insulin resistant humans and rodents [15-17]. Moreover, there is growing evidence that inflammation and oxidative stress are closely related, creating a vicious circle that may contribute to impaired glucose homeostasis installation [18]. Undeniably, one question that comes up is when oxidative stress occurs in muscle tissue, and whether it contributes to, or is simply a consequence of, obesity and impaired glucose homeostasis installation and development in our obese animal model. Although some teams have addressed this issue, the obtained results are conflictual [19, 20] and the question remains open.

This study aimed at determining the time-course of skeletal muscle lipid accumulation and composition as well as mitochondrial function and oxidative stress in parallel to the development of obesity and glucose intolerance in high fat/high fructose (HFHFr) diet-fed rats.

2. Materials and Methods

2.1 Animals and diets

Seventy five 6-weeks old male Wistar rats (Charles River, L'Arbresle, France) were housed (2 per cage) under conditions of constant temperature (20-22°C), humidity (45-50%) and a standard dark cycle (20.00-08.00 hours). The rats were randomized into two groups of animals and fed for 4 to 20 weeks one of the two following semi-purified diets: 1) control diet, or 2) high fat/high fructose diet (HFHFr). Seven rats from control group and 8 rats from HFHFr group were sacrificed after 4, 8, 12, 16 or 20 weeks of diet treatment.

The detailed composition of these two diets is given in the supplementary Table 1. Roughly, the control diet contains 4% lipids as soybean oil, and the HFHFr diet contains 35% lipids (1% soybean oil+8.5% lard+17% palm oil+8.5% cocoa butter and 0.2% cholesterol) and 25% fructose. As shown in the supplementary Table 2, the lipid fraction of the control diet was composed of 17.4% saturated fatty acids (SFA), 28.1% monounsaturated fatty acids (MUFA) and 54.5% polyunsaturated fatty acids (PUFA), and that of the HFHFr diet was composed of 44.2% SFA, 43.2% MUFA and 12.6% PUFA. Rats were given free access to food and tap water. Rat body weight was determined weekly and food

consumption was determined every two-three days. Our institution guidelines for the care and use of laboratory animals were observed and all experimental procedures were approved by the local ethical committee in Montpellier, France (Reference CEEA-LR-12002).

2.2 Oral glucose tolerance test

The oral glucose tolerance test (OGTT) was completed four to five days before rat sacrifice and performed as previously described [21]. Briefly, 6-hours fasting rats received by gavage 2.5 g glucose/kg body weight. Blood was sampled through the tail vein of conscious rats immediately prior to the gavage, and 20, 40, 60, 90, 120 and 180 min afterward and blood glucose was measured using glucose strips and a commercial glucometer (AccuChek Active, Roche Diagnostics, USA). The area under the curve (AUC) was calculated by the trapezium method [22]. The AUC values are expressed as g glucose/L/180 min.

2.3 Sampling and routine biochemical analyses

Four to five days after the OGTT, 9 hours-fasted rats (23h:00–08h:00) were anaesthetized with pentobarbital (40 mg/kg b.w.) (Ceva Santé Animale, Libourne, Fr) and blood was drawn from the abdominal artery with a heparinized syringe and distributed into dry and heparinized tubes. Blood tubes were centrifuged at 1000 g for 10 min at 4°C, plasma or serum were collected and stored at –80°C until analysis. Liver, heart, muscles, adipose tissue and kidney were removed, rinsed with 0.9% NaCl, weighed and kept at -80°C until analysis, except a gastrocnemius that has been used for the isolation of fresh mitochondria.

2.4 Muscle neutral lipids and diacylglycerols and ceramide species

A part of a gastrocnemius muscle was homogenized in NaCl (9 g/L) and Triton X-100 (0.1%) and free fatty acids (FFA), triglycerides (TG) and total cholesterol levels were quantified on the muscle homogenate by enzymatic methods with commercial kits (Wako-NEFA-C kit, Oxoid, Dardilly, France; Cholesterol RTU kit and triglycerides PAP kit, Biomérieux, Lyon, France).

The muscle diacylglycerols and ceramide contents were measured according to the methods described by Blachnio-Zabielska et al. [24, 25]. Briefly, lipids were extracted from ~20 mg of soleus muscle by the extraction mixture isopropanol/water/ethyl acetate (35/5/60; v/v/v). Quantitative measurement of ceramides and diacylglycerols was made using an Agilent 6460 triple quadrupole mass spectrometer. Both ceramides and diacylglycerols were analyzed using positive ion electrospray ionization source with multiple reaction monitoring. The chromatographic separation was performed using an Agilent 1290 Infinity Ultra High Performance Liquid Chromatography. A mixture of d17:1/8:0, d17:1/18:0, d17:1/18:1(9Z), d17:1/20:0, d17:1/24:0 and d17:1/24:1(15Z) was used as internal standards for ceramides and Deuterated DAG Mixture I and Mixture II were used as internal standards for diacylglycerols (Avanti Polar Lipids, Alabaster, AL, USA). Concentration of each compound was assessed against standard calibration curves.

2.5 Phospholipids analysis of gastrocnemius muscle

Lipids from gastrocnemius muscle were extracted by a Folch mixture of chloroform/methanol (2:1 v/v) in the presence of butylated hydroxytoluene (50 mg/L). The application of Folch lipid extracts on silica gel 60 HPTLC plates 250 μ m, 20*10 cm, pretreated with 2.3% w/v boric acid in ethanol (100%), was automatically performed on a 4 mm band width using a ATS4 apparatus (CAMAG, Muttenz, Switzerland). The development was performed with methanol/acetic acid/pentane/chloroform (15/10/30/45, by volume), which allowed the separation of phospholipids and neutral lipids on a 60 mm total migration distance. The scanning of the plates was carried out using a TLC scanner 3 (CAMAG, Muttenz, Switzerland), operating in the reflectance mode. The plates were scanned at 715 nm after dipping in a solution of Blue Spray (Blue Spray/H₂SO₄ 4.2 M/acetone, 1/2/3 v/v/v) and heating for 3 min at 55 °C. The different classes of phospholipids (sphingomyelin, lysophosphatidylcholine, phosphatidylcholine, phosphatidylinositol, phosphatidyl-serine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidic acid, cardiolipin) (SM, LPC, PC, PI, PS, PE, PG, PA, CL respectively) were finally identified by comparing their retention factor (R_f) to authentic standards and quantified using calibration curves of the same standards [26].

2.6 Fatty acids composition of gastrocnemius muscle

An internal standard (12,5 µg C17:0/mL) was added to the Folch lipid extracts and fatty acid methyl esters were prepared by incubation with acidified methanol, according to the method of Lepage and Roy [27]. Briefly, the methylation reagent was generated by mixing sulphuric acid with methanol and butylated hydroxytoluen as antioxidant (50 mg/L), and the sample was heated at 90°C for 45 min to get the fatty acid methyl esters. After addition of sodium bicarbonate, distilled water and hexane, the sample was vortexed, centrifuged, and the upper hexane layer was transferred to a glass vial, evaporated under a nitrogen stream at 37°C, and dissolved in 0.5 ml isooctane/ BHT for gas chromatography analysis. Individual fatty acid methyl esters were identified according to the retention times of standards by gas liquid chromatography with a Supelco Vax capillary column, an AS-3000 autosampler and flame-ionization detector (Thermo Fisher Scientific, Courtaboeuf, France). The fatty acid methyl esters were quantified using the chromatographic peak area according to the internal standard method.

2.7 Desaturase activity and unsaturation indices of gastrocnemius muscle

Because it is very tricky to directly measure the activity of the enzymes that catalyze the desaturation and elongation reactions in the fatty acid metabolic pathways, results in animals have supported the use of surrogate measures of desaturase activity such as use of desaturase indices [28]. These indices are based on the ratio product to precursor of individual fatty acids. The desaturase indices were calculated as follows: $\Delta 9$ desaturase = [16:1 n-7/16:0], $\Delta 6$ desaturase = [18:3 n-6/18:2 n-6] and $\Delta 5$ desaturase = [20:4 (n-6)/20:3 (n-6)]. In addition, we have determined the gene expression of the stearoyl-CoA desaturase 1 (SCD1= $\Delta 9$ desaturase) in the muscle. The unsaturation index (UI) was calculated from the relative percentage of each type of mono- and polyunsaturated fatty acid multiplied for the number of double bonds present in the molecule [29].

2.8 Mitochondrial isolation and characteristics of gastrocnemius muscle

Mitochondrial isolation: muscle mitochondria were extemporaneously isolated by the differential centrifugation technique [30]. Briefly, a whole gastrocnemius muscle (2-3 g) was homogenized on ice (1 g wet tissue for 9 volumes of sucrose buffer: Sucrose 0.25 M, Tris 10 mM, EDTA 0.5 mM, pH 7.5) using a Polytron homogenizer. The homogenate was centrifuged at 900 g for 10 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 10 000 g for 10 min at 4°C. The obtained mitochondrial pellet was suspended and washed with 2 ml of sucrose buffer and centrifuged at 10 000 g for 10 min at 4°C. The final mitochondrial pellet was suspended in 1 ml MIRO5 buffer (Sucrose 110 mM, Hepes 20 mM, KH₂PO₄ 10 mM, Taurine 20 mM, K-Lactobionate 60 mM, MgCl₂ 3 mM EGTA 0.5 mM, BSA 1 g/l, pH 7.1) and aliquoted for mitochondrial function measurements. Protein content was determined according to Bradford with bovine serum albumin as the standard [31].

Membrane fluidity measurement: muscle mitochondria underwent three cycles of freezing/thawing to fragment and expose the mitochondrial membranes. The muscle mitochondrial membrane fluidity was assessed by electron spin resonance as previously described [30], using two spin labels: 5-nitroxide stearate (5-NS) and 16-nitroxide stearate (16-NS). These both labels allow the measurement of the fluidity at both the surface of- and inside the lipid bilayer membrane of mitochondria, respectively.

Membrane potential measurement: mitochondrial membrane potential was monitored according to the method described by Baracca et al [32] using Rhodamine (RH-123) as a probe. Mitochondrial potential ($\Delta\psi$) changes have been evaluated by measuring RH-123 fluorescence quenching at the following steps: with RH-123 label alone (2 μ M) in MIRO5 buffer plus muscle fresh mitochondria (0.25 mg proteins), plus glutamate/malate/succinate (2.5/5/5 mM), plus ADP (0.5 mM). RH-123 fluorescence was measured at 495 nm (excitation) and 525 nm (emission) under continuous stirring.

Mitochondrial oxygen consumption: mitochondrial respiration was determined by measuring mitochondrial oxygen consumption in two 2 ml air-tight thermo-stated chambers of high resolution Oxygraph (Oroboros Oxygraph^{2k}, Autriche) [30]. The chambers were equilibrated with the MIRO5 respiration buffer at 37°C and fresh mitochondria were then added (0.4 mg protein) followed by

substrates malate/glutamate/succinate 2.5/5/5 mM (state 4) and ADP 0.5 mM (state 3). Respiratory coefficient ratio (RCR) was calculated as the ratio of state 3 to state 4 rates of respiration.

Mitochondrial ROS production: mitochondrial ROS production was measured on fresh mitochondria as previously described [33]. Briefly, reaction mixtures (2 ml), containing MIRO5 buffer, dihydrodichlorofluorescein (10 μ M) and peroxidase (2 IU/ml), were incubated with 60 μ g of mitochondrial proteins with or without substrates (malate/glutamate/succinate 2.5/5/5 mM, respectively) and with or without ADP (0.5 mM). Fluorescence was measured 60 minutes after reaction initiation at 490/525nm excitation and emission, respectively.

2.9 Mitochondrial enzymatic activities of gastrocnemius muscle

Citrate synthase (CS) activity was measured according to Srere [34]: the activity of the enzyme is measured by following the color of 5-thio-2-nitrobenzoic acid, which is generated from 5,5'-dithiobis-2-nitrobenzoic acid present in the reaction of citrate synthesis, and caused by the deacetylation of acetyl-CoA. The activity of the different mitochondrial respiratory complexes was determined as previously described [35]. Mitochondrial complex I activity was measured spectrophotometrically at 600 nm during 45 sec by following the reduction of 2,6-dichloroindophenol by electrons accepted from decylubiquinol, itself reduced after oxidation of NADH by complex I [36]. Complex II (CII) activity was measured spectrophotometrically at 600 nm by following the reduction of 2,6-dichloroindophenol by the succinate during 120 sec. Complex II+III (CII+CIII) activities were measured spectrophotometrically by following the reduction of cytochrome C at 550 nm during 90 sec [37]. Cytochrome c oxidase (CIV) activity was measured spectrophotometrically by following the oxidation of reduced cytochrome C at 550 nm during 30 sec [38].

In addition, mitochondrial β -hydroxyacyl-CoA dehydrogenase (β -HAD), a marker of last step of mitochondrial β -oxidation activity, was determined spectrophotometrically according to Clayton et al. [39]. Briefly, muscle mitochondria suspension (25 μ g protein) were mixed with NADH and acetoacetyl CoA in 1 ml of 50 mM Tris-HCl buffer, pH 7.0, and absorbance was followed at 340 nm for 60 seconds at 30°C.

