



HAL
open science

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

Yang Wang, Gilles Fouret, Béatrice Bonafos, Agnieszka Blachnio-Zabielska, Thibault Leroy, David Crouzier, Bruno Barea, Sylvie Gaillet, Cédric Moro, Jérôme Lecomte, et al.

► To cite this version:

Yang Wang, Gilles Fouret, Béatrice Bonafos, Agnieszka Blachnio-Zabielska, Thibault Leroy, et al.. 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. *Journal of Nutritional Biochemistry*, 2019, 64, pp.182-197. 10.1016/j.jnutbio.2018.10.021 . hal-02154358

HAL Id: hal-02154358

<https://hal.science/hal-02154358>

Submitted on 21 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

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

4
5 Yang Wang^{1,2}, Gilles Fouret¹, Beatrice Bonafos¹, Agnieszka Blachnio-Zabielska^{3,4}, Thibault
6 Leroy⁵, David Crouzier⁶, Bruno Barea⁷, Sylvie Gaillet¹, Cedric Moro⁵, Jerome Lecomte⁷,
7 Charles Coudray¹, Christine Feillet-Coudray^{1*}

8
9 ¹ DMEM, INRA, Univ. Montpellier, Montpellier, France

10 ² College of animal science & veterinary medicine, Zhejiang agricultural university, Hangzhou, China

11 ³ Department of Physiology, Medical University of Bialystok, Poland

12 ⁴ Hygiene, Epidemiology Metabolic Disorders Department, Medical University of Bialystok, Poland

13 ⁵ INSERM, UMR1048, Institute of Metabolic and Cardiovascular Diseases, Toulouse, France

14 ⁶ Institut de Recherche Biomédicale des Armées, Paris, France

15 ⁷ IATE, CIRAD, Montpellier, France

16
17
18
19 **Running title:** Muscle lipid accumulation and glucose homeostasis in HFHFr diet-fed rats

20

21

22

23 *Corresponding author

24 Dr Christine FEILLET-COUDRAY, INRA UMR 866, 34060 Montpellier, France.

25 Tel: 33 4 99 61 30 38 / Fax: 33 4 67 54 56 94

26 Email: christine.coudray@inra.fr

27

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

51 **1. Introduction**

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

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

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

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

89

90 **2. Materials and Methods**

91 *2.1 Animals and diets*

92 Seventy five 6-weeks old male Wistar rats (Charles River, L'Arbresle, France) were housed (2 per
93 cage) under conditions of constant temperature (20-22°C), humidity (45-50%) and a standard dark
94 cycle (20.00-08.00 hours). The rats were randomized into two groups of animals and fed for 4 to 20
95 weeks one of the two following semi-purified diets: 1) control diet, or 2) high fat/high fructose diet
96 (HFHFr). Seven rats from control group and 8 rats from HFHFr group were sacrificed after 4, 8, 12,
97 16 or 20 weeks of diet treatment.

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

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

108

109 *2.2 Oral glucose tolerance test*

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

117

118 *2.3 Sampling and routine biochemical analyses*

119 Four to five days after the OGTT, 9 hours-fasted rats (23h:00–08h:00) were anaesthetized with
120 pentobarbital (40 mg/kg b.w.) (Ceva Santé Animale, Libourne, Fr) and blood was drawn from the
121 abdominal artery with a heparinized syringe and distributed into dry and heparinized tubes. Blood
122 tubes were centrifuged at 1000 g for 10 min at 4°C, plasma or serum were collected and stored at –
123 80°C until analysis. Liver, heart, muscles, adipose tissue and kidney were removed, rinsed with 0.9%
124 NaCl, weighed and kept at -80°C until analysis, except a gastrocnemius that has been used for the
125 isolation of fresh mitochondria.

126

127 *2.4 Muscle neutral lipids and diacylglycerols and ceramide species*

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

132 The muscle diacylglycerols and ceramide contents were measured according to the methods
133 described by Blachnio-Zabielska et al. [24, 25]. Briefly, lipids were extracted from ~20 mg of soleus
134 muscle by the extraction mixture isopropanol/water/ethyl acetate (35/5/60; v/v/v). Quantitative
135 measurement of ceramides and diacylglycerols was made using an Agilent 6460 triple quadrupole
136 mass spectrometer. Both ceramides and diacylglycerols were analyzed using positive ion electrospray
137 ionization source with multiple reaction monitoring. The chromatographic separation was performed
138 using an Agilent 1290 Infinity Ultra High Performance Liquid Chromatography. A mixture of
139 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
140 internal standards for ceramides and Deuterated DAG Mixture I and Mixture II were used as internal
141 standards for diacylglycerols (Avanti Polar Lipids, Alabaster, AL, USA). Concentration of each
142 compound was assessed against standard calibration curves.

143

144 *2.5 Phospholipids analysis of gastrocnemius muscle*

145 Lipids from gastrocnemius muscle were extracted by a Folch mixture of chloroform/methanol (2:1
146 v/v) in the presence of butylated hydroxytoluene (50 mg/L). The application of Folch lipid extracts on
147 silica gel 60 HPTLC plates 250 μ m, 20*10 cm, pretreated with 2.3% w/v boric acid in ethanol (100%),
148 was automatically performed on a 4 mm band width using a ATS4 apparatus (CAMAG, Muttenz,
149 Switzerland). The development was performed with methanol/acetic acid/pentane/chloroform
150 (15/10/30/45, by volume), which allowed the separation of phospholipids and neutral lipids on a 60
151 mm total migration distance. The scanning of the plates was carried out using a TLC scanner 3
152 (CAMAG, Muttenz, Switzerland), operating in the reflectance mode. The plates were scanned at 715
153 nm after dipping in a solution of Blue Spray (Blue Spray/H₂SO₄ 4.2 M/acetone, 1/2/3 v/v/v) and
154 heating for 3 min at 55 °C. The different classes of phospholipids (sphingomyelin,
155 lysophosphatidylcholine, phosphatidylcholine, phosphatidylinositol, phosphatidyl-serine,
156 phosphatidylethanolamine, phosphatidylglycerol, phosphatidic acid, cardiolipin) (SM, LPC, PC, PI,
157 PS, PE, PG, PA, CL respectively) were finally identified by comparing their retention factor (R_f) to
158 authentic standards and quantified using calibration curves of the same standards [26].

159

160 *2.6 Fatty acids composition of gastrocnemius muscle*

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

173

174 *2.7 Desaturase activity and unsaturation indices of gastrocnemius muscle*

175 Because it is very tricky to directly measure the activity of the enzymes that catalyze the
176 desaturation and elongation reactions in the fatty acid metabolic pathways, results in animals have
177 supported the use of surrogate measures of desaturase activity such as use of desaturase indices [28].
178 These indices are based on the ratio product to precursor of individual fatty acids. The desaturase
179 indices were calculated as follows: $\Delta 9$ desaturase = [16:1 n-7/16:0], $\Delta 6$ desaturase = [18:3 n-6/18:2 n-
180 6] and $\Delta 5$ desaturase = [20:4 (n-6)/20:3 (n-6)]. In addition, we have determined the gene expression of
181 the stearoyl-CoA desaturase 1 (SCD1= $\Delta 9$ desaturase) in the muscle. The unsaturation index (UI) was
182 calculated from the relative percentage of each type of mono- and polyunsaturated fatty acid
183 multiplied for the number of double bonds present in the molecule [29].

184

185 *2.8 Mitochondrial isolation and characteristics of gastrocnemius muscle*

186 *Mitochondrial isolation:* muscle mitochondria were extemporaneously isolated by the differential
187 centrifugation technique [30]. Briefly, a whole gastrocnemius muscle (2-3 g) was homogenized on ice
188 (1 g wet tissue for 9 volumes of sucrose buffer: Sucrose 0.25 M, Tris 10 mM, EDTA 0.5 mM, pH 7.5)
189 using a Polytron homogenizer. The homogenate was centrifuged at 900 g for 10 min at 4°C. The pellet
190 was discarded and the supernatant was centrifuged at 10 000 g for 10 min at 4°C. The obtained
191 mitochondrial pellet was suspended and washed with 2 ml of sucrose buffer and centrifuged at 10 000
192 g for 10 min at 4°C. The final mitochondrial pellet was suspended in 1 ml MIRO5 buffer (Sucrose 110
193 mM, Hepes 20 mM, KH₂PO₄ 10 mM, Taurine 20 mM, K-Lactobionate 60 mM, MgCl₂ 3 mM EGTA
194 0.5 mM, BSA 1 g/l, pH 7.1) and aliquoted for mitochondrial function measurements. Protein content
195 was determined according to Bradford with bovine serum albumin as the standard [31].

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

202 *Membrane potential measurement:* mitochondrial membrane potential was monitored according to
203 the method described by Baracca et al [32] using Rhodamine (RH-123) as a probe. Mitochondrial
204 potential ($\Delta\psi$) changes have been evaluated by measuring RH-123 fluorescence quenching at the
205 following steps: with RH-123 label alone (2 μ M) in MIRO5 buffer plus muscle fresh mitochondria
206 (0.25 mg proteins), plus glutamate/malate/succinate (2.5/5/5 mM), plus ADP (0.5 mM). RH-123
207 fluorescence was measured at 495 nm (excitation) and 525 nm (emission) under continuous stirring.