2.10 *Oxidative stress status of gastrocnemius muscle*

A part of a gastrocnemius muscle was homogenized in phosphate buffer (50 mM, pH 7) 1 g for 9 ml buffer, using a Polytron homogenizer. The muscle thiobarbituric acid reactive substances (TBARS) and total glutathione (GSH) levels were measured in muscle homogenate according to Sunderman [40] and Griffith [41], respectively. The remaining homogenate was centrifuged at 1000 g for 10 min at 4°C and the supernatant was used for the other analyses of oxidative stress. Protein oxidation was assessed in muscle supernatant by measurement of thiol groups as previously described [42]. Catalase activity was measured in muscle supernatant according to Beers and Sizer [43]. Glutathione peroxidase (GPx) was measured in muscle supernatant according to Flohe and Gunzler [44]. Total superoxide dismutase (SOD) was measured in muscle supernatant according to Marklund [45].

2.11 *Protein isolation and western blotting analysis of gastrocnemius muscle*

Frozen gastrocnemius muscle samples were homogenized using an Ultra Turax homogenizer in an ice cold extraction buffer containing 20 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% SDS, 1 mM PMSF, 10 µM leupeptin, and 1 µM pepstatin. Proteins (50 µg) were separated with 10% SDS-PAGE and then transferred to a nitrocellulose membrane (45 min, 200 V). Membranes were blocked in 5% fat-free milk for 1 hour at room temperature. Then, membranes were incubated overnight with primary antibody against one of the following proteins: acetyl-CoA carboxylase (ACC), phosphorylated acetyl-CoA carboxylase (ser⁷⁹p-ACC), acyl-CoA synthetase long chain family member 1 (ACSL1), cardiolipin synthase (CLS), muscle-Carnitine palmitoyltransferase I (m-CPT1), fatty acid synthase (FAS), cluster of differentiation 36 (fatty acid translocase) (FAT/CD36), fatty-acid-transporter protein 1 (FATP1) and peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) in blocking buffer (supplementary Table 3).

After washes in TBS/Tween under gentle agitation, membranes were incubated for 1 hour with horseradish peroxidase-labeled antibody. After further washes, blots were treated with enhanced chemiluminescence detection reagents (ThermoFischer Scientific, Illkirch, France). β-actin was used

as loading references, and blot intensities were measured using Image Lab™ Software 5.2.1 (Bio-Rad Laboratories, France). The blots showing the strips scanned by densitometry for the gastrocnemius muscle proteins of the ten investigated groups are provided in the supplementary Figure 1.

2.12 Total RNA isolation and real-time RT-PCR analysis of gastrocnemius & soleus muscles

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) was used to measure target genes mRNA expression in the *gastrocnemius* & *soleus* muscles. Total RNA was extracted with Trizol reagent (Invitrogen Life Technologies, Cergy Pontoise, France). Reverse transcription reaction was performed with 2 µg total RNA. cDNA was synthesized with the use of SuperScript II Reverse Transcriptase for first strand cDNA synthesis and Oligo (dT) primers (Invitrogen Life Technologies, Cergy Pontoise, France). The mRNA expressions of target genes were determined by RT-qPCR, using IQTM SYBR Green Supermix with a MiniOpticon detection system (Biorad, Hercules, CA, USA). Data were normalized to RPS9 (*gastrocnemius* muscle) or Hprt (*soleus* muscle) used as reference genes. The mean expression levels and the standard deviations of the housekeeping RPS9 rRNA gene were as follows: 23.7±0.8; 24.9±1.4; 24.5±1.4; 24.0±1.7; 24.3±1.9; for control groups (4, 8, 12, 16 & 20 weeks, respectively) and 23.7±1.0; 24.4±1.5; 23.7±1.5; 23.7±1.6 and 23.9±2.0 for HFHFr groups (4, 8, 12, 16 & 20 weeks, respectively). The mean expression levels and the standard deviations of the housekeeping Hprt rRNA gene were as follows: 25.0±0.7; 24.8±1.1; 24.7±1.6; 24.3±0.5; 24.2±0.3 for control groups (4, 8, 12, 16 & 20 weeks, respectively) and 24.8±1.2; 24.5±0.9; 24.2±0.4; 24.7±0.9 and 24.1±0.2 for HFHFr groups (4, 8, 12, 16 & 20 weeks, respectively).-The primer sequences used for RT-qPCR analysis are shown in the supplementary Table 4.

2.13 Statistical analysis

The sample size was calculated from the expected difference in the AUC of the OGTT between the control group and the HFHFr groups. We expected an AUC about 1.20 times greater in the HFHFr groups than in the control group. A standard deviation of 15% of the higher value could be allowed. For a type 1 risk α of 0.05 and power (1- β) of 80%, the sample size required was eight per group.

Results were expressed as means \pm SD, n=7 animals per control group and 8 animals per HFHFr group per each treatment duration.

All the groups were tested for the effects of diet, treatment duration, and their interaction using a two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyze the effect of HFHFr diet compared to control diet within each diet treatment duration. The OGTT, mitochondrial characteristics parameters and the western blot analyses were carried out separately for each diet treatment duration. So statistical analysis for these parameters was made only between HFHFr and control diets, inside of each diet treatment duration, by the unpaired student's *t*-test. Correlations between parameters were performed with the Spearman method. The limit of statistical significance was set at $p<0.05$. HFHFr diet vs control diet: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Statistical analyses were performed using the StatView program (SAS Institute, Cary, NC, USA).

3. Results

3.1 Rat characteristics and blood biochemical parameters

Both dietary and energy intakes were increased throughout the study in both rat groups but the dietary intake was largely lower from the HFHFr diet compared to the control diet. Nevertheless, if the energy intake in both groups was overall similar, it was lower on 4 wks and higher on 12 and 16 wks from the HFHFr diet compared to the control diet (Table 1). In addition to a moderate but significant increase in body weight of HFHFr diet-fed rats (Table 1), the HFHFr rats exhibited higher liver and adipose tissue weights with lower gastrocnemius weight than control rats (Figure 1). These effects were further accentuated when expressed in relation to rat body weight (Figure 1). In addition, the two-way ANOVA analysis showed a slight but significant decrease in the weight of soleus, tibialis and extensor digitorum longus in the HFHFr rats compared to the control rats, although no significant difference was observed at any diet treatment duration between control and HFHFr rats. Finally, the heart and kidney weights remained unchanged (Table 1).

The time-course of OGTT for control and HFHFr rats is shown in Figure 2A and 2B respectively. The AUC of the OGTT showed that impaired glucose homeostasis was increased in the HFHFr rats compared to the control rats, reaching a maximum at 12 wks of diet intake (Figure 2B).

Serum levels of both TG and total cholesterol were gradually and significantly increased in both diet groups whereas HFHFr diet had no significant effect on these parameters. Surprisingly, serum FFA levels were significantly decreased with the HFHFr diet compared to control diet (Figure 3).

3.2 *Gastrocnemius muscle neutral lipids and soleus muscle DAG and ceramides contents*

The gastrocnemius muscle levels of FFA and TG were increased with the HFHFr diet, whereas that of cholesterol remained unchanged (Figure 4A). Specifically, FFA and TG levels were significantly increased at 12 wks of HFHFr diet by comparison to control diet. Indeed, the muscle triglycerides content was highly increased (2.2 times increased compared to control rats at 12 wks of diet), whereas free fatty acids were only moderately increased (+24% increase compared to control rats at 12 wks of diet).

Total DAG content in the soleus muscle was increased since the 4th wk of diet with the HFHFr diet compared to controls. In particular, it was significantly increased at the 16th wk of HFHFr diet by comparison to control diet (Figure 4B). All muscle DAG species C16- C16, C16- C18.1, C16- C18, C18.1- C18.1 and C18- C18.1 were significantly increased, except C16- C18.2 (decreased) and C18.1- 18.2 (unchanged), with the HFHFr diet compared to the control diet (Table 2).

Total ceramide content in the soleus muscle was also increased with the HFHFr diet compared to controls. In particular, it was significantly increased at 4 and 12 wks of HFHFr diet by comparison to control diet (Figure 4B). This increase was due the increase in four important ceramide species, the sphingosine and sphinganine, as well as ceramides C18 and C20, despite the fact that the content of the five other ceramides, namely, C16, C18.1, C22, C24.1 and C24 remained unchanged (Table 3).

Because lipid droplets are the major cellular organelles for the storage of neutral lipids, we have investigated the gene expression level of key players involved in lipid droplets dynamics in the soleus, e.i., PLIN2, DGAT1, ATGL, CD36 and LPL. As shown in Figure 5, the two-way ANOVA test

showed that only the gene expression of CD36 was significantly increased with the HFHFr diet compared to the control diet, particularly on the 16th wk of diet. In addition, when only student's *t*-test was applied on the 16th treatment duration, the gene expression of both PLIN2 and ATGL appeared also increased with the HFHFr diet compared to the control diet.

3.3 Phospholipids distribution and fatty acid composition in the gastrocnemius muscle

The muscle content of total phospholipids remained statistically similar with HFHFr and control diet. Individually, the percentage of different phospholipid classes remained also statistically similar with HFHFr and control diet (Supplementary Table 5). In particular, the percentage of cardiolipin, that is considered as a biomarker of mitochondrial content [46], was not modified.

As expected, the percentage of muscle total SFA was increased under the HFHFr diet, which was due to the increase in both palmitic and stearic acid, except at 8 wks and 12 wks of diet where SFA percentage was unchanged between controls and HFHFr rats. (Figure 6, Table 4). In addition, the percentage of muscle total MUFAs was also increased with the HFHFr diet, in particular on 8 and 12 wks of diet intake (Figure 6). Indeed, the percentage of the minor MUFAs, namely, C16:1 n-7 and C18:1 n-7, was significantly decreased whereas that of the major MUFA, C18:1 n-9, has more than doubled (Table 4). Finally, the percentage of muscle total PUFA was markedly decreased under the HFHFr diet compared to the control diet (Figure 6). Specifically, the percentage of linoleic acid (18:2 n-6), a major PUFA, and docosahexaenoic acid (22:6n-3) was decreased whereas that of another major PUFA, arachidonic acid (20:4n-6), remained unchanged with the HFHFr diet compared to the control diet (Table 4).

Consequently, the unsaturation index of muscle fatty acids was decreased with the HFHFr diet compared to the control diet (Table 4). Indeed, this decrease in the unsaturation index was in agreement with the decrease in the percentage of PUFAs under the HFHFr diet compared to the control diet. In addition, the fatty acid desaturase indices of the three important desaturases ($\Delta 9 = [16:1n-7/16:0]$, $\Delta 6 = [18:3n-6/18:2n-6]$ and $\Delta 5 = [20:4n-6/20:3n-6]$) were all decreased with the HFHFr diet compared to the control diet (Table 4). This is in agreement with the above reported

decreases in the percentage of PUFAs and in the unsaturation index. Moreover, this was confirmed with gene expression of SCD1, the stearoyl-CoA desaturase (or Δ -9-desaturase) that catalyzes the rate-limiting step in the formation of oleate and palmitoleate from stearoyl-CoA and palmitoyl-CoA, respectively. In fact, SCD1 gene expression was significantly decreased with the HFHFr diet compared to the control diet (Table 4).

3.4 Lipid signalling pathways and mitochondrial activity in the gastrocnemius muscle

In order to explain the obtained results, we studied the muscle expression of several actors of lipid metabolism. To begin with the lipid uptake, the protein abundance of both FAT/CD36 and FATP1, two fatty acid transporters, remained unchanged (Tables 5), whereas the gene expression of FABP3, another fatty acid transporter, was increased with the HFHFr diet compared to the control diet (Table 6). Protein expression of the long chain acyl-CoA synthetase (ACSL1), that activates the conversion of 12 to 20 carbon fatty acids into acyl-CoA thioesters, remained unchanged, except on 12 wks of diet. The muscle gene expression of PPAR α , PPAR γ and Srebp-1c, three key regulators of lipid metabolism, as well as the gene and protein expression of PGC-1 α , a key regulator of energy metabolism, remained unchanged (Tables 5, 6). In addition, protein abundance of mCPT-1, a mitochondrial fatty acids transporter, remained also unchanged. Regarding lipogenesis, the muscle protein abundance of total acetyl CoA carboxylase (ACC), a lipogenic enzyme providing the malonyl-CoA substrate for fatty acid synthesis and ser⁷⁹p-ACC, its inactivated form, remained unchanged whereas protein abundance of muscle fatty acid synthase (FAS), a multifunctional enzyme that synthesizes palmitic acid, was significantly and markedly decreased all along the study with the HFHFr diet compared to the control diet (Table 5).

3.5 Mitochondrial characteristics and enzymatic activity in the gastrocnemius muscle

The main muscle gastrocnemius mitochondrial parameters characterising mitochondrial function, that is, membrane potential, membrane fluidity and respiration, remained unchanged in the HFHFr diet-fed rats compared to the control rats (Table 7). In addition, the enzymatic activities of citrate

synthase, a renowned biomarker of mitochondrial content [46], and mitochondrial respiratory chain complexes (complex I, complex II, complex II+III and complex IV) as well as β -hydroxybutyryl coenzyme A dehydrogenase (β -HAD), involved in fatty acid β -oxidation, remained unchanged with the HFHF diet compared to the control diet (Table 8). Because cardiolipin play a key role in the mitochondrial bioenergetic, in particular in mitochondrial respiratory chain complexes assembly and activity, we have determined the gene expression and the protein abundance of the cardiolipin synthase (CLS) as well as the gene expression of the CDP-Diacylglycerol Synthase 1 (CDS1), a critical enzyme that catalyzes the formation of CDP-diacylglycerol from phosphatidic acid for de novo cardiolipin synthesis. The gene expression but not the protein abundance of CLS was slightly but significantly decreased with the HFHF diet compared to the control diet, whereas the gene expression of CDS1 remained unchanged (Table 8).

3.6 Mitochondrial ROS production and oxidative stress status in the gastrocnemius muscle

Mitochondrial ROS production of gastrocnemius muscle challenged with substrates alone (malate+glutamate+succinate) or with substrates plus ADP, remained unchanged throughout the study between HFHF and control rats (Table 9). In addition, several long-established oxidative and anti-oxidative markers were determined in this study. The gastrocnemius muscle levels of TBARS (marker of lipid peroxidation), sulfhydryl groups (marker of protein oxidation) and GSH (a major cellular antioxidant) as well as the enzymatic activities of the antioxidant enzymes SOD, catalase and GPx remained unchanged with the HFHF diet compared to the control diet (Table 9).

3.7 Correlation of glucose intolerance with lipid composition, mitochondrial activity and oxidative stress parameters

The TG, cholesterol and FFA contents of gastrocnemius muscle were only slightly positively correlated with glucose intolerance. However, a moderate positive correlation was observed between soleus muscle DAGs content and glucose intolerance but not with soleus muscle ceramides content (Figure 7). Specifically, several major DAG species (C16/C18:1, C16/C18, C18:1/C18:1) were

positively correlated with glucose intolerance. Regarding muscle FA composition, the muscle SFAs were not correlated whereas both MUFAs and PUFAs were respectively positively and negatively correlated with glucose intolerance (Figure 8). The determination of mitochondrial activity and ROS production showed that the enzymatic activities of the respiratory chain complexes I and IV were positively, whereas ROS production was negatively correlated with glucose intolerance (Figure 9). Finally, we observed no significant correlation between glucose intolerance and several measured oxidative stress parameters in the gastrocnemius muscle, namely, TBARS, sulfhydryl groups, GSH, SOD, catalase and GPx, monitored in this study (data not shown).

4. Discussion

It is well known that fatty acid composition of the diet can influence membrane fatty acid composition and then cellular activity and homeostasis [47]. Indeed, many reports have shown that high intake of dietary saturated fat significantly impaired glucose homeostasis and thus worsen insulin-resistance [7]. However, changes over time in skeletal muscle lipid accumulation, mitochondrial function and oxidative stress, along with the onset of obesity and impaired glucose homeostasis, remain crucial and interesting questions in animal models of obesity. In this study, rats were fed an experimental diet with a blend of fats with a high percentage of saturated fats, in particular palmitic acid. This diet was efficient in inducing moderate overweight, increasing liver and adipose tissue weights. These marked modifications of rat characteristics were clearly accompanied with muscle lipid accumulation and impaired glucose tolerance since the 8th week of diet.

4.1 Muscle lipid accumulated with HFHF diet with a maximum at 12th week of diet

Muscle lipid accumulation and/or lipid metabolism alteration is known to play an important role in the development of glucose intolerance in HF feeding models [48]. However, there is an ongoing debate on which of the lipid species plays the major role in inducing muscle insulin resistance and thus impaired glucose homeostasis. Although many studies have reported increased muscle lipid accumulation in mice/rats fed HF diets, it is the intracellular lipotoxic metabolites (fatty acyl CoA,

DAG and ceramides) that are considered the true culprits responsible for the installation and development of muscle metabolic perturbations and thus impaired glucose homeostasis. Indeed, in addition to the increased muscle TG, cholesterol and FFA contents, our results indicated that muscle DAG and ceramides were markedly increased with the HFHFr diet compared to the control diet. This could result from fatty acid storage within lipid droplets of skeletal muscle reflected by a sustained higher gene expression of FAT/CD36 during the course of HFHFr diet. In addition, total DAG and several individual species (C16/C18 and C18:1/C18:1), as well as TG, cholesterol and FFA, were positively correlated with glucose intolerance in this study. In accordance, muscle DAG accumulation in both animals and humans has been linked to insulin resistance [49]. Although the cellular mechanisms underlying this relationship are still in debates, the connection between muscle DAG accumulation and insulin resistance could be attributed to activation of protein kinase C signaling [50]. In addition, ceramides are important second messengers that interact with several pathways involved in insulin resistance, oxidative stress, inflammation and apoptosis [51]. Indeed, the ceramides have been shown to inhibit several distinct intermediates in the insulin signaling pathway, by regulating IRS-1, PI3-kinase, and Akt/PKB [52]. It was shown that the muscle ceramide (C18:1/C18:0) was the only higher lipid in insulin resistance independent of overweight/obesity [53]. In addition, muscle insulin resistance was predominantly associated with higher C18:0 ceramide [5]. However, although the accumulation of muscle stearoyl-ceramide species (C18:0) is considered having the greatest impact on skeletal muscle insulin sensitivity [53], no ceramide species was correlated with impaired glucose homeostasis in the present study.

Interestingly, maximal lipid accumulation under the HFHFr diet was observed at 12th/16th wk of diet. This is particularly notable for TG and DAG, but also for cholesterol and FFA. This was associated with higher gene expression of muscle FAT/CD36, a major player of cellular lipid storage and trafficking. In accordance, other parameters were modified maximally at this time of the diet, in particular OGTT, plasma glucose and HOMA-IR index, reflecting maximal impaired glucose tolerance. A previous study from our laboratory also demonstrated that hepatic steatosis and many biochemical modifications plateaued at 8–12 weeks of HFHFR diet in rats with slight amelioration

afterwards [54]. It is likely that adaptive mechanisms are initiated to attenuate the effect of the HFHFr diet after some weeks of diet. However, none of the proteins from the lipid metabolism signaling pathways we have explored has allowed us to explain such observation.

4.2 Muscle fatty acid composition was altered as soon as 4 weeks of HFHFr diet

The fatty acid composition of the gastrocnemius muscle was modified as early as 4 weeks of diet. The percentage of both major SFAs, C16 and C18, was increased with HFHFr diet compared to the control diet. However, there was no significant correlation between total SFA or individual SFAs with glucose intolerance. The intake of the HFHFr diet led also to an overall increase in muscle MUFA, in particular the major MUFA, C18:1 n-9. This results from the dietary C18:1 n-9 and not from *in vivo* conversion of saturated fatty acids into monounsaturated fatty acids, which serve as the preferred substrate for synthesis of stored lipids [55, 56], as SCD1 gene and activity were decreased with the HFHFr diet. Such a result may contradict the idea that MUFAs are rather beneficial in improving insulin sensitivity compared to a high-saturated-fat diet [57]. Another consequence of this HFHFr diet was a significant decrease in the percentage of muscle PUFAs, in particular in the metabolically important linoleic acid whereas the arachidonic acid percent remained unchanged. Interestingly, the muscle total PUFA as well as many of individuals PUFAs, among which n-6 and n-3 PUFA, were negatively correlated with glucose intolerance. This observation reinforces the concept that PUFAs are beneficial for insulin sensitivity [58]. Indeed, diets rich in SFAs induce impaired glucose homeostasis with concomitant increases in muscle DAG whereas diets rich in PUFAs appear to prevent glucose intolerance by directing fat into TG formation rather than in other toxic lipid metabolites [59].

4.3 Dysfunction of the muscle mitochondria is not mainly involved in glucose intolerance

Skeletal muscle mitochondria may play a key role in high fat or HF/HFr diet induced insulin resistance [60]. Two mechanisms by which mitochondrial function could be involved in skeletal muscle IR are debated [61]. The first suggests that mitochondrial dysfunction causes intramyocellular lipid accumulation leading to IR, while the second involves excess fuel within mitochondria in the

absence of increased energy demand, stimulating mitochondrial oxidant production and emission, and ultimately leading to the development of IR. The first mechanism may be more privileged but not in a significant way as both the content and characteristics of muscle mitochondria and mitochondrial activity remained unchanged in this study. However, the study of mitochondrial activity showed that the enzymatic activities of respiratory chain complexes I and IV were positively correlated with impaired glucose homeostasis, probably indicating that mitochondrial activity was impacted by the HFHFr diet although this was not appearing with the statistical analysis between the control group and the HFHFr group. Moreover, our results do not exclude possible important modifications of mitochondrial content and function in other tissues, in particular pancreas. In agreement with our results, Holloszy [62] has reported that the observed reduction in muscle content of mitochondria in patients with types 2 diabetes is not responsible for insulin resistance.

The other mechanism involving production and release of mitochondrial oxidants that may induce insulin resistance is not supported by our present results. Indeed, the muscle mitochondrial ROS production was not modified by the HFHFr diet. In addition, the muscle oxidative and anti-oxidative stress parameters remained unchanged under the HFHFr diet compared to the control diet and this throughout the study. ROS production from muscle mitochondria were even negatively correlated with impaired glucose homeostasis. These results are consistent with many of our earlier findings of marked oxidative stress in the blood and/or liver, but not in skeletal muscle with HFHFr diet [54, 63]. Moreover, many studies have reported oxidative stress onset in liver, intestine, adipose tissue or pancreas but rarely in skeletal muscle and/or heart in rats fed high-caloric diets [64, 65]. In line with this and in a very nice proteomic study, Mendez et al [66] have reported that long-term HFHS diet consumption increased protein oxidation in plasma and liver; meanwhile protein carbonyls from skeletal muscle did not change. Unlike the liver, skeletal muscle is a second target of nutrients, and is therefore less exposed than the liver to direct dietary changes, including high calorie diets.

5. Conclusion

The present results indicate that muscle lipid alterations, as well as impaired glucose homeostasis occur as early as the 4-8th week of HFHFr diet, increase to reach a plateau around the 12th weeks, and then attenuate towards the end of study (20th weeks). In addition, at these diet treatment durations, muscle mitochondrial activity and oxidative stress remained unchanged and do not seem to play a major role in the development of glucose intolerance, while accumulation of lipids and intracellular lipotoxic metabolites may rather be involved with probable species specificity.

Author contributions

Author contributions were as follows: study design (CC, CF-C), data collection (YW, GF, BB, AB-Z, BB, JL, TL, CM), statistical analysis (CC, CF-C), data interpretation (CC, CF-C, SG), manuscript preparation (CC, CF-C, CM), literature search (CC & CF-C) and funds collection (CF-C).

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Conflict of interest

The authors have no conflicts of interest to declare.

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Abbreviations

ACC: acetyl-CoA carboxylase; ser⁷⁹p-ACC: phosphorylated acetyl-CoA carboxylase; ACSL1: acyl-CoA synthetase long chain family member 1; ADP: Adenine diphosphate; ATGL: adipose

triglyceride lipase; AUC: area under the curve; CDP: Cytidine diphosphate; CL: cardiolipin; CLS: cardiolipin synthase; DGAT1: diacylglycerol O-acyltransferase 1; FAS: fatty acid synthase; FAT/CD36: cluster of differentiation 36 (fatty acid translocase); FATP1: fatty-acid-transporter protein 1; GPx: Glutathione peroxidase; GSH: Glutathione; β -HAD: β -hydroxyacyl-CoA dehydrogenase; HFHFr: high fat high fructose; HPTLC: High performance thin layer chromatography; LPC: lysophosphatidylcholine; LPL: lipoprotein lipase; m-CPT1: muscle-Carnitine palmitoyltransferase I; MUFA: monounsaturated fatty acids; NAFLD: non-alcoholic fatty liver disease; 5-NS: 5-nitroxide stearate; 16-NS, 16-nitroxide stearate; OGTT: oral glucose tolerance test; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PGC-1 α : peroxisome proliferator-activated receptor-gamma coactivator-1 α ; PI: phosphatidylinositol; PLIN2: perilipin 2; PS: phosphatidyl-serine; PUFA: polyunsaturated fatty acids; Rf: retention factor; qPCR: quantitative-polymerase chain reaction; RNS: reactive nitrogen species; ROS: reactive oxygen species; SCD1: stearoyl-CoA desaturase 1; SFA: saturated fatty acids; SM: sphingomyelin; SOD: superoxide dismutase; TBARS: Thiobarbituric acid reactive substances; TLC: thin layer chromatography; WB: western blotting.

Figure legends

Figure 1: Weight of liver, adipose tissue and gastrocnemius and their ratios to rat body weight in rats fed control or HFHFr diets for 4 to 20 weeks

Results were expressed as means \pm SD, n=6–8 animals per group per diet treatment duration, rats liver (A), adipose tissue (B) and gastrocnemius (C) weights and their ratio to rat body weight. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each treatment duration. The limit of statistical significance was set at $p < 0.05$. HFHFr diet vs control diet: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2: Time-course of oral glucose tolerance test in rats fed control or HFHFr diets for 4–20 weeks

A period of 6-h fasting rats received by gavage 2.5 g glucose/kg body weight. Blood was sampled through the tail vein of conscious rats immediately before the gavage, and 20, 40, 60, 90, 120 and 180 min afterwards and blood glucose was measured using glucose strips and a commercial glucometer. Changes in blood glucose after oral glucose load are shown for control (A) and HFHFr (Abis) rat groups. Total cumulative glucose levels for each treatment group are reported as AUC and were calculated by the trapezium method and expressed as g glucose/l per 180 min (B). Results were expressed as means \pm SD, n=6–8 animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each treatment duration. The limit of statistical significance was set at $p < 0.05$. HFHFr diet vs control diet: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. AUC: area under the curve; OGTT: oral glucose tolerance test.