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

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

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

220

221 *2.9 Mitochondrial enzymatic activities of gastrocnemius muscle*

222 Citrate synthase (CS) activity was measured according to Srere [34]: the activity of the enzyme is
223 measured by following the color of 5-thio-2-nitrobenzoic acid, which is generated from 5,5'-dithiobis-
224 2-nitrobenzoic acid present in the reaction of citrate synthesis, and caused by the deacetylation of
225 acetyl-CoA. The activity of the different mitochondrial respiratory complexes was determined as
226 previously described [35]. Mitochondrial complex I activity was measured spectrophotometrically at
227 600 nm during 45 sec by following the reduction of 2,6-dichloroindophenol by electrons accepted
228 from decylubiquinol, itself reduced after oxidation of NADH by complex I [36]. Complex II (CII)
229 activity was measured spectrophotometrically at 600 nm by following the reduction of 2,6-
230 dichloroindophenol by the succinate during 120 sec. Complex II+III (CII+CIII) activities were
231 measured spectrophotometrically by following the reduction of cytochrome C at 550 nm during 90 sec
232 [37]. Cytochrome c oxidase (CIV) activity was measured spectrophotometrically by following the
233 oxidation of reduced cytochrome C at 550 nm during 30 sec [38].

234 In addition, mitochondrial β -hydroxyacyl-CoA dehydrogenase (β -HAD), a marker of last step of
235 mitochondrial β -oxidation activity, was determined spectrophotometrically according to Clayton et al.
236 [39]. Briefly, muscle mitochondria suspension (25 μ g protein) were mixed with NADH and
237 acetoacetyl CoA in 1 ml of 50 mM Tris-HCl buffer, pH 7.0, and absorbance was followed at 340 nm
238 for 60 seconds at 30°C.

239

240 *2.10 Oxidative stress status of gastrocnemius muscle*

241 A part of a gastrocnemius muscle was homogenized in phosphate buffer (50 mM, pH 7) 1 g for 9
242 ml buffer, using a Polytron homogenizer. The muscle thiobarbituric acid reactive substances (TBARS)
243 and total glutathione (GSH) levels were measured in muscle homogenate according to Sunderman [40]
244 and Griffith [41], respectively. The remaining homogenate was centrifuged at 1000 g for 10 min at
245 4°C and the supernatant was used for the other analyses of oxidative stress. Protein oxidation was
246 assessed in muscle supernatant by measurement of thiol groups as previously described [42]. Catalase
247 activity was measured in muscle supernatant according to Beers and Sizer [43]. Glutathione
248 peroxidase (GPx) was measured in muscle supernatant according to Flohe and Gunzler [44]. Total
249 superoxide dismutase (SOD) was measured in muscle supernatant according to Marklund [45].

250

251 *2.11 Protein isolation and western blotting analysis of gastrocnemius muscle*

252 Frozen gastrocnemius muscle samples were homogenized using an Ultra Turax homogenizer in an
253 ice cold extraction buffer containing 20 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-
254 100, 0.1% SDS, 1 mM PMSF, 10 µM leupeptin, and 1 µM pepstatin. Proteins (50 µg) were separated
255 with 10% SDS-PAGE and then transferred to a nitrocellulose membrane (45 min, 200 V). Membranes
256 were blocked in 5% fat-free milk for 1 hour at room temperature. Then, membranes were incubated
257 overnight with primary antibody against one of the following proteins: acetyl-CoA carboxylase
258 (ACC), phosphorylated acetyl-CoA carboxylase (ser⁷⁹p-ACC), acyl-CoA synthetase long chain family
259 member 1 (ACSL1), cardiolipin synthase (CLS), muscle-Carnitine palmitoyltransferase I (m-CPT1),
260 fatty acid synthase (FAS), cluster of differentiation 36 (fatty acid translocase) (FAT/CD36), fatty-acid-
261 transporter protein 1 (FATP1) and peroxisome proliferator-activated receptor-gamma coactivator-1α
262 (PGC-1α) in blocking buffer (supplementary Table 3).

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

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

269

270 2.12 Total RNA isolation and real-time RT-PCR analysis of gastrocnemius & soleus muscles

271 Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) was used to measure target genes
272 mRNA expression in the *gastrocnemius & soleus* muscles. Total RNA was extracted with Trizol
273 reagent (Invitrogen Life Technologies, Cergy Pontoise, France). Reverse transcription reaction was
274 performed with 2 µg total RNA. cDNA was synthesized with the use of SuperScript II Reverse
275 Transcriptase for first strand cDNA synthesis and Oligo (dT) primers (Invitrogen Life Technologies,
276 Cergy Pontoise, France). The mRNA expressions of target genes were determined by RT-qPCR, using
277 IQTM SYBR Green Supermix with a MiniOpticon detection system (Biorad, Hercules, CA, USA).
278 Data were normalized to RPS9 (*gastrocnemius* muscle) or Hprt (*soleus* muscle) used as reference
279 genes. The mean expression levels and the standard deviations of the housekeeping RPS9 rRNA gene
280 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 &
281 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
282 (4, 8, 12, 16 & 20 weeks, respectively). The mean expression levels and the standard deviations of the
283 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
284 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
285 24.1±0.2 for HFHFr groups (4, 8, 12, 16 & 20 weeks, respectively).-The primer sequences used for
286 RT-qPCR analysis are shown in the supplementary Table 4.

287

288 2.13 Statistical analysis

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

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

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

304

305 **3. Results**

306 *3.1 Rat characteristics and blood biochemical parameters*

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

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

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

324

325 *3.2 Gastrocnemius muscle neutral lipids and soleus muscle DAG and ceramides contents*

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

332 Total DAG content in the soleus muscle was increased since the 4th wk of diet with the HFHFr
333 diet compared to controls. In particular, it was significantly increased at the 16th wk of HFHFr diet by
334 comparison to control diet (Figure 4B). All muscle DAG species C16- C16, C16- C18.1, C16- C18,
335 C18.1- C18.1 and C18- C18.1 were significantly increased, except C16- C18.2 (decreased) and C18.1-
336 18.2 (unchanged), with the HFHFr diet compared to the control diet (Table 2).

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

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

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

349

350 *3.3 Phospholipids distribution and fatty acid composition in the gastrocnemius muscle*

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

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

366 Consequently, the unsaturation index of muscle fatty acids was decreased with the HFHFr diet
367 compared to the control diet (Table 4). Indeed, this decrease in the unsaturation index was in
368 agreement with the decrease in the percentage of PUFAs under the HFHFr diet compared to the
369 control diet. In addition, the fatty acid desaturase indices of the three important desaturases ($\Delta 9 =$
370 $[16:1n-7/16:0]$, $\Delta 6 = [18:3n-6/18:2n-6]$ and $\Delta 5 = [20:4n-6/20:3n-6]$) were all decreased with the
371 HFHFr diet compared to the control diet (Table 4). This is in agreement with the above reported

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

377

378 *3.4 Lipid signalling pathways and mitochondrial activity in the gastrocnemius muscle*

379 In order to explain the obtained results, we studied the muscle expression of several actors of lipid
380 metabolism. To begin with the lipid uptake, the protein abundance of both FAT/CD36 and FATP1,
381 two fatty acid transporters, remained unchanged (Tables 5), whereas the gene expression of FABP3,
382 another fatty acid transporter, was increased with the HFHFr diet compared to the control diet (Table
383 6). Protein expression of the long chain acyl-CoA synthetase (ACSL1), that activates the conversion of
384 12 to 20 carbon fatty acids into acyl-CoA thioesters, remained unchanged, except on 12 wks of diet.
385 The muscle gene expression of PPAR α , PPAR γ and Srebp-1c, three key regulators of lipid
386 metabolism, as well as the gene and protein expression of PGC-1a, a key regulator of energy
387 metabolism, remained unchanged (Tables 5, 6). In addition, protein abundance of mCPT-1, a
388 mitochondrial fatty acids transporter, remained also unchanged. Regarding lipogenesis, the muscle
389 protein abundance of total acetyl CoA carboxylase (ACC), a lipogenic enzyme providing the malonyl-
390 CoA substrate for fatty acid synthesis and ser⁷⁹p-ACC, its inactivated form, remained unchanged
391 whereas protein abundance of muscle fatty acid synthase (FAS), a multifunctional enzyme that
392 synthesizes palmitic acid, was significantly and markedly decreased all along the study with the
393 HFHFr diet compared to the control diet (Table 5).