Figure 3: Blood lipid levels in rats fed control or HFHFr diets for 4 to 20 weeks

Results were expressed as means \pm SD, n=6–8 animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each treatment duration. The limit of statistical significance was set at $p < 0.05$. HFHFr diet vs control diet: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4: Muscle neutral lipid accumulation in rats fed control or HFHFr diets for 4 to 20 weeks

Results were expressed as means \pm SD, n=6–8 animals per group per diet treatment duration. Muscle gastrocnemius TG, cholesterol and FFA (A), and muscle soleus DAG and Ceramides putative mediators of lipid-induced hepatic insulin resistance (B). All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each

treatment duration. The limit of statistical significance was set at $p < 0.05$. HFHF diet vs control diet: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. DAG: diacylglycerols; FFA: free fatty acids; TG: triglycerides.

Figure 5: Gene expression of the major players in the formation of lipid droplets in the soleus muscle in rats fed control or HFHF diets for 4 to 20 weeks

Results were expressed as means \pm SD, $n=6-8$ animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHF diet within each treatment duration. The limit of statistical significance was set at $p < 0.05$. HFHF diet vs control diet: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ATGL: adipose triglyceride lipase; CD36: cluster of differentiation 36 (fatty acid translocase); DGAT1: diacylglycerol O-acyltransferase 1; LPL: lipoprotein lipase; PLIN2: perilipin 2.

Figure 6: Fatty acid classes in the gastrocnemius muscle from rats fed control or HFHF diets for 4 to 20 weeks

Results were expressed as means \pm SD, $n=6-8$ animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHF diet within each treatment duration. The limit of statistical significance was set at $p < 0.05$. HFHF diet vs control diet: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids.

Figure 7: Spearman correlations between glucose tolerance test and accumulation of key lipids in gastrocnemius and soleus muscles

Results were expressed as means \pm SD, $n=6-8$ animals per group per diet treatment duration. Correlations were performed with the Spearman method. Glucose tolerance test was negatively correlated with muscle TG levels but positively correlated with muscle DAG levels. AUC: area under the curve; DAG: diacylglycerols; TG: triglycerides.

Figure 8: Spearman correlations between glucose tolerance test and gastrocnemius muscle fatty acid composition

Results were expressed as means \pm SD, n=6–8 animals per group per diet treatment duration. Correlations were performed with the Spearman method. AUC: area under the curve; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Figure 9: Spearman correlations between glucose tolerance test and mitochondrial activity markers in the gastrocnemius muscle

Results were expressed as means \pm SD, n=6–8 animals per group per diet treatment duration. Correlations were performed with the Spearman method. ADP: adenosine diphosphate; AUC: area under the curve; CI: complex I; COX: complex IV/cytochrome oxidase; MGS: malate/glutamate/succinate; ROS: reactive oxygen species.

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Figure 1: Weight of liver, adipose tissue and gastrocnemius and their ratios to rat body weight in rats fed control or HFHFr diets for 4 to 20 weeks

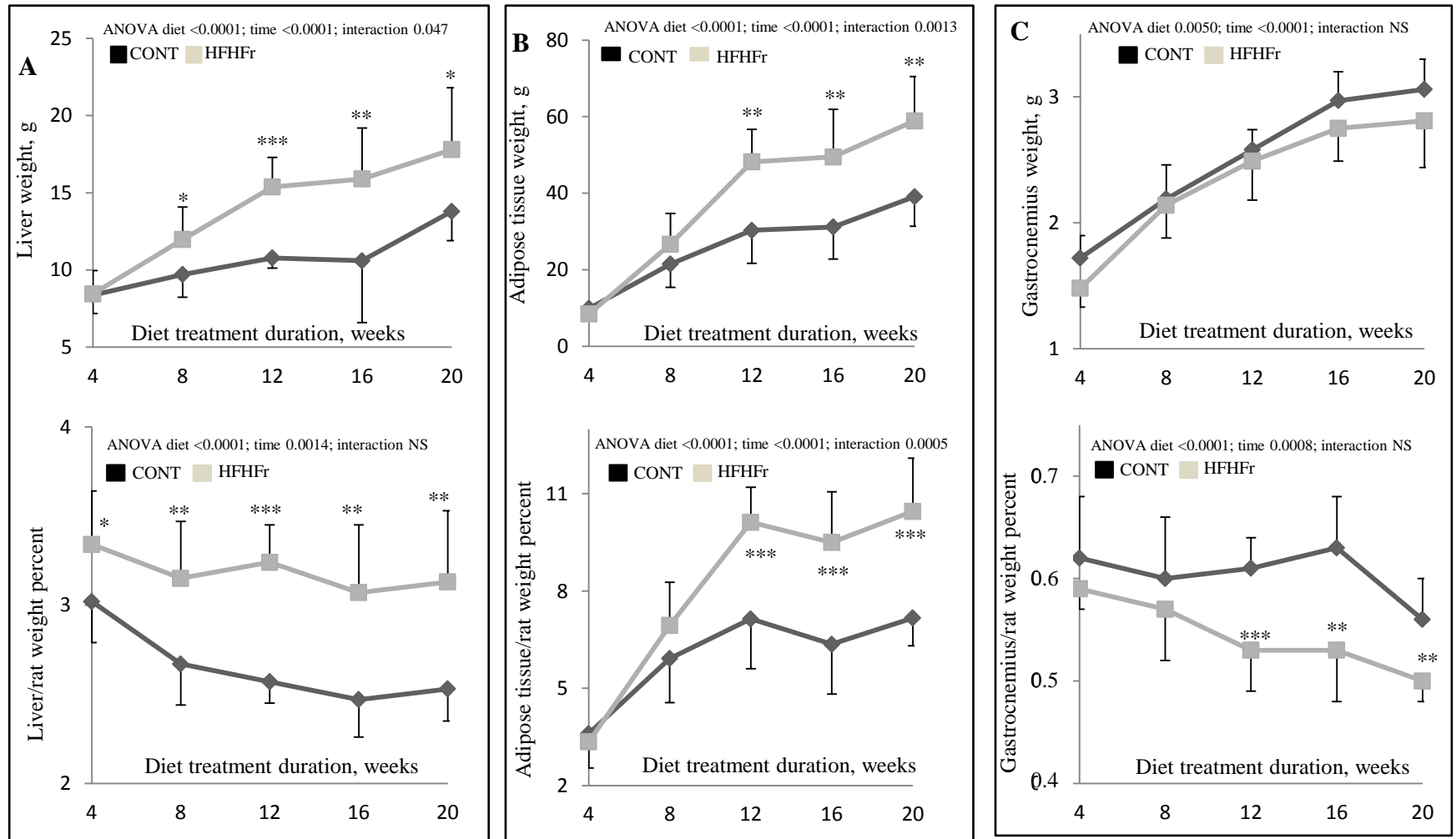


Figure 2: Time-course of oral glucose tolerance test in rats fed control or HFHFr diets for 4–20 weeks

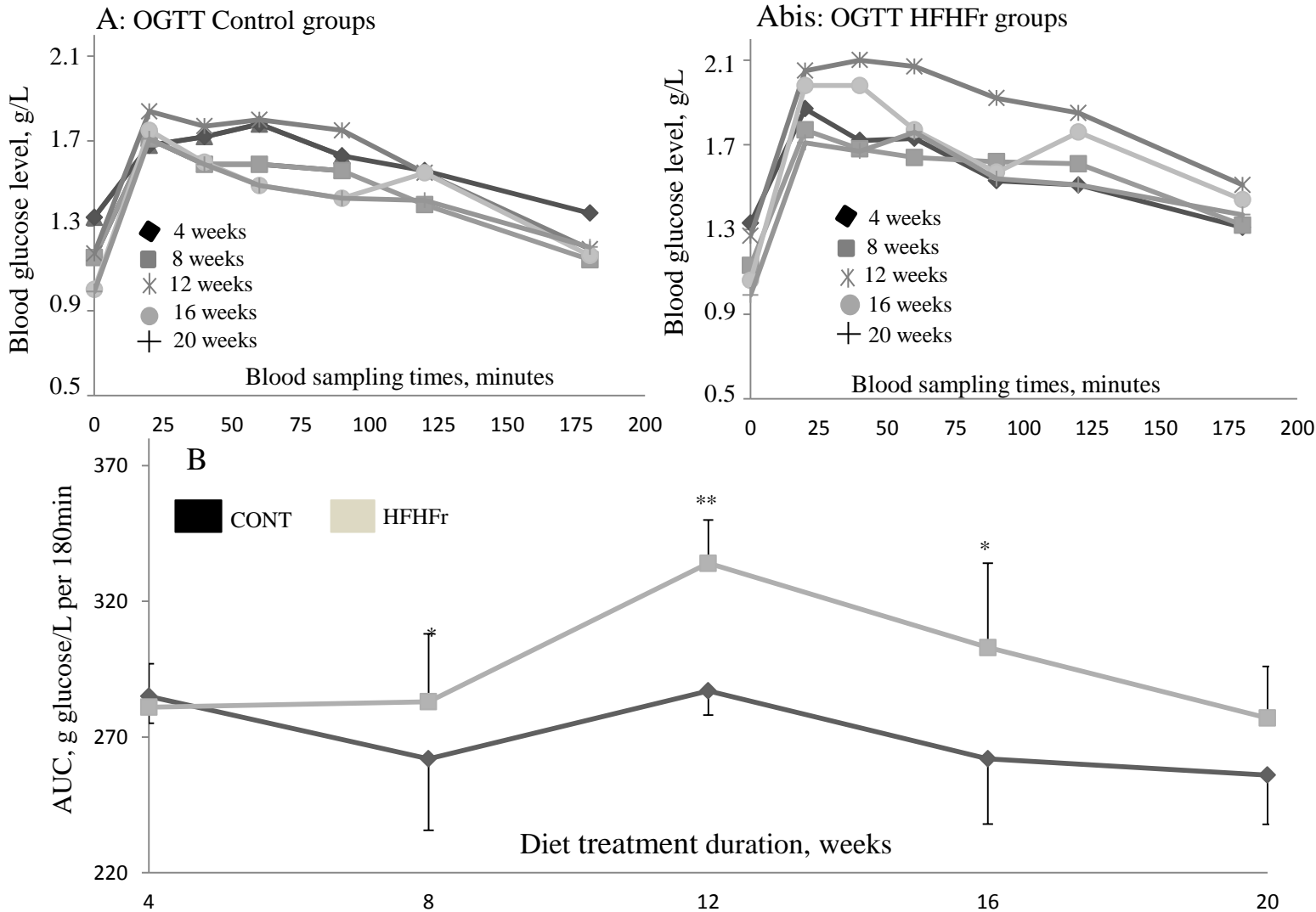


Figure 3: Blood lipid levels in rats fed control or HFHFr diets for 4 to 20 weeks

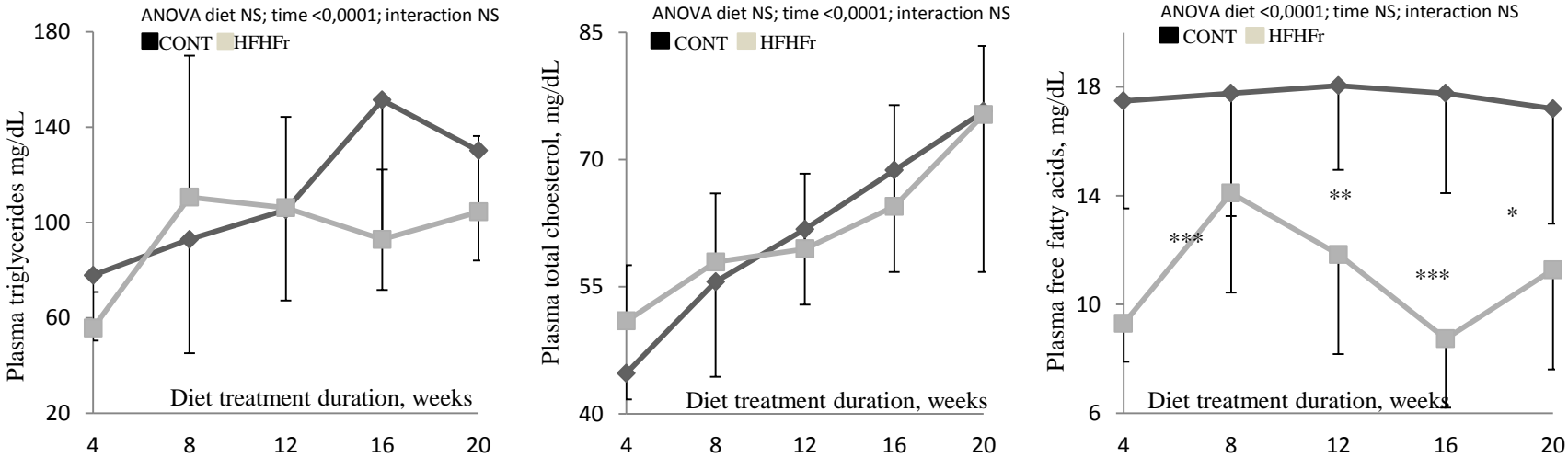


Figure 4: Muscle neutral lipid accumulation in rats fed control or HFHFr diets for 4 to 20 weeks

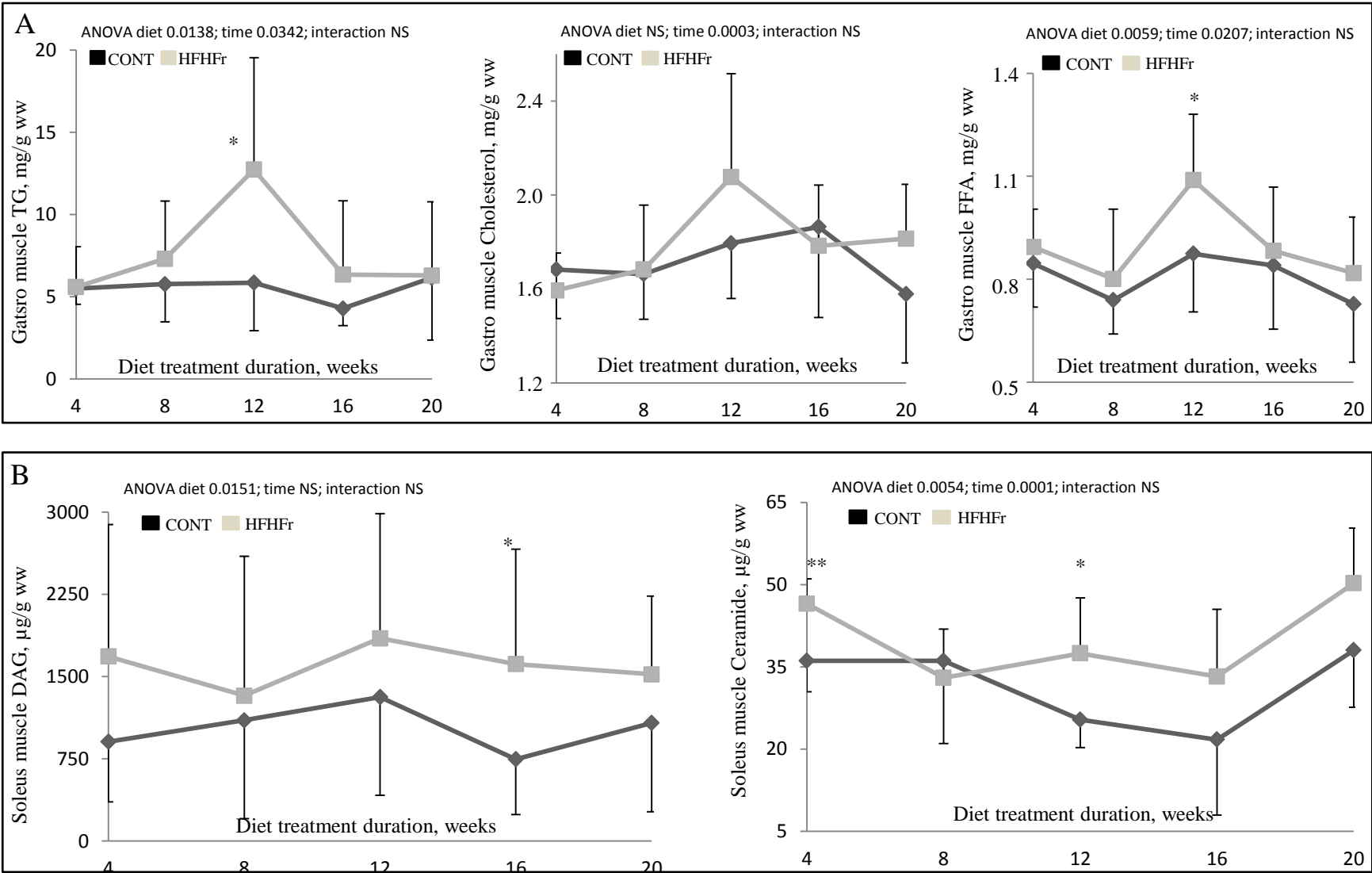


Figure 5: Gene expression of the major players in the formation of lipid droplets in the soleus muscle in rats fed control or HFHFr diets for 4 to 20 weeks

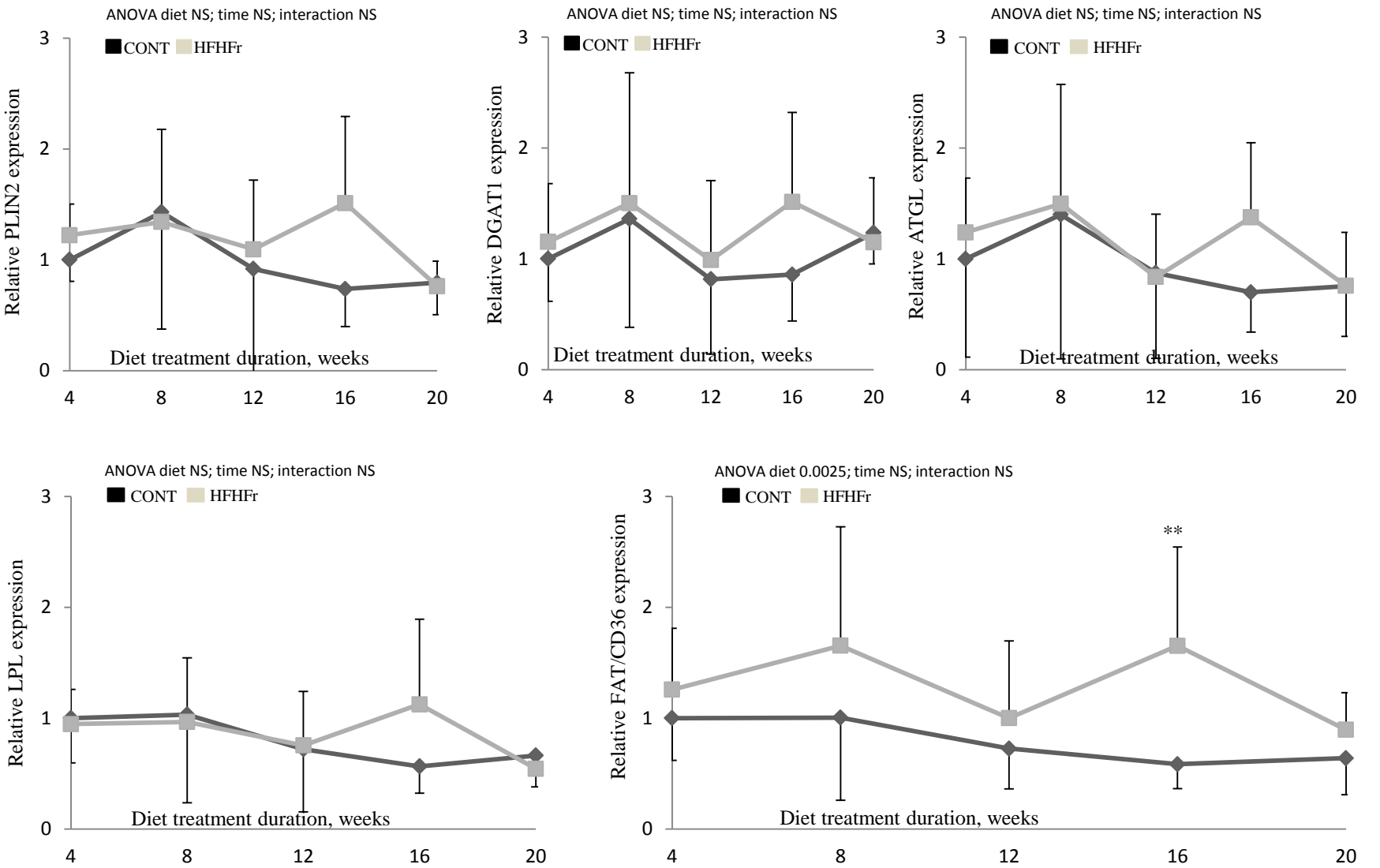


Figure 6: Fatty acid classes in the gastrocnemius muscle from rats fed control or HFHFr diets for 4 to 20 weeks

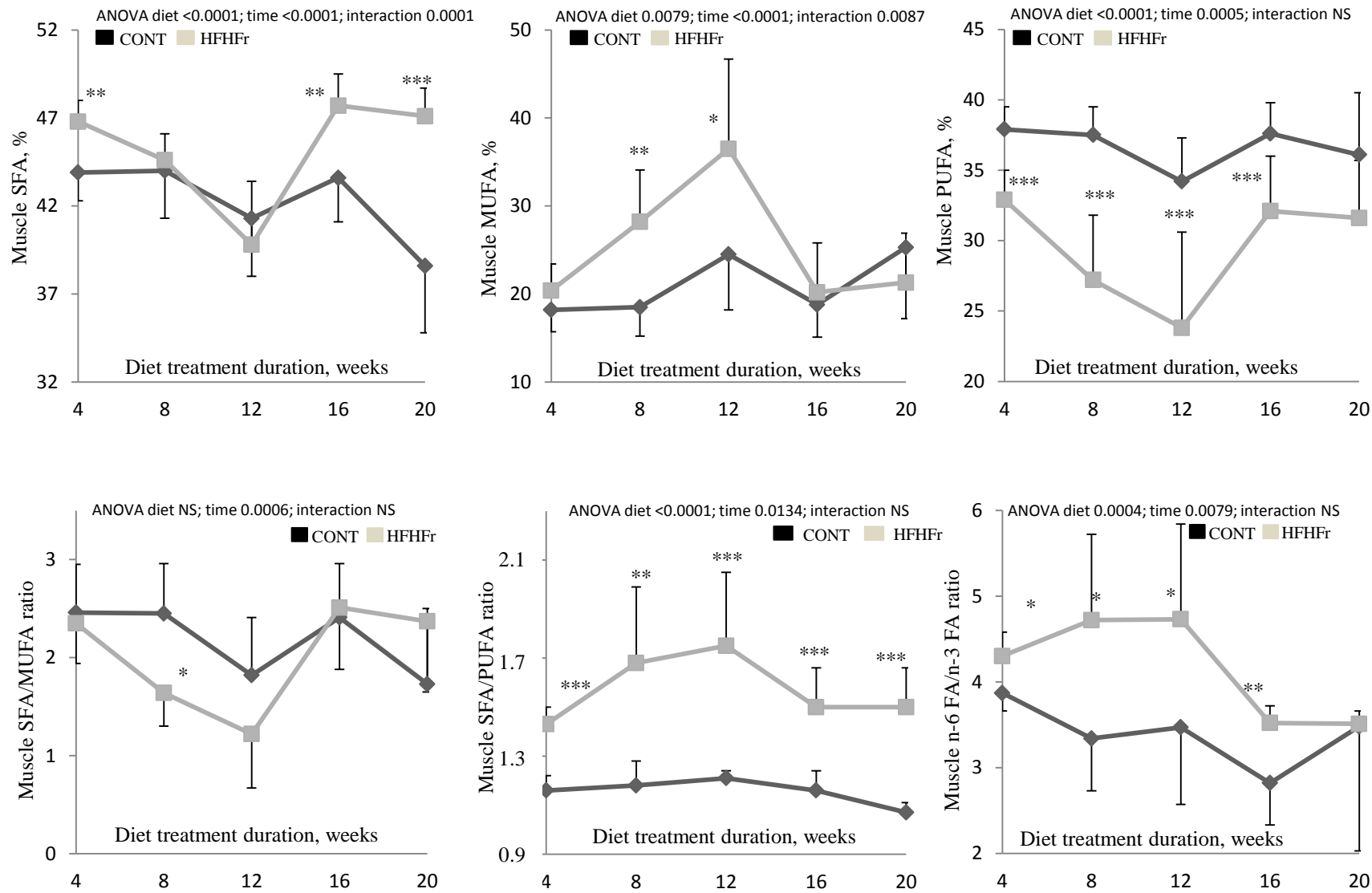


Figure 7: Spearman correlations between glucose tolerance test and accumulation of key lipids in gastrocnemius and soleus muscles