394

395 *3.5 Mitochondrial characteristics and enzymatic activity in the gastrocnemius muscle*

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

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

410

411 *3.6 Mitochondrial ROS production and oxidative stress status in the gastrocnemius muscle*

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

419

420 *3.7 Correlation of glucose intolerance with lipid composition, mitochondrial activity and oxidative* 421 *stress parameters*

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

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

434

435 **4. Discussion**

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

446

447 *4.1 Muscle lipid accumulated with HFHF diet with a maximum at 12th week of diet*

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

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

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

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

483

484 *4.2 Muscle fatty acid composition was altered as soon as 4 weeks of HFHFr diet*

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

501

502 *4.3 Dysfunction of the muscle mitochondria is not mainly involved in glucose intolerance*

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

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

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

531

532 **5. Conclusion**

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

539

540 **Author contributions**

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

544

545 **Acknowledgments**

546 The authors acknowledge the financial support of the National Institute for Agronomic Research
547 and in particular the Human nutrition Department (Alim-H department). The authors wish also to
548 thank the animal staff from Metamus DMEM facility which belongs to Montpellier animal facilities
549 network (RAM, Biocampus).

550

551 **Conflict of interest**

552 The authors have no conflicts of interest to declare.

553

554 **Funding**

555 Yang Wang thanks for the support from PhD program of the China Scholarship Council.

556

557 **Abbreviations**

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

560 triglyceride lipase; AUC: area under the curve; CDP: Cytidine diphosphate; CL: cardiolipin; CLS:
561 cardiolipin synthase; DGAT1: diacylglycerol O-acyltransferase 1; FAS: fatty acid synthase;
562 FAT/CD36: cluster of differentiation 36 (fatty acid translocase); FATP1: fatty-acid-transporter protein
563 1; GPx: Glutathione peroxidase; GSH: Glutathione; β -HAD: β -hydroxyacyl-CoA dehydrogenase;
564 HFHFr: high fat high fructose; HPTLC: High performance thin layer chromatography; LPC:
565 lysophosphatidylcholine; LPL: lipoprotein lipase; m-CPT1: muscle-Carnitine palmitoyltransferase I;
566 MUFA: monounsaturated fatty acids; NAFLD: non-alcoholic fatty liver disease; 5-NS: 5-nitroxide
567 stearate; 16-NS, 16-nitroxide stearate; OGTT: oral glucose tolerance test; PA: phosphatidic acid; PC:
568 phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PGC-1 α : peroxisome
569 proliferator-activated receptor-gamma coactivator-1 α ; PI: phosphatidylinositol; PLIN2: perilipin 2;
570 PS: phosphatidyl-serine; PUFA: polyunsaturated fatty acids; Rf: retention factor; qPCR: quantitative-
571 polymerase chain reaction; RNS: reactive nitrogen species; ROS: reactive oxygen species; SCD1:
572 stearyl-CoA desaturase 1; SFA: saturated fatty acids; SM: sphingomyelin; SOD: superoxide
573 dismutase; TBARS: Thiobarbituric acid reactive substances; TLC: thin layer chromatography; WB:
574 western blotting.

575

576

577 **Figure legends**

578 **Figure 1: Weight of liver, adipose tissue and gastrocnemius and their ratios to rat body weight in** 579 **rats fed control or HFHFr diets for 4 to 20 weeks**

580 Results were expressed as means \pm SD, n=6–8 animals per group per diet treatment duration, rats liver
581 (A), adipose tissue (B) and gastrocnemius (C) weights and their ratio to rat body weight. All the
582 groups were tested for the effects of diet, treatment duration, and their interaction by two-way
583 ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse
584 the effect of HFHFr diet within each treatment duration. The limit of statistical significance was set at
585 $p < 0.05$. HFHFr diet vs control diet: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

586

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

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

601

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

603 Results were expressed as means \pm SD, n=6–8 animals per group per diet treatment duration. All the
604 groups were tested for the effects of diet, treatment duration, and their interaction by two-way
605 ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse
606 the effect of HFHFr diet within each treatment duration. The limit of statistical significance was set at
607 $p < 0.05$. HFHFr diet vs control diet: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

608

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

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

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

617

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

620 Results were expressed as means \pm SD, $n = 6-8$ animals per group per diet treatment duration. All the
621 groups were tested for the effects of diet, treatment duration, and their interaction by two-way
622 ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse
623 the effect of HFHF diet within each treatment duration. The limit of statistical significance was set at
624 $p < 0.05$. HFHF diet vs control diet: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ATGL: adipose triglyceride
625 lipase; CD36: cluster of differentiation 36 (fatty acid translocase); DGAT1: diacylglycerol O-
626 acyltransferase 1; LPL: lipoprotein lipase; PLIN2: perilipin 2.

627

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

630 Results were expressed as means \pm SD, $n = 6-8$ animals per group per diet treatment duration. All the
631 groups were tested for the effects of diet, treatment duration, and their interaction by two-way
632 ANOVA test. When the effect of diet was significant, the unpaired student's *t*-test was used to analyse
633 the effect of HFHF diet within each treatment duration. The limit of statistical significance was set at
634 $p < 0.05$. HFHF diet vs control diet: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. MUFA: monounsaturated fatty
635 acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids.

636

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

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

643

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

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

649

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

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

656

657

658 **References**

659 [1] Barness LA, Opitz JM, Gilbert-Barness E. Obesity: genetic, molecular, and environmental
660 aspects. *Am J Med Genet A*. 2007;143:3016-34.

661 [2] DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type
662 2 diabetes. *Diabetes Care*. 2009;32 Suppl 2:S157-63.

663 [3] Kraegen EW, Cooney GJ, Turner N. Muscle insulin resistance: a case of fat
664 overconsumption, not mitochondrial dysfunction. *Proc Natl Acad Sci U S A*. 2008;105:7627-
665 8.

666 [4] Holland WL, Summers SA. Sphingolipids, insulin resistance, and metabolic disease: new
667 insights from in vivo manipulation of sphingolipid metabolism. *Endocr Rev*. 2008;29:381-
668 402.

669 [5] Tonks KT, Coster AC, Christopher MJ, Chaudhuri R, Xu A, Gagnon-Bartsch J, et al.
670 Skeletal muscle and plasma lipidomic signatures of insulin resistance and overweight/obesity
671 in humans. *Obesity (Silver Spring)*. 2016;24:908-16.

672 [6] Borkman M, Storlien LH, Pan DA, Jenkins AB, Chisholm DJ, Campbell LV. The relation
673 between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. *N*
674 *Engl J Med*. 1993;328:238-44.

675 [7] Riccardi G, Giacco R, Rivellese AA. Dietary fat, insulin sensitivity and the metabolic
676 syndrome. *Clin Nutr*. 2004;23:447-56.

677 [8] Clore JN, Li J, Gill R, Gupta S, Spencer R, Azzam A, et al. Skeletal muscle
678 phosphatidylcholine fatty acids and insulin sensitivity in normal humans. *Am J Physiol*.
679 1998;275:E665-70.

680 [9] Funai K, Lodhi IJ, Spears LD, Yin L, Song H, Klein S, et al. Skeletal Muscle
681 Phospholipid Metabolism Regulates Insulin Sensitivity and Contractile Function. *Diabetes*.
682 2016;65:358-70.

683 [10] Newsom SA, Brozinick JT, Kiseljak-Vassiliades K, Strauss AN, Bacon SD, Kerege AA,
684 et al. Skeletal muscle phosphatidylcholine and phosphatidylethanolamine are related to
685 insulin sensitivity and respond to acute exercise in humans. *J Appl Physiol (1985)*.
686 2016;120:1355-63.

687 [11] Victor VM, Rocha M, Herance R, Hernandez-Mijares A. Oxidative stress and
688 mitochondrial dysfunction in type 2 diabetes. *Curr Pharm Des*. 2011;17:3947-58.

689 [12] Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol*.
690 2003;552:335-44.

691 [13] Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and
692 antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol*.
693 2007;39:44-84.

- 694 [14] Montgomery MK, Turner N. Mitochondrial dysfunction and insulin resistance: an
695 update. *Endocr Connect.* 2015;4:R1-R15.
- 696 [15] Keung W, Ussher JR, Jaswal JS, Raubenheimer M, Lam VH, Wagg CS, et al. Inhibition
697 of carnitine palmitoyltransferase-1 activity alleviates insulin resistance in diet-induced obese
698 mice. *Diabetes.* 2013;62:711-20.
- 699 [16] Ahn J, Lee H, Im SW, Jung CH, Ha TY. Allyl isothiocyanate ameliorates insulin
700 resistance through the regulation of mitochondrial function. *J Nutr Biochem.* 2014;25:1026-
701 34.
- 702 [17] Martins AR, Crisma AR, Masi LN, Amaral CL, Marzuca-Nassr GN, Bomfim LHM, et
703 al. Attenuation of obesity and insulin resistance by fish oil supplementation is associated with
704 improved skeletal muscle mitochondrial function in mice fed a high-fat diet. *J Nutr Biochem.*
705 2018;55:76-88.
- 706 [18] Bai J, Zheng S, Jiang D, Han T, Li Y, Zhang Y, et al. Oxidative stress contributes to
707 abnormal glucose metabolism and insulin sensitivity in two hyperlipidemia models. *Int J Clin*
708 *Exp Pathol.* 2015;8:13193-200.
- 709 [19] Das N, Mandala A, Bhattacharjee S, Mukherjee D, Bandyopadhyay D, Roy SS. Dietary
710 fat proportionately enhances oxidative stress and glucose intolerance followed by impaired
711 expression of the genes associated with mitochondrial biogenesis. *Food Funct.* 2017;8:1577-
712 86.
- 713 [20] Lionetti L, Mollica MP, Crescenzo R, D'Andrea E, Ferraro M, Bianco F, et al. Skeletal
714 muscle subsarcolemmal mitochondrial dysfunction in high-fat fed rats exhibiting impaired
715 glucose homeostasis. *Int J Obes (Lond).* 2007;31:1596-604.
- 716 [21] Feillet-Coudray C, Fouret G, Ebabe Elle R, Rieusset J, Bonafos B, Chabi B, et al. The
717 mitochondrial-targeted antioxidant MitoQ ameliorates metabolic syndrome features in

718 obesogenic diet-fed rats better than Apocynin or Allopurinol. *Free Radic Res.* 2014;48:1232-
719 46.

720 [22] Jenkins DJ, Wolever TM, Kalmusky J, Giudici S, Giordano C, Wong GS, et al. Low
721 glycemic index carbohydrate foods in the management of hyperlipidemia. *Am J Clin Nutr.*
722 1985;42:604-17.

723 [23] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC.
724 Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma
725 glucose and insulin concentrations in man. *Diabetologia.* 1985;28:412-9.

726 [24] Blachnio-Zabielska AU, Persson XM, Koutsari C, Zabielski P, Jensen MD. A liquid
727 chromatography/tandem mass spectrometry method for measuring the in vivo incorporation
728 of plasma free fatty acids into intramyocellular ceramides in humans. *Rapid Commun Mass*
729 *Spectrom.* 2012;26:1134-40.

730 [25] Blachnio-Zabielska AU, Zabielski P, Jensen MD. Intramyocellular diacylglycerol
731 concentrations and [U-(1)(3)C]palmitate isotopic enrichment measured by LC/MS/MS. *J*
732 *Lipid Res.* 2013;54:1705-11.

733 [26] Coudray C, Fouret G, Lambert K, Ferreri C, Rieusset J, Blachnio-Zabielska A, et al. A
734 mitochondrial-targeted ubiquinone modulates muscle lipid profile and improves
735 mitochondrial respiration in obesogenic diet-fed rats. *Br J Nutr.* 2016;115:1155-66.

736 [27] Lepage G, Roy CC. Direct transesterification of all classes of lipids in a one-step
737 reaction. *J Lipid Res.* 1986;27:114-20.

738 [28] Warensjo E, Riserus U, Gustafsson IB, Mohsen R, Cederholm T, Vessby B. Effects of
739 saturated and unsaturated fatty acids on estimated desaturase activities during a controlled
740 dietary intervention. *Nutr Metab Cardiovasc Dis.* 2008;18:683-90.

741 [29] Aoun M, Fouret G, Michel F, Bonafos B, Ramos J, Cristol JP, et al. Dietary fatty acids
742 modulate liver mitochondrial cardiolipin content and its fatty acid composition in rats with
743 non alcoholic fatty liver disease. *J Bioenerg Biomembr.* 2012;44:439-52.

744 [30] Aoun M, Feillet-Coudray C, Fouret G, Chabi B, Crouzier D, Ferreri C, et al. Rat liver
745 mitochondrial membrane characteristics and mitochondrial functions are more profoundly
746 altered by dietary lipid quantity than by dietary lipid quality: effect of different nutritional
747 lipid patterns. *Br J Nutr.* 2011;107:647-59.

748 [31] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities
749 of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-54.

750 [32] Baracca A, Sgarbi G, Solaini G, Lenaz G. Rhodamine 123 as a probe of mitochondrial
751 membrane potential: evaluation of proton flux through F(0) during ATP synthesis. *Biochim*
752 *Biophys Acta.* 2003;1606:137-46.

753 [33] Barja G. The quantitative measurement of H₂O₂ generation in isolated mitochondria. *J*
754 *Bioenerg Biomembr.* 2002;34:227-33.

755 [34] Srere P. Citrate synthase. *Methods enzymol.* 1969;13:3-11.

756 [35] Feillet-Coudray C, Sutra T, Fouret G, Ramos J, Wrutniak-Cabello C, Cabello G, et al.
757 Oxidative stress in rats fed a high-fat high-sucrose diet and preventive effect of polyphenols:
758 Involvement of mitochondrial and NAD(P)H oxidase systems. *Free Radic Biol Med.*
759 2009;46:624-32.

760 [36] Janssen AJ, Trijbels FJ, Sengers RC, Smeitink JA, van den Heuvel LP, Wintjes LT, et al.
761 Spectrophotometric assay for complex I of the respiratory chain in tissue samples and
762 cultured fibroblasts. *Clin Chem.* 2007;53:729-34.

763 [37] Rustin P, Chretien D, Bourgeron T, Gerard B, Rotig A, Saudubray JM, et al.
764 Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta.*
765 1994;228:35-51.

766 [38] Wharton D, Tzagoloff A. Cytochrome oxidase from beef heart mitochondria. *Methods*
767 *enzymol.* 1967;10:245-50.

768 [39] Clayton PT, Eaton S, Aynsley-Green A, Edginton M, Hussain K, Krywawych S, et al.
769 Hyperinsulinism in short-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency reveals the
770 importance of beta-oxidation in insulin secretion. *J Clin Invest.* 2001;108:457-65.

771 [40] Sunderman FW, Jr., Marzouk A, Hopfer SM, Zaharia O, Reid MC. Increased lipid
772 peroxidation in tissues of nickel chloride-treated rats. *Ann Clin Lab Sci.* 1985;15:229-36.

773 [41] Griffith OW. Determination of glutathione and glutathione disulfide using glutathione
774 reductase and 2-vinylpyridine. *Anal Biochem.* 1980;106:207-12.

775 [42] Faure P, Lafond J. Measurement of plasma sulfhydryl and carbonyl groups as a possible
776 indicator of protein oxidation. In: Favier A, Cadet J, Kalyanaraman B, Fontecave M, Pierre J,
777 editors. *Analysis of Free Radicals in Biological Systems.* Basel (Switzerland): Birkhauser
778 Verlag 1995. p. 237-48.

779 [43] Beers RF, Jr., Sizer IW. A spectrophotometric method for measuring the breakdown of
780 hydrogen peroxide by catalase. *J Biol Chem.* 1952;195:133-40.

781 [44] Flohe L, Gunzler WA. Assays of glutathione peroxidase. *Methods Enzymol.*
782 1984;105:114-21.

783 [45] Marklund S. Spectrophotometric study of spontaneous disproportionation of superoxide
784 anion radical and sensitive direct assay for superoxide dismutase. *J Biol Chem.*
785 1976;251:7504-7.

786 [46] Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, et al. Biomarkers of
787 mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol.*
788 2012;590:3349-60.

789 [47] Abbott SK, Else PL, Hulbert AJ. Membrane fatty acid composition of rat skeletal muscle
790 is most responsive to the balance of dietary n-3 and n-6 PUFA. *Br J Nutr.* 2009:1-8.

791 [48] van Herpen NA, Schrauwen-Hinderling VB. Lipid accumulation in non-adipose tissue
792 and lipotoxicity. *Physiol Behav.* 2008;94:231-41.

793 [49] Finck BN, Hall AM. Does Diacylglycerol Accumulation in Fatty Liver Disease Cause
794 Hepatic Insulin Resistance? *Biomed Res Int.* 2015;2015:104132.

795 [50] Jornayvaz FR, Shulman GI. Diacylglycerol activation of protein kinase Cepsilon and
796 hepatic insulin resistance. *Cell Metab.* 2012;15:574-84.

797 [51] Pagadala M, Kasumov T, McCullough AJ, Zein NN, Kirwan JP. Role of ceramides in
798 nonalcoholic fatty liver disease. *Trends Endocrinol Metab.* 2012;23:365-71.

799 [52] Summers SA. Ceramides in insulin resistance and lipotoxicity. *Prog Lipid Res.*
800 2006;45:42-72.

801 [53] Blachnio-Zabielska AU, Chacinska M, Vendelbo MH, Zabielski P. The Crucial Role of
802 C18-Cer in Fat-Induced Skeletal Muscle Insulin Resistance. *Cell Physiol Biochem.*
803 2016;40:1207-20.

804 [54] Fouret G, Gaillet S, Lecomte J, Bonafos B, Djohan F, Barea B, et al. 20-Week follow-up
805 of hepatic steatosis installation and liver mitochondrial structure and activity and their
806 interrelation in rats fed a high-fat-high-fructose diet. *Br J Nutr.* 2018;119:368-80.