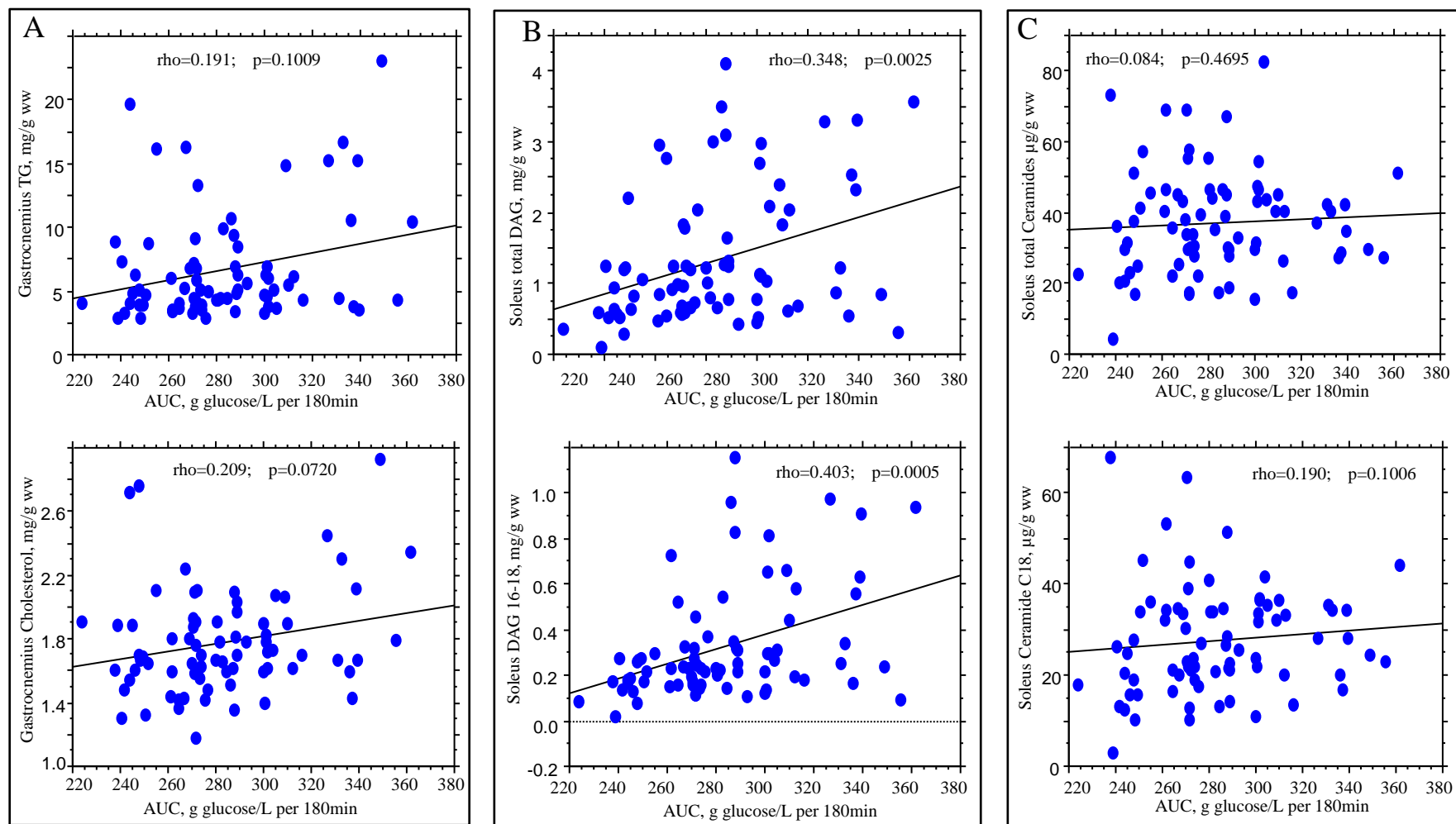


Figure 8: Spearman correlations between glucose tolerance test and gastrocnemius muscle fatty acid composition

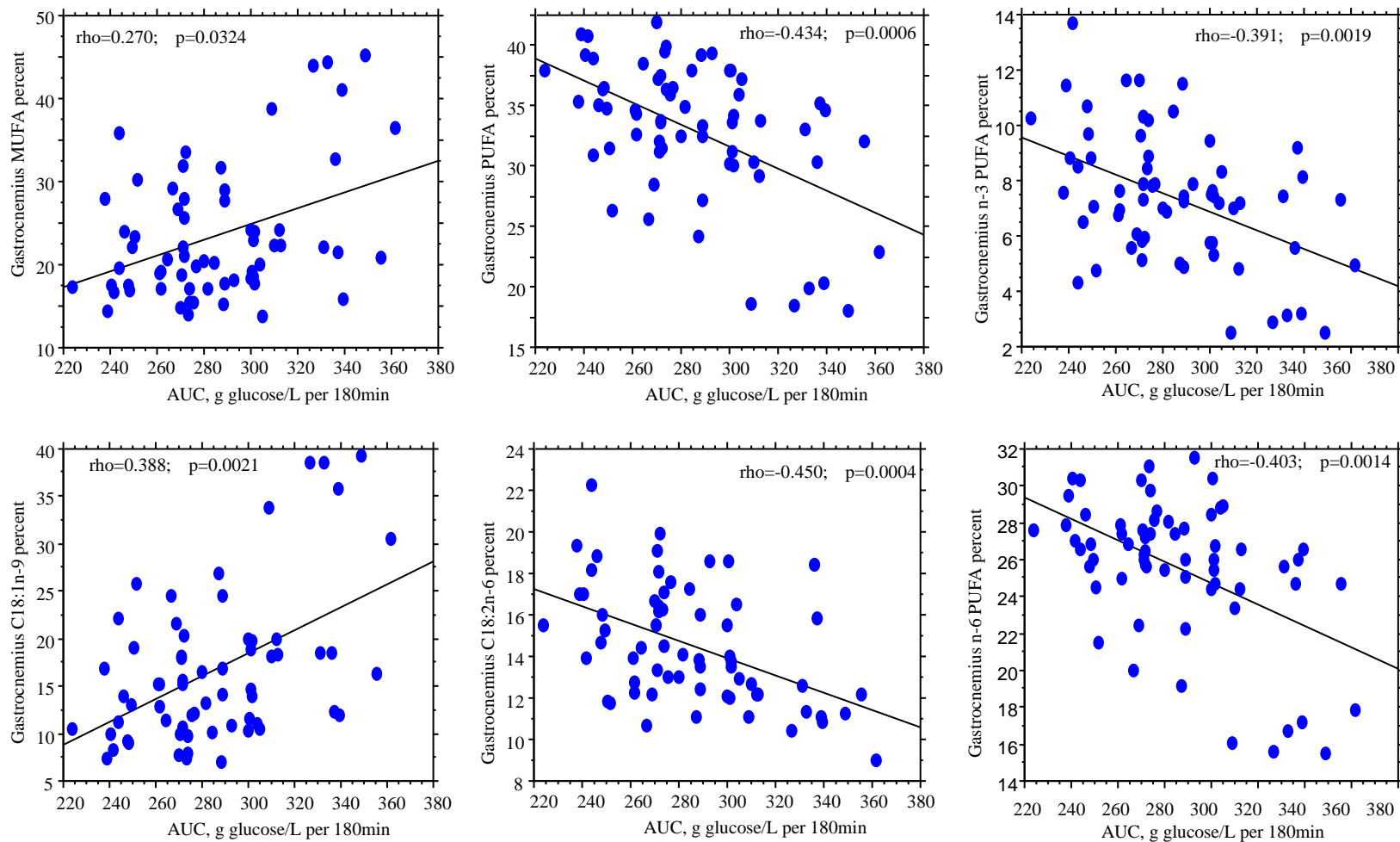


Figure 9: Spearman correlations between glucose tolerance test and mitochondrial activity markers in the gastrocnemius muscle

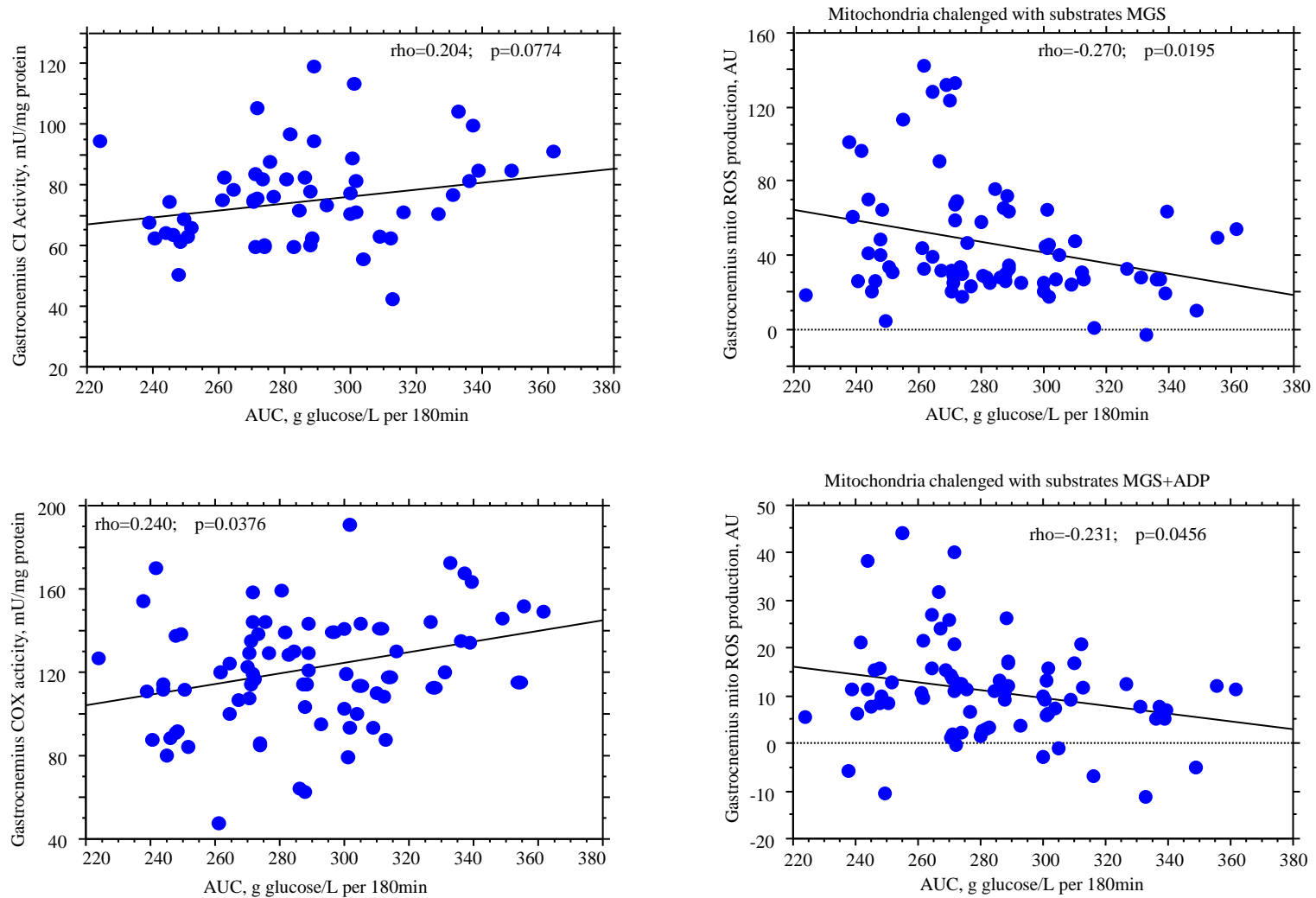


Table 1: Food consumption and rat body and organ weights.

Parameters	Diet	4 wks	8 wks	12 wks	16 wks	20 wks	p Diet	p Time	p time*diet
Dietary intake, g/d	Control	19.1±0.3	19.5±0.6	19.3±0.4	19.9±0.6	21.4±0.9			
	HFHFr	12.2±0.1***	14.1±0.1***	15.0±0.5***	15.1±0.3***	15.3±0.8***	<0.0001	<0.0001	0.0002
Energy intake, kCal/d	Control	73.6±1.3	75.2±2.2	74.6±1.6	76.7±2.4	82.6±3.5			
	HFHFr	65.1±0.4***	75.3±0.5	80.3±2.6*	80.5±1.5*	81.7±4.2	NS	0.0001	<0.0001
Initial body weight, g	Control	102±8	106±10	96±6	101±9	105±6			
	HFHFr	102±9	105±3	99±9	101±6	103±11	NS	NS	NS
Final body weight, g	Control	276±21	362 ± 32	421±41	471±35	543±53			
	HFHFr	252±23	379±44	474±40*	517±46*	561±60	0.0300	<0.0001	NS
Tibialis, g ww	Control	0.73±0.06	0.94±0.08	1.03±0.10	1.27±0.19	1.44±0.15			
	HFHFr	0.64±0.08	0.90±0.13	1.01±0.14	1.24±0.12	1.32±0.18	0.0347	<0.0001	NS
Soleus, mg ww	Control	109±6	151±23	158±15	192±18	215±21			
	HFHFr	94±13	130±16	163±28	181±25	205±30	0.0396	<0.0001	NS
EDL, mg ww	Control	120±10	155±15	172±16	197±25	220±26			
	HFHFr	108±13	143±25	169±27	195±21	198±32	0.0465	<0.0001	NS
Heart, g ww	Control	0.85±0.08	1.03±0.22	1.07±0.12	1.11±0.13	1.25±0.12			
	HFHFr	0.76±0.06	0.96±0.11	1.08±0.13	1.10±0.07	1.24±0.16	NS	<0.0001	NS
Kidney, g ww	Control	0.98±0.09	1.05±0.16	1.11±0.06	1.13±0.10	1.40±0.15			
	HFHFr	0.84±0.09	1.07±0.19	1.22±0.15	1.32±0.25	1.30±0.20	NS	<0.0001	0.0269

Results were expressed as means±SD, n=6–8 animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each diet treatment duration. The limit of statistical significance was set at $p<0.05$. HFHFr diet vs control diet: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NS: not significant. EDL: extensor digitorum longus; ww: wet weight.