807 [55] Dobrzyn A, Ntambi JM. The role of stearoyl-CoA desaturase in the control of
808 metabolism. *Prostaglandins Leukot Essent Fatty Acids.* 2005;73:35-41.

809 [56] Dobrzyn P, Jazurek M, Dobrzyn A. Stearoyl-CoA desaturase and insulin signaling--what
810 is the molecular switch? *Biochim Biophys Acta.* 2010;1797:1189-94.

811 [57] Riccardi G, Rivellese AA. Dietary treatment of the metabolic syndrome--the optimal
812 diet. *Br J Nutr.* 2000;83 Suppl 1:S143-8.

813 [58] Riserus U. Fatty acids and insulin sensitivity. *Curr Opin Clin Nutr Metab Care.*
814 2008;11:100-5.

815 [59] Lee JS, Pinnamaneni SK, Eo SJ, Cho IH, Pyo JH, Kim CK, et al. Saturated, but not n-6
816 polyunsaturated, fatty acids induce insulin resistance: role of intramuscular accumulation of
817 lipid metabolites. *J Appl Physiol*. 2006;100:1467-74.

818 [60] Lark DS, Fisher-Wellman KH, Neuffer PD. High-fat load: mechanism(s) of insulin
819 resistance in skeletal muscle. *Int J Obes Suppl*. 2012;2:S31-S6.

820 [61] Putti R, Migliaccio V, Sica R, Lionetti L. Skeletal Muscle Mitochondrial Bioenergetics
821 and Morphology in High Fat Diet Induced Obesity and Insulin Resistance: Focus on Dietary
822 Fat Source. *Front Physiol*. 2015;6:426.

823 [62] Holloszy JO. "Deficiency" of mitochondria in muscle does not cause insulin resistance.
824 *Diabetes*. 2013;62:1036-40.

825 [63] Feillet-Coudray C, Aoun M, Fouret G, Bonafos B, Ramos J, Casas F, et al. Effects of
826 long-term administration of saturated and n-3 fatty acid-rich diets on lipid utilisation and
827 oxidative stress in rat liver and muscle tissues. *Br J Nutr*. 2013;110:1789-802.

828 [64] Johnson AR, Wilkerson MD, Sampey BP, Troester MA, Hayes DN, Makowski L.
829 Cafeteria diet-induced obesity causes oxidative damage in white adipose. *Biochem Biophys*
830 *Res Commun*. 2016;473:545-50.

831 [65] Pirozzi C, Lama A, Simeoli R, Paciello O, Pagano TB, Mollica MP, et al.
832 Hydroxytyrosol prevents metabolic impairment reducing hepatic inflammation and restoring
833 duodenal integrity in a rat model of NAFLD. *J Nutr Biochem*. 2016;30:108-15.

834 [66] Mendez L, Pazos M, Molinar-Toribio E, Sanchez-Martos V, Gallardo JM, Rosa Nogues
835 M, et al. Protein carbonylation associated to high-fat, high-sucrose diet and its metabolic
836 effects. *J Nutr Biochem*. 2014;25:1243-53.

837

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

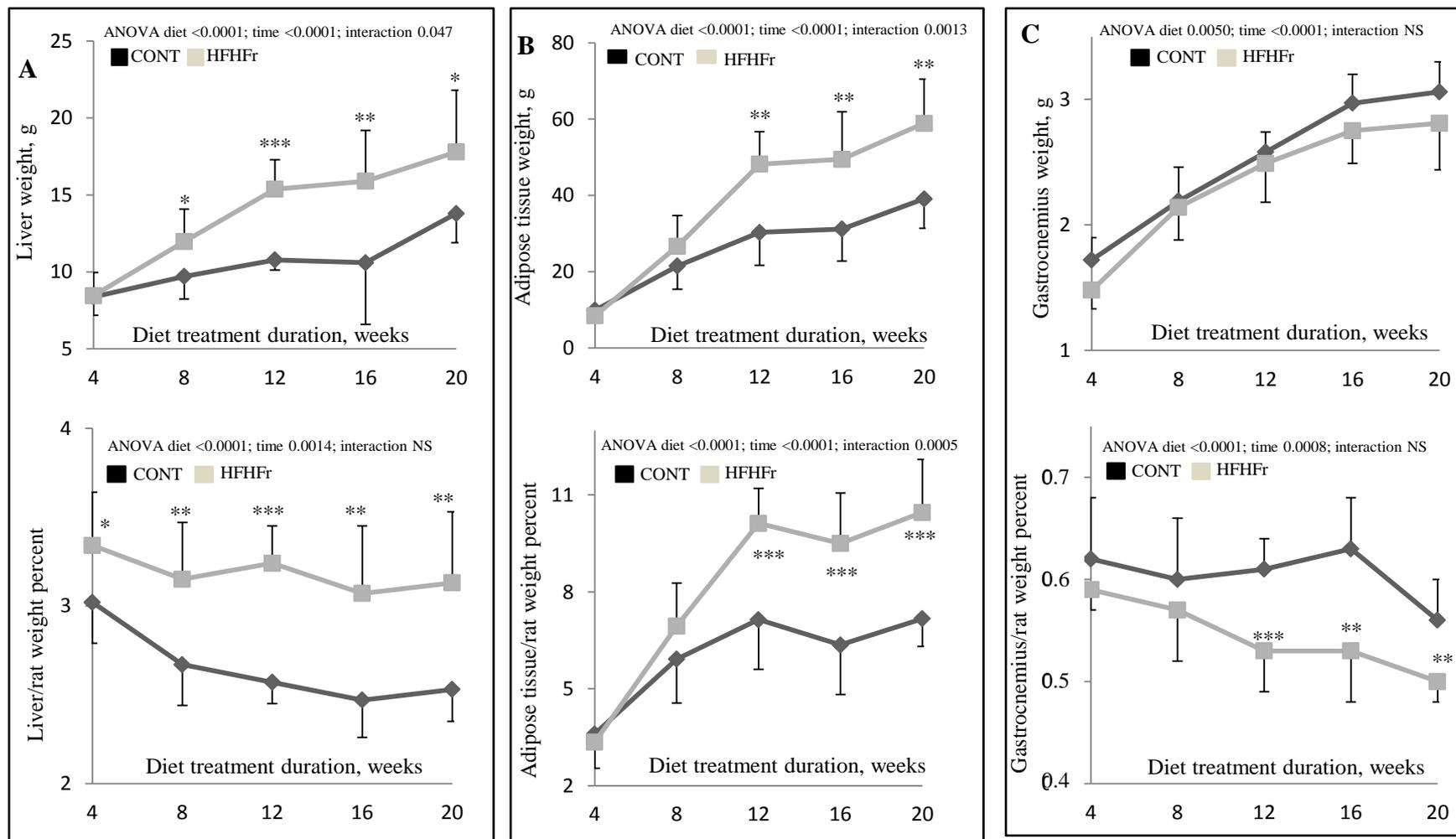


Figure 2: Time-course of oral glucose tolerance test in rats fed control or HFHFr diets for 4–20 weeks

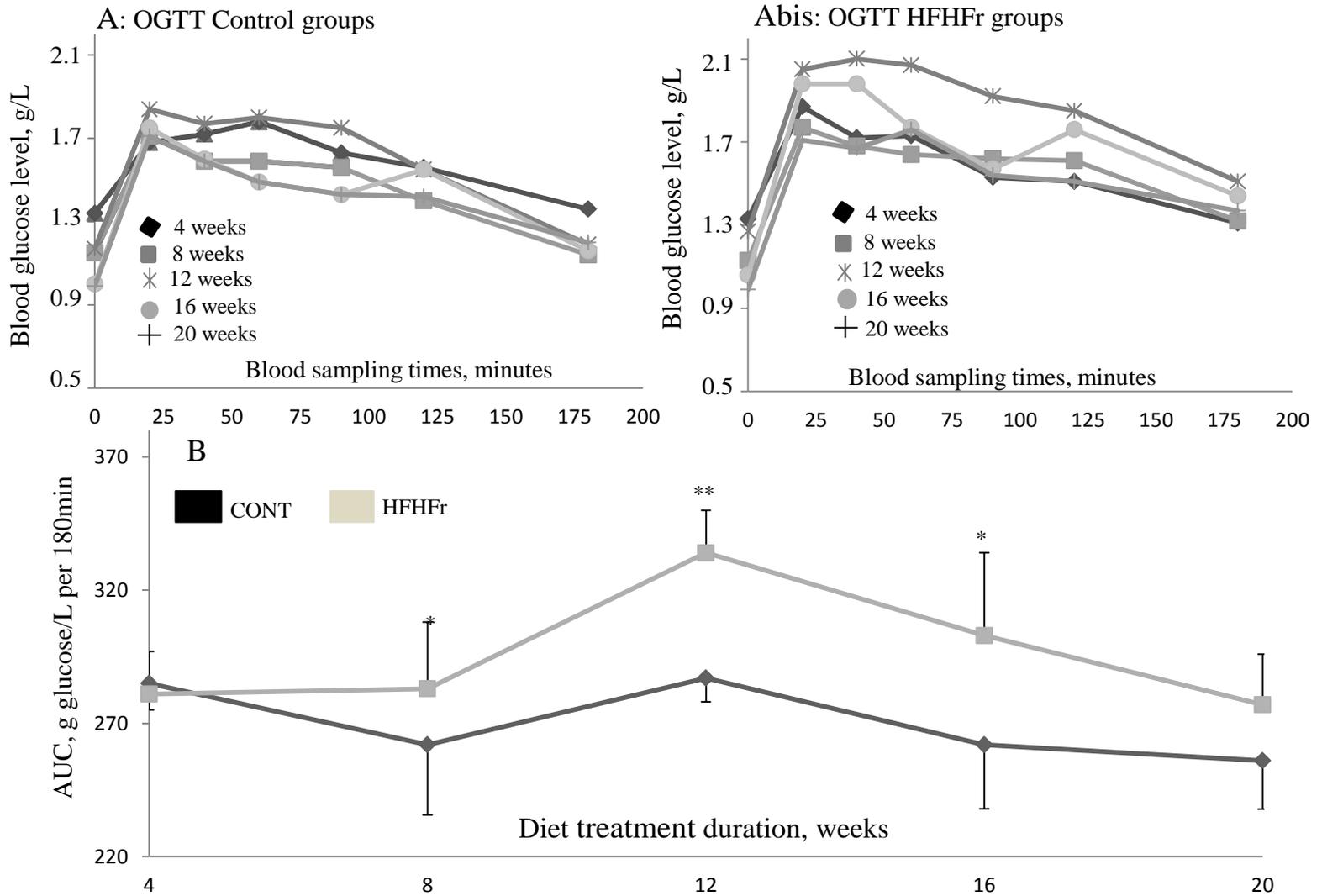


Figure 3: Blood lipid levels in rats fed control or HFHF diets for 4 to 20 weeks

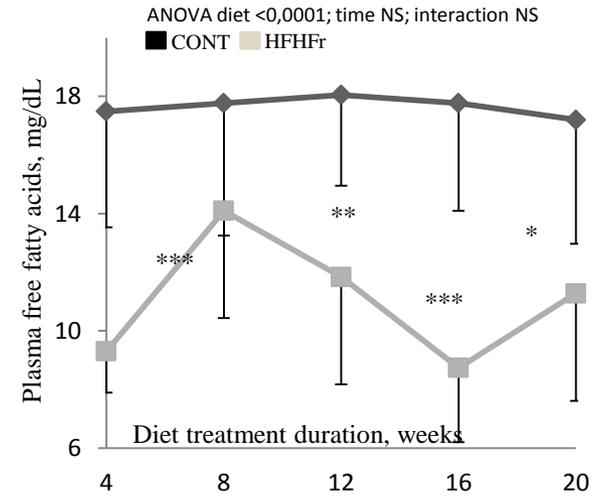
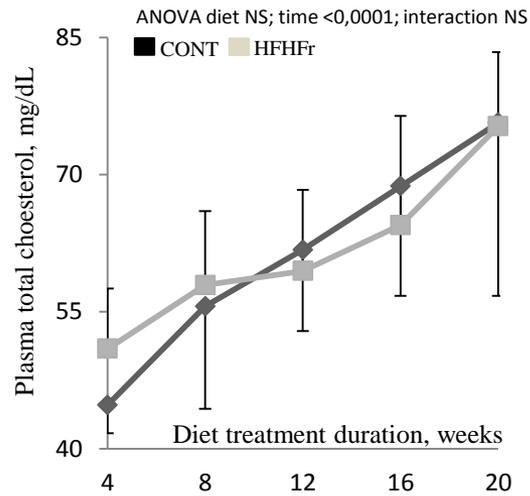
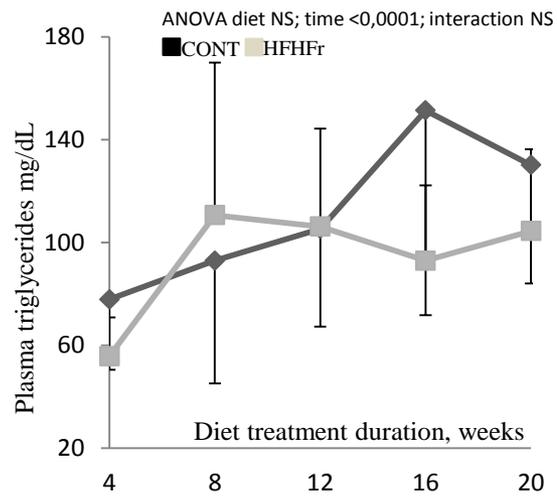


Figure 4: Muscle neutral lipid accumulation in rats fed control or HFHF_r diets for 4 to 20 weeks

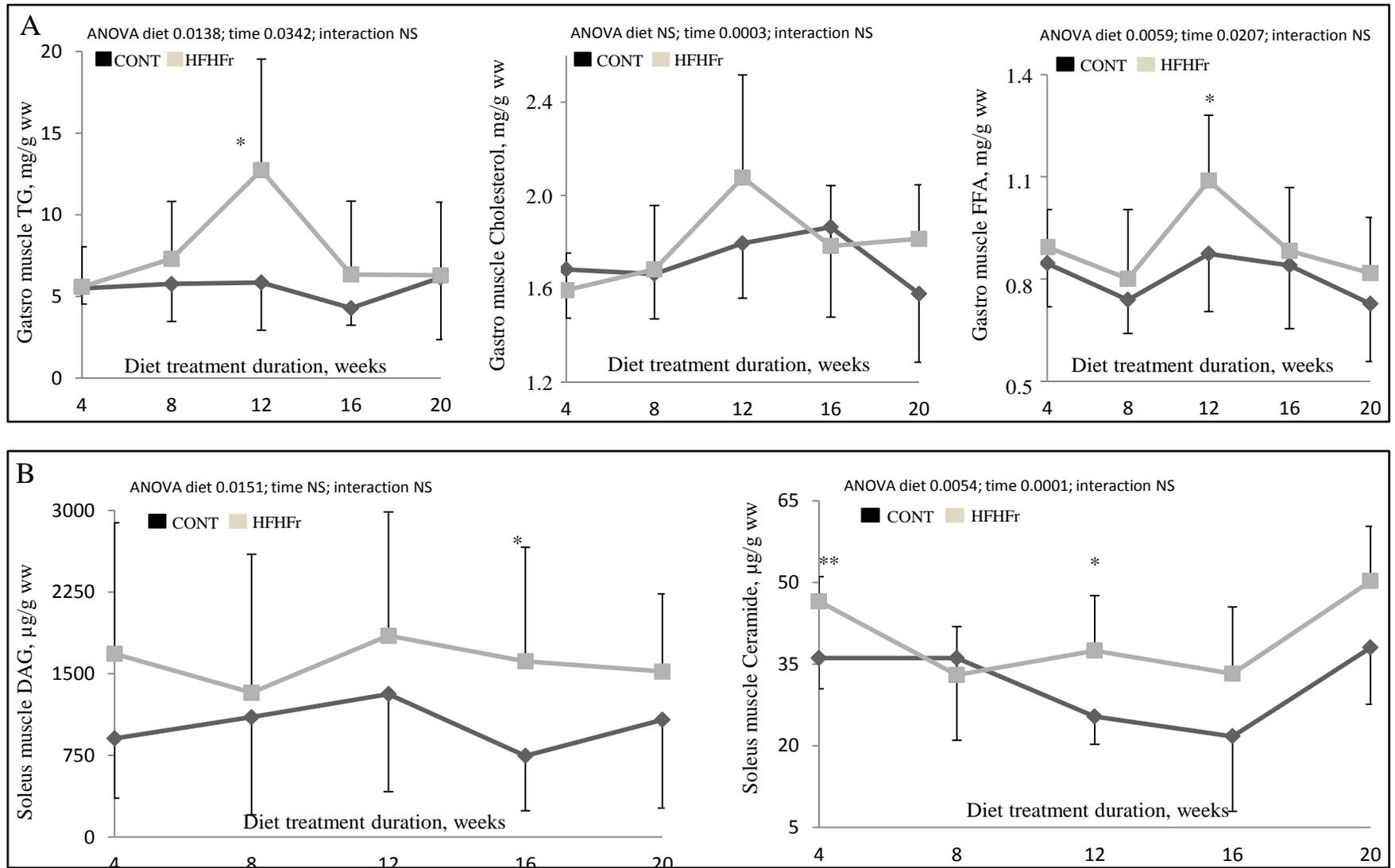


Figure 5: Gene expression of the major players in the formation of lipid droplets in the soleus muscle in rats fed control or HFHF_r diets for 4 to 20 weeks