Table 2: Content of diacylglycerols species in the soleus muscle ($\mu\text{g/g ww}$)

Parameters	Diet	4 wks	8 wks	12 wks	16 wks	20 wks	p Diet	p Time	p time*diet
<i>Diacylglycerols species</i>									
C16-18.2	Control	119 \pm 89	171 \pm 164	219 \pm 181	133 \pm 104	177 \pm 157	0.0367	NS	NS
(PM=592.5)	HFHFr	115 \pm 101	75 \pm 81	110 \pm 72	89 \pm 52	149 \pm 87			
C18.1-18.2	Control	212 \pm 207	267 \pm 338	299 \pm 243	178 \pm 124	260 \pm 276	NS	NS	NS
(PM=618.5)	HFHFr	187 \pm 171	126 \pm 131	155 \pm 96	184 \pm 111	319 \pm 278			
C16-16	Control	53 \pm 23	51 \pm 34	52 \pm 34	28 \pm 17	48 \pm 28	0.0267	NS	NS
(PM=568.4)	HFHFr	63 \pm 24	53 \pm 28	61 \pm 18	59 \pm 26*	65 \pm 25			
C16-18.1	Control	195 \pm 89	232 \pm 141	298 \pm 224	147 \pm 97	249 \pm 131	0.0012	NS	NS
(PM=594.5)	HFHFr	426 \pm 289	347 \pm 322	492 \pm 301	408 \pm 269*	316 \pm 174			
C16-18	Control	199 \pm 92	239 \pm 142	252 \pm 159	153 \pm 100	268 \pm 144	0.0004	NS	NS
(PM=596.4)	HFHFr	452 \pm 313	373 \pm 356	517 \pm 316	431 \pm 284*	332 \pm 175			
C18.1-18.1	Control	104 \pm 66	122 \pm 89	169 \pm 120	94 \pm 64	143 \pm 89	0.0002	NS	NS
(PM=620.5)	HFHFr	325 \pm 270	259 \pm 294	392 \pm 278	335 \pm 258*	225 \pm 186			
C18-18.1	Control	23 \pm 8	18 \pm 6	24 \pm 12	12 \pm 7	22 \pm 6	<0.0001	NS	NS
(PM=622.5)	HFHFr	117 \pm 68**	92 \pm 79*	123 \pm 80**	106 \pm 65*	77 \pm 81			

Results were expressed as means \pm SD, n=6–8 animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each diet treatment duration. The limit of statistical significance was set at $p < 0.05$. HFHFr diet vs control diet: * $p < 0.05$, ** $p < 0.01$. NS: not significant. ww: wet weight.

Table 3: Content of ceramides species in the soleus muscle ($\mu\text{g/g ww}$)

Parameters	Diet	4 wks	8 wks	12 wks	16 wks	20 wks	p Diet	p Time	p time*diet
<i>Ceramides species</i>									
Sphingosine (PM=299.3)	Control	0.31 \pm 0.04	0.30 \pm 0.04	0.27 \pm 0.04	0.13 \pm 0.04	0.30 \pm 0.15	0.0023	<0.0001	NS
	HFHFr	0.41 \pm 0.10*	0.29 \pm 0.08	0.30 \pm 0.05	0.24 \pm 0.06***	0.33 \pm 0.49			
Sphinganine (PM=301.3)	Control	0.05 \pm 0.01	0.04 \pm 0.01	0.04 \pm 0.01	0.04 \pm 0.02	0.05 \pm 0.05	0.0003	NS	NS
	HFHFr	0.07 \pm 0.03	0.05 \pm 0.02	0.07 \pm 0.02**	0.05 \pm 0.02	0.07 \pm 0.14			
C16 (PM=537.3)	Control	4.91 \pm 1.29	4.64 \pm 2.21	3.85 \pm 1.86	3.31 \pm 1.55	5.17 \pm 4.65	NS	0.0186	NS
	HFHFr	5.30 \pm 1.06	3.99 \pm 3.12	3.06 \pm 1.06	3.40 \pm 1.90	5.53 \pm 3.10			
C18.1 (PM=563.3)	Control	0.05 \pm 0.01	0.04 \pm 0.01	0.03 \pm 0.02	0.07 \pm 0.01	0.04 \pm 0.01	NS	NS	NS
	HFHFr	0.07 \pm 0.06	0.03 \pm 0.01*	0.03 \pm 0.01	0.04 \pm 0.05	0.04 \pm 0.02			
C18 (PM=565.3)	Control	25.9 \pm 4.4	27.4 \pm 16.3	17.8 \pm 3.7	12.7 \pm 5.6	33.3 \pm 30.9	0.0012	0.0002	NS
	HFHFr	34.8 \pm 2.3***	25.3 \pm 6.3	30.9 \pm 9.0**	25.3 \pm 8.3**	39.0 \pm 13.0			
C20 (PM=593.3)	Control	0.14 \pm 0.05	0.10 \pm 0.04	0.10 \pm 0.03	0.08 \pm 0.06	0.11 \pm 0.07	<0.0001	0.0131	NS
	HFHFr	0.20 \pm 0.09	0.13 \pm 0.03	0.14 \pm 0.03*	0.15 \pm 0.06*	0.20 \pm 0.11*			
C22 (PM=621.3)	Control	1.50 \pm 0.35	1.01 \pm 0.25	0.97 \pm 0.32	1.02 \pm 1.11	1.12 \pm 0.66	NS	0.0210	NS
	HFHFr	1.58 \pm 0.71	0.97 \pm 0.16	0.89 \pm 0.19	1.09 \pm 0.57	1.49 \pm 0.71			
C24.1 (PM=647.3)	Control	1.97 \pm 0.50	1.31 \pm 0.52	1.35 \pm 0.52	1.53 \pm 1.74	1.44 \pm 0.65	NS	NS	NS
	HFHFr	1.89 \pm 0.84	1.26 \pm 0.58	1.00 \pm 0.28	1.36 \pm 0.82	1.75 \pm 0.81			
C24 (PM=2649.3)	Control	1.72 \pm 0.27	1.57 \pm 0.19	1.31 \pm 0.29	3.32 \pm 6.56	1.67 \pm 0.38	NS	NS	NS
	HFHFr	2.68 \pm 1.40	1.27 \pm 0.25*	1.29 \pm 0.38	1.99 \pm 2.50	2.16 \pm 1.89			

Results were expressed as means \pm SD, n=6–8 animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each diet treatment duration. The limit of statistical significance was set at $p < 0.05$. HFHFr diet vs control diet: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NS: not significant. ww: wet weight.

Table 4: Fatty acid composition (%) of muscle lipids in the gastrocnemius muscle

Parameters	Diet	4 wks	8 wks	12 wks	16 wks	20 wks	p Diet	p Time	p time*diet
C14:0	Control	1.65±0.21	2.20±1.37	1.92±0.44	2.05±0.76	1.66±0.31			
	HFHFr	1.15±0.11	2.00±1.06	1.21±0.30	1.43±0.24	1.30±0.14	0.0032	NS	NS
C16:0	Control	27.9±0.7	28.1±1.2	27.2±0.7	27.7±1.2	26.2±0.9			
	HFHFr	29.0±0.8*	28.9±0.6	26.8±0.6	30.0±0.7***	29.9±0.5***	<0.0001	<0.0001	<0.0001
C18:0	Control	11.7±1.1	10.8±1.2	9.32±2.52	10.5±1.6	8.4±2.6			
	HFHFr	13.6±1.0*	11.0±1.2	9.72±2.19	12.7±1.3*	12.4±1.4**	0.0002	0.007	NS
C16:1 n-7	Control	2.55±0.87	3.07±1.34	4.51±2.56	3.16±1.09	4.70±2.23			
	HFHFr	0.78±0.23***	1.32±0.49*	1.74±0.63*	0.84±0.36***	0.99±0.49**	<0.0001	0.0403	NS
C18:1 n-9	Control	10.6±1.9	10.3±2.2	14.2±3.6	9.95±2.53	14.6±5.7			
	HFHFr	16.3±3.0**	23.8±5.6***	31.4±9.3***	16.2±5.2*	17.2±5.1	<0.0001	<0.0001	0.0023
C18:1 n-7	Control	3.85±0.15	3.88±0.16	4.10±0.38	4.06±0.39	4.28±0.33			
	HFHFr	2.17±0.11***	2.14±0.15***	2.08±0.13***	1.95±0.11***	1.94±0.09***	<0.0001	NS	0.0076
C18:2 n-6	Control	17.5±1.1	16.6±1.7	16.5±1.6	16.4±1.5	17.5±3.1			
	HFHFr	13.6±0.5***	11.9±0.5***	11.1±1.2***	12.4±1.1***	12.2±0.8**	<0.0001	NS	NS
C18:3 n-6	Control	0.066±0.009	0.060±0.010	0.049±0.012	0.056±0.012	0.066±0.005			
	HFHFr	0.040±0.006***	0.032±0.004***	0.029±0.004**	0.030±0.006***	0.032±0.004***	<0.0001	0.0005	NS
C18:3 n-3	Control	0.460±0.121	0.462±0.169	0.581±0.295	0.421±0.0178	0.727±0.392			
	HFHFr	0.142±0.048***	0.188±0.050**	0.214±0.067**	0.111±0.038***	0.112±0.03**	<0.0001	NS	NS
C20:3 n-6	Control	0.530±0.035	0.428±0.054	0.359±0.098	0.391±0.034	0.307±0.120			
	HFHFr	0.622±0.081***	0.537±0.158	0.409±0.212	0.607±0.104***	0.597±0.138**	<0.0001	0.0039	NS
C20:4 n-6	Control	11.2±1.4	10.9±1.4	8.73 ± 2.30	10.6±1.7	9.06±3.55			
	HFHFr	11.2±1.3	8.96±2.57	7.09 ± 3.74	11.8±2.5	11.6±2.3	NS	0.0064	NS
C22:6 n-3	Control	5.05±0.41	6.17±1.20	5.68 ± 1.89	7.27±1.48	6.54±3.18			
	HFHFr	4.54±0.59	3.46±1.22**	3.14 ± 1.50*	5.25±0.79**	5.21±0.75	<0.0001	0.0130	NS
n-6	Control	30.1±1.3	28.7±1.3	26.2±1.3	27.7±1.6	27.1±1.6			
	HFHFr	26.6±1.4	22.2±3.3	19.3±4.7	25.0±3.0	24.6±3.1	<0.0001	<0.0001	NS
n-3	Control	7.78±0.48	8.80±1.39	7.98±1.98	9.99±1.45	8.94±3.43			
	HFHFr	6.23±0.70	4.96±1.49	4.46±2.09	7.13±1.01	7.03±1.04	<0.0001	0.0074	NS
Unsaturation Index	Control	145±5	148±9	140±13	152±9	148±22			
	HFHFr	134±7*	120±14**	116±19*	135±11**	134±11	<0.0001	0.0233	NS
$\Delta 9$ -desaturase (C16:1n-7/C16:0) x 100	Control	9.12±2.96	10.88±4.66	16.64±9.69	11.45±4.02	18.13±8.86			
	HFHFr	2.70±0.80***	4.55±1.76*	6.50±2.42*	2.81±1.24***	3.28±1.62**	<0.0001	0.0260	NS
$\Delta 6$ -desaturase (C18:3n-6/C18:2n-6) x 100	Control	0.37±0.05	0.34±0.04	0.30±0.07	0.35±0.08	0.38±0.04			
	HFHFr	0.30±0.05*	0.28±0.04*	0.25±0.06	0.25±0.04***	0.27±0.04***	<0.0001	0.0538	NS
$\Delta 5$ -desaturase (C20:4n-6/C20:3n-6)	Control	21.0±2.3	25.9±4.7	24.6±3.6	27.0±2.6	29.1±2.7			
	HFHFr	18.0±1.3*	16.9±1.8***	17.4±3.4**	19.5±3.2***	19.7±2.2***	<0.0001	0.0009	NS
SCD1/RPS9, qPCR	Control	100±52	252±142	159±102	161±83	225±84			
	HFHFr	116±59	185±101	92±25	107±37	140±75*	0.0085	0.0028	NS

Results were expressed as means±SD, n=6–8 animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each diet treatment duration. The limit of statistical significance was set at $p < 0.05$. HFHFr diet vs control diet: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NS: not significant. MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; qPCR: quantitative-polymerase chain reaction; SCD1: stearoyl-CoA desaturase 1; SFA: saturated fatty acids.

Unsaturation index = (% monoenoic x1) + (% dienoic x2) + (% trienoic x3) + (% tetraenoic x4) + (% pentaenoic x5) + (% hexaenoic x6).