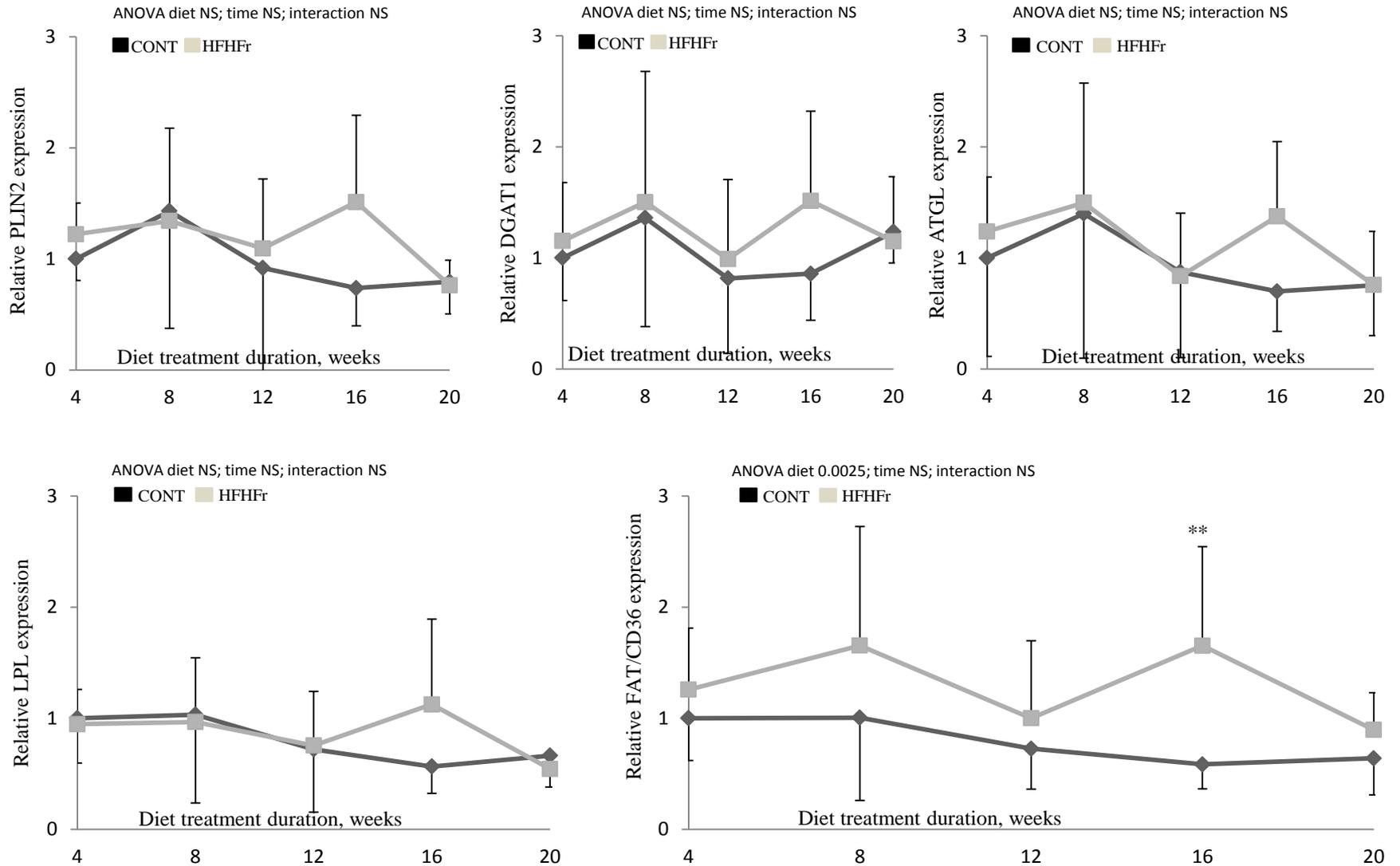


Figure 6: Fatty acid classes in the gastrocnemius muscle from rats fed control or HFHF diets for 4 to 20 weeks

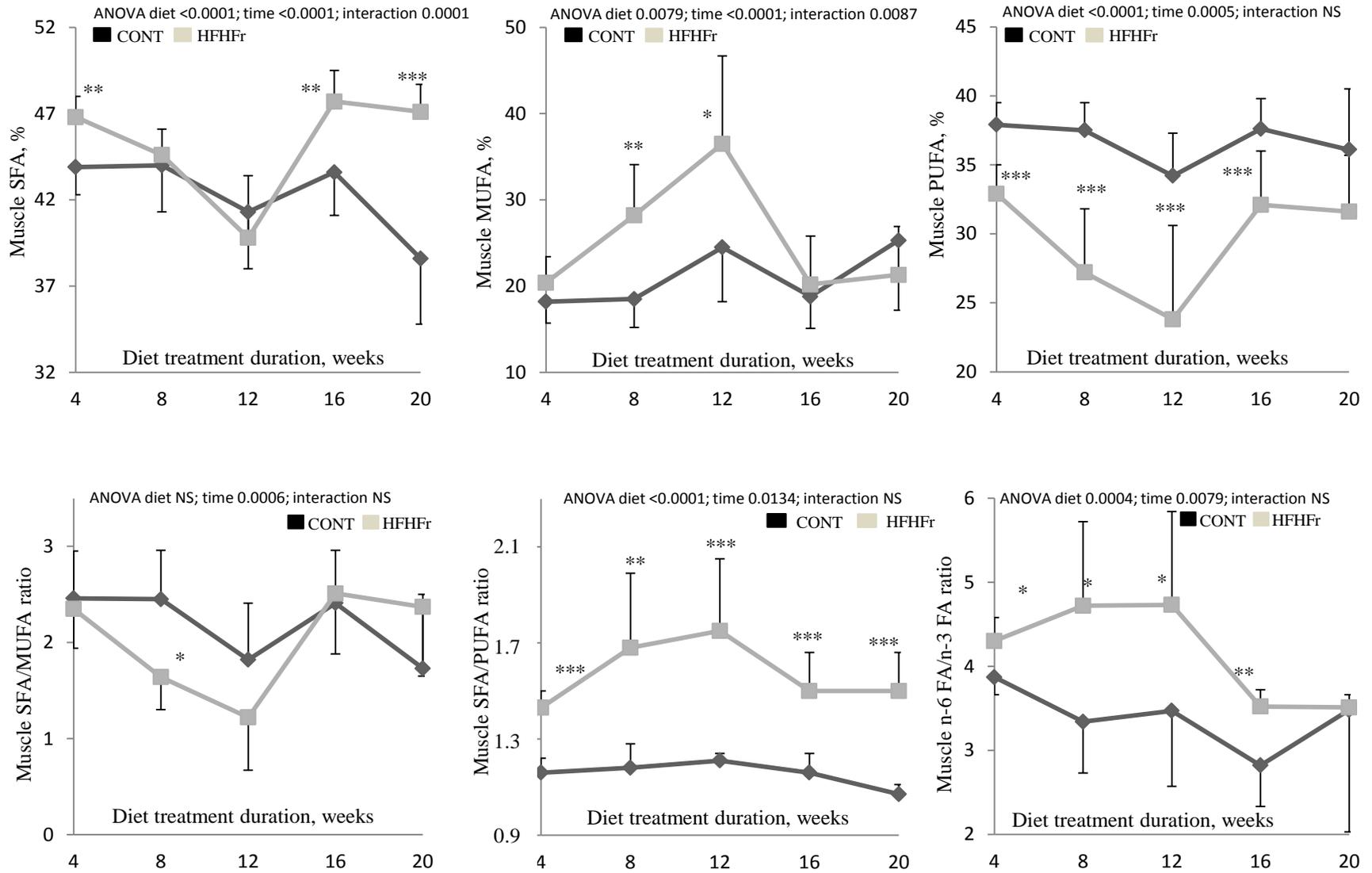


Figure 7: Spearman correlations between glucose tolerance test and accumulation of key lipids in gastrocnemius and soleus muscles

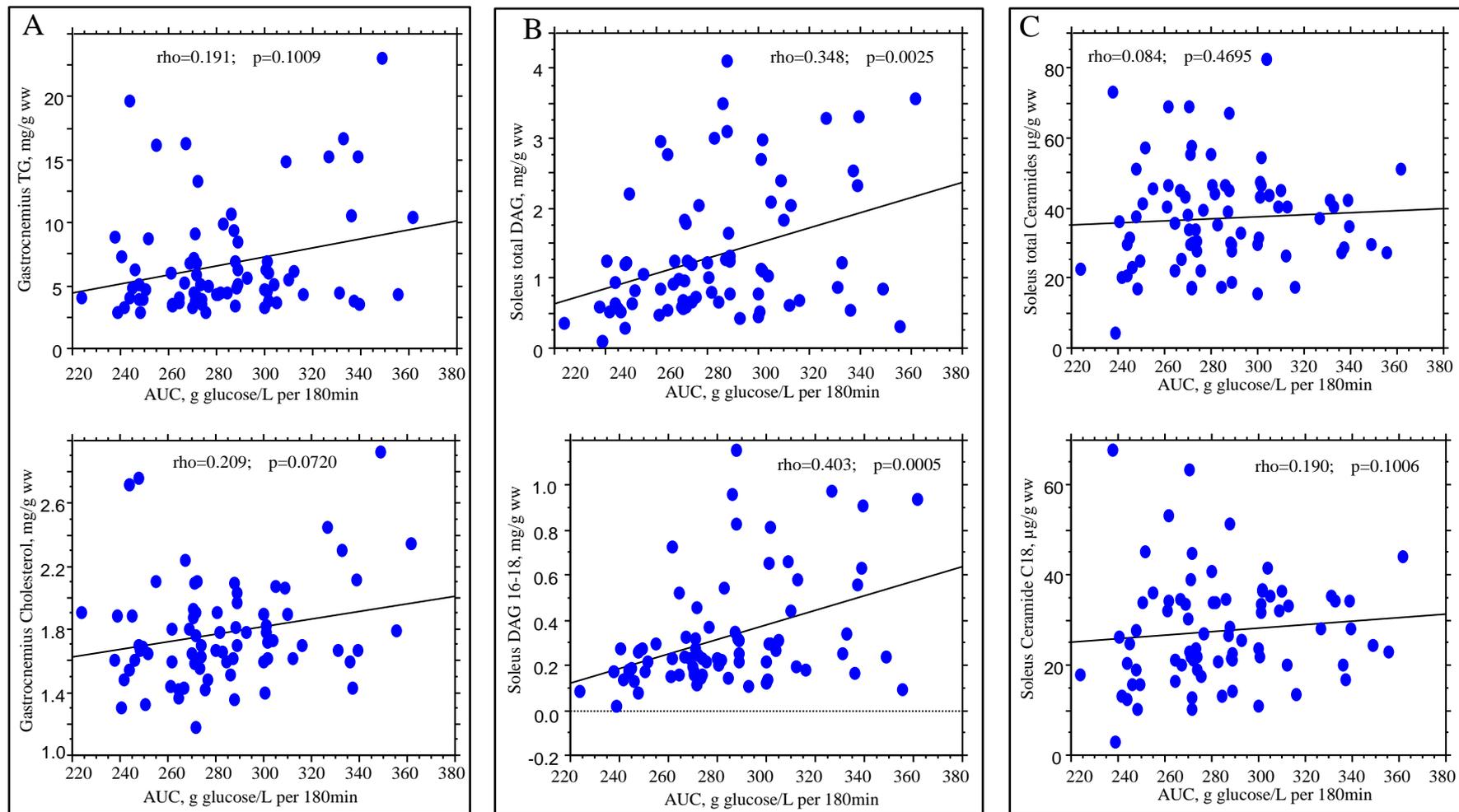


Figure 8: Spearman correlations between glucose tolerance test and gastrocnemius muscle fatty acid composition

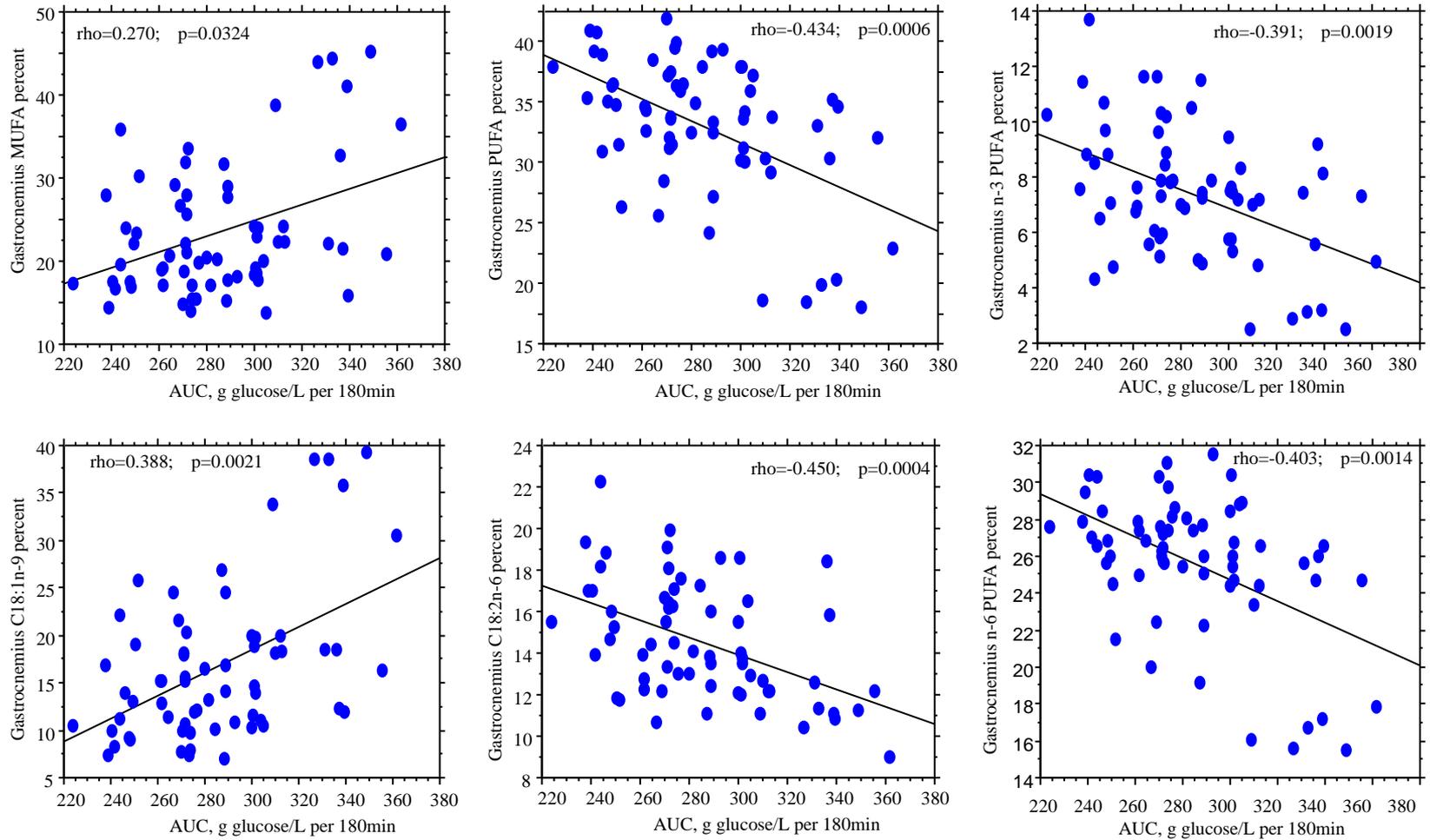


Figure 9: Spearman correlations between glucose tolerance test and mitochondrial activity markers in the gastrocnemius muscle

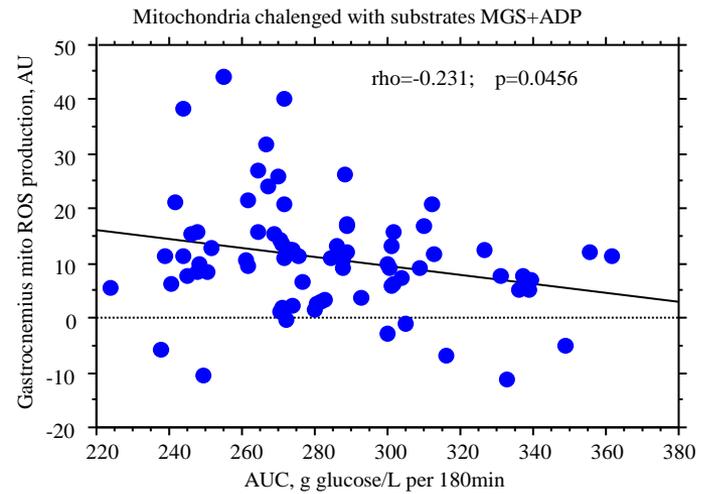
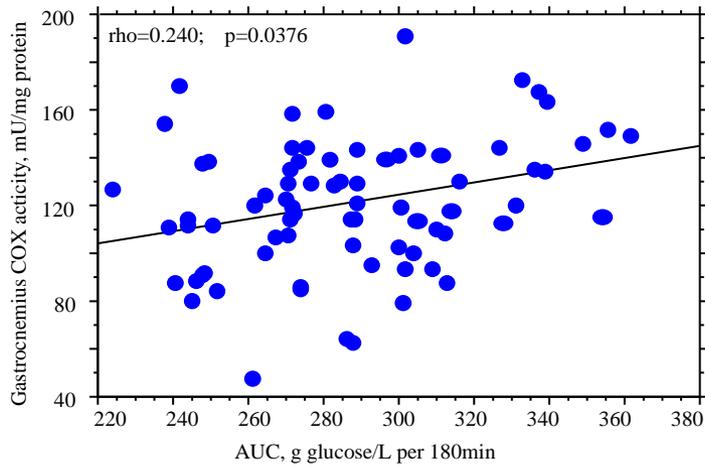