Table 5: Protein abundance of major actors of lipid metabolism in the gastrocnemius muscle

Parameters	Diet	4 wks	8 wks	12 wks	16 wks	20 wks
CD36/ β -actin	Control	1.00 \pm 0.17	1.00 \pm 0.15	1.00 \pm 0.36	1.00 \pm 0.36	1.00 \pm 0.12
	HFHFr	1.09 \pm 0.34	1.20 \pm 0.47	0.97 \pm 0.39	0.85 \pm 0.25	0.85 \pm 0.26
FATP1/ β -actin	Control	1.00 \pm 0.20	1.00 \pm 0.8	1.00 \pm 0.16	1.00 \pm 0.18	1.00 \pm 0.70
	HFHFr	0.97 \pm 0.38	1.04 \pm 0.20	1.24 \pm 0.33	1.20 \pm 0.39	0.54 \pm 0.41
ACSL1/ β -actin	Control	1.00 \pm 0.28	1.00 \pm 0.21	1.00 \pm 0.16	1.00 \pm 0.20	1.00 \pm 0.12
	HFHFr	0.89 \pm 0.16	0.82 \pm 0.30	1.39 \pm 0.38*	1.05 \pm 0.39	1.10 \pm 0.31
PGC1 α / β -actin	Control	1.00 \pm 0.12	1.00 \pm 0.11	1.00 \pm 0.30	1.00 \pm 0.32	1.00 \pm 0.76
	HFHFr	0.99 \pm 0.18	0.79 \pm 0.35	1.34 \pm 0.47	0.77 \pm 0.32	0.81 \pm 0.48
mCPT1/ β -actin	Control	1.00 \pm 0.27	1.00 \pm 0.37	1.00 \pm 0.28	1.00 \pm 0.09	1.00 \pm 0.22
	HFHFr	0.98 \pm 0.24	1.04 \pm 0.38	0.90 \pm 0.38	1.10 \pm 0.44	0.91 \pm 0.16
ACC/ β -actin	Control	1.00 \pm 0.07	1.00 \pm 0.07	1.00 \pm 0.24	1.00 \pm 0.36	1.00 \pm 0.33
	HFHFr	0.88 \pm 0.21	0.84 \pm 0.27	1.27 \pm 0.30	0.93 \pm 0.35	1.07 \pm 0.18
p-ACC/ β -actin	Control	1.00 \pm 0.36	1.00 \pm 0.37	1.00 \pm 0.35	1.00 \pm 0.55	1.00 \pm 0.39
	HFHFr	0.99 \pm 0.38	0.63\pm0.32*	1.20 \pm 0.25	0.89 \pm 0.41	0.96 \pm 0.55
FAS/ β -actin	Control	1.00 \pm 0.73	1.00 \pm 0.55	1.00 \pm 1.27	1.00 \pm 1.29	1.00 \pm 0.35
	HFHFr	0.68 \pm 0.35	0.36\pm0.35*	0.18 \pm 0.16	0.50 \pm 0.27	0.58\pm0.46*

Results were expressed as means \pm SD, n=6–8 animals per group per diet treatment duration. Because western blotting assay was performed separately on each treatment duration, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each diet treatment duration. The limit of statistical significance was set at $p < 0.05$. * $p < 0.05$. ACC: acetyl-CoA carboxylase; ACSL1: acyl-CoA synthetase long chain family member 1; CD36/FAT: cluster of differentiation 36 (fatty acid translocase); FAS: fatty acid synthase; FATP1: fatty-acid-transporter protein; mCPT1: muscle carnitine palmitoyltransferase I; PGC-1 α : peroxisome proliferator-activated receptor γ coactivator-1 α .

The WB results were obtained as blot intensity of the studied protein divided by blot intensity of β -actin and normalised by the blot intensity of one control reference in the same gel: (protein blot intensity/actin blot intensity)/(protein blot intensity/actin blot intensity)_{control reference}. Then all the results were normalized by the mean value obtained for the control group inside of each diet treatment duration.

Table 6: Gene expression of major actors of lipid metabolism in the gastrocnemius muscle

Parameters	Diet	4 wks	8 wks	12 wks	16 wks	20 wks	p Diet	p Time	P time*diet
FABP3/RPS9	Control	1.00±0.55	0.76±0.23	0.79±0.28	0.75±0.46	0.64±0.34	0.0419	NS	NS
	HFHFr	1.26±0.65	0.82±0.28	0.96±0.38	0.84±0.20	1.06±0.39*			
PPAR α /RPS9	Control	1.00±0.27	0.71±0.37	0.97±0.31	1.00±0.31	0.91±0.27	NS	0.0167	NS
	HFHFr	1.22±0.27	0.79±0.21	0.88±0.29	0.89±0.19	0.86±0.22			
PPAR γ /RPS9	Control	1.00±0.48	0.60±0.35	0.75±0.30	0.80±0.17	0.64±0.17	NS	0.0017	NS
	HFHFr	1.43±0.65	0.80±0.32	0.85±0.40	0.74±0.29	0.76±0.33			
PGC-1 α /RPS9	Control	1.00±0.36	1.68±0.41	1.22±0.27	1.17±0.46	1.31±0.40	0.0145	0.0040	NS
	HFHFr	1.08±0.33	1.33±0.32	0.95±0.32	0.86±0.19	1.10±0.51			
Srebp-1c/RPS9	Control	1.00±0.45	0.74±0.36	1.46±0.52	1.52±0.78	1.60±0.91	NS	NS	NS
	HFHFr	1.22±0.34	1.40±0.83	1.52±0.47	1.59±0.46	1.33±0.38			

Results were expressed as means±SD, n=6–8 animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each diet treatment duration. The limit of statistical significance was set at *p<0.05. NS: not significant. FABP3: fatty-acid-binding protein 3; PGC-1 α : peroxisome proliferator-activated receptor γ coactivator-1 α ; PPAR α : peroxisome proliferator activated receptor alpha; PPAR γ : peroxisome proliferator activated receptor gamma; Srebp-1c: Sterol regulatory element-binding transcription factor 1c.

The qPCR results were obtained as: $(1+E_{\text{gene}})^{(C(t) \text{ of GENE standard}-C(t) \text{ of GENE})}/(1+E_{\text{RPS9}})^{(C(t) \text{ of RPS9 standard}-C(t) \text{ of RPS9})}$ with E: efficiency of the gene and C(t): cycle threshold obtained by relative qPCR. Then all the results were normalized by the mean value obtained for the control group at 4 weeks. In such a case, all the other groups were relative to the control group at 4 weeks of diet.

Table 7: Characterization of mitochondrial functionality in the gastrocnemius muscle

Parameters	Diet	4 wks	8 wks	12 wks	16 wks	20 wks	p Diet	p Time	p time*diet
Membrane fluidity									
With 5-NS label, AU	Control	1.77±0.05	1.74±0.02	1.97±0.06	1.99±0.06	1.82±0.14	NS	<0.0001	NS
	HFHF _r	1.82±0.12	1.82±0.05	1.99±0.08	2.02±0.12	1.78±0.06			
With 16-NS label, AU	Control	0.99±0.07	1.18±0.09	1.69±0.37	1.64±0.53	1.05±0.11	NS	0.0006	NS
	HFHF _r	1.15±0.10	1.63±0.78	1.60±0.48	1.68±0.41	1.10±0.07			
Membrane potential									
Mitoch alone, AU	Control	0.25±0.04	0.28±0.07	0.32±0.07	0.27±0.08	0.21±0.08	NS	NS	NS
	HFHF _r	0.23±0.06	0.26±0.05	0.31±0.03	0.27±0.06	0.19±0.06			
Mitoch+MGS, AU	Control	0.45±0.04	0.53±0.06	0.52±0.08	0.57±0.06	0.43±0.08	NS	NS	NS
	HFHF _r	0.43±0.10	0.51±0.07	0.55±0.04	0.55±0.09	0.44±0.08			
Mitoch+MGS+ADP, AU	Control	0.33±0.02	0.38±0.03	0.35±0.06	0.34±0.05	0.27±0.05	NS	NS	NS
	HFHF _r	0.32±0.05	0.37±0.04	0.37±0.03	0.33±0.07	0.27±0.04			
Mitoch. respiration									
Mito+MGS, pmol O ₂ /sec/μg protein	Control	0.97±0.29	1.23±0.40	1.13±0.37	1.34±0.33	1.60±0.46	NS	NS	NS
	HFHF _r	0.79±0.22	1.15±0.42	0.94±0.44	1.1.26±0.35	1.65±0.26			
Mito+MGS+ADP, pmol O ₂ /sec/μg prot.	Control	2.33±0.81	3.97±1.28	4.12±1.47	5.31±1.86	3.66±1.48	NS	NS	NS
	HFHF _r	1.88±0.71	3.36±1.15	3.15±1.78	4.80±1.39	3.80±1.27			
Mitochondrial RCR	Control	2.38±0.43	3.29±0.50	3.65±0.95	3.95±1.08	2.31±0.85	NS	NS	NS
	HFHF _r	2.33±0.56	3.14±0.97	3.03±0.98	3.89±0.89	2.302±0.74			

Results were expressed as means±SD, n=6–8 animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each diet treatment duration. The limit of statistical significance was set at $p < 0.05$. NS: not significant. ADP: adenosine diphosphate; AU: arbitray units; MGS: malate/glutamate/succinate; 5-NS: 5-nitroxide stearate; 16-NS, 16-nitroxide stearate; RCR: respiratory coefficient ratio.

Table 8: Gastrocnemius mitochondrial respiratory chain complexes activities and cardiolipin synthesis

Parameters	Diet	4 wks	8 wks	12 wks	16 wks	20 wks	p Diet	p Time	p time*diet
Citrate synthase, mU/mg protein	Control	549±80	539±61	529±59	469±70	518±84	NS	0.0007	NS
	HFHFr	576±61	591±105	486±91	482±51	425±77			
Complex I, mU/mg protein	Control	76.0±10.5	64.9±6.9	82.3±14.9	69.7±16.2	77.5±18.4	NS	NS	NS
	HFHFr	85.3±13.6	72.7±19.6	78.2±18.2	75.4±14.1	59.2±17.0			
Complex II, mU/mg protein	Control	116±21	90±7	137±15	115±27	140±31	NS	0.0059	NS
	HFHFr	124±20	104±33	126±28	122±23	111±29			
Complexes II+III, mU/mg protein	Control	18.3±3.2	14.2±2.7	19.8±5.4	20.1±5.9	20.5±5.3	NS	0.0138	NS
	HFHFr	16.5±3.4	15.1±3.2	17.7±2.5	18.7±4.0	18.2±3.7			
Complex IV, mU/mg protein	Control	124±22	95±17	138±17	119±24	132±20	NS	<0.0001	0.0426
	HFHFr	93±35	104±13	135±29	138±30	127±14			
β-HAD, mU/mg protein	Control	169±29	178±29	205±21	185±38	171±42	NS	NS	NS
	HFHFr	196±32	216±46	205±44	215±34	203±45			
CDS1/RPS9, qPCR	Control	1.00±0.42	0.34±0.16	0.81±0.38	0.75±0.45	0.82±0.43	NS	0.0240	NS
	HFHFr	1.09±0.42	0.72±0.44	0.97±0.42	0.82±0.43	0.71±0.38			
CLS/RPS9, qPCR	Control	1.00±0.33	1.52±0.33	1.32±0.42	1.27±0.50	1.59±0.70	0.0196	NS	NS
	HFHFr	1.06±0.25	1.29±0.37	0.94±0.22	0.99±0.37	1.24±0.63			
CLS/β-actin, WB	Control	1.00±0.24	1.00±0.46	1.00±0.49	1.00±0.41	1.00±0.32	t-test	t-test	t-test
	HFHFr	1.40±0.66	0.99±0.42	1.32±0.49	1.00±0.46	1.00±0.44			

Results were expressed as means±SD, n=6–8 animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each treatment duration. Because western blotting assay of CLS was performed separately on each diet treatment duration, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each diet treatment duration. The limit of statistical significance was set at $p < 0.05$. NS: not significant. CDS1: CDP-diacylglycerol synthase 1; CLS: cardiolipin synthase; β-HAD: β-hydroxyacyl coenzyme A dehydrogenase; qPCR: quantitative-polymerase chain reaction; WB: western blotting.

Table 9: ROS production and oxidative stress parameters in the gastrocnemius muscle

Parameters	Diet	4 wks	8 wks	12 wks	16 wks	20 wks	p Diet	p Time	p time*diet
<i>Mito ROS production</i>									
Mito+MGS, AU	Control	29.1±8.1	25.3±4.8	26.7±5.3	60.1±13.3	96.0±31.2			
	HFHFr	30.6±9.1	29.9±5.6	32.1±13.2	51.6±11.5	91.5±39.1	NS	NS	NS
Mito+MGS+ADP, AU	Control	6.1±4.0	8.8±5.3	8.8±3.9	15.7±5.9	19.4±17.0			
	HFHFr	8.5±4.3	12.9±4.6	9.7±3.1	14.4±5.2	17.0±16.7	NS	NS	NS
<i>Oxidative stress</i>									
TBARS, nmol/g ww	Control	41.2±3.6	45.0±3.4	46.9±3.0	42.7±4.9	37.2±6.4			
	HFHFr	40.9±3.3	43.2±4.5	45.6±9.3	39.2±3.2b	35.7±6.3	NS	<0.0001	NS
Thiols, nmol/mg protein	Control	176±22	183±13	194±15	184±19	185±22			
	HFHFr	175±9	182±13	191±24	193±23	175±23	NS	NS	NS
Total GSH, nmol/g ww	Control	832±160	706±69	671±80	693±57	708±80			
	HFHFr	821±80	712±99	668±99	695±94	684±82	NS	0.0002	NS
SOD, U/mg protein	Control	5.75±0.75	7.71±1.05	9.24±0.58	8.83±1.17	8.96±0.97			
	HFHFr	6.20±0.60	7.37±0.56	9.10±1.41	9.01±1.05	7.95±1.18	NS	<0.0001	NS
Catalase, U/mg protein	Control	24.3±3.9	21.0±3.8	21.4±4.2	21.0±4.8	23.2±7.3			
	HFHFr	24.7±5.2	25.3±4.0	21.9±4.1	25.7±5.3	24.0±8.0	NS	NS	NS
GPx, mU/mg protein	Control	521±64	756±92	1072±147	1069±262	1097±235			
	HFHFr	594±88	759±138	1019±200	1044±199	913±240b	NS	<0.0001	NS

Results were expressed as means ± SD, n=6–8 animals per group per diet treatment duration. All the groups were tested for the effects of diet, treatment duration, and their interaction by two-way ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse the effect of HFHFr diet within each diet treatment duration. The limit of statistical significance was set at $p < 0.05$. NS: not significant. ADP: adenosine diphosphate; GSH: glutathione; GPx: glutathione peroxidase; MGS: malate/glutamate/succinate; SOD: superoxide dismutase; TBARS: thiobarbituric acid reactive substances; Thiols: sulfhydryl groups; ww: wet weight.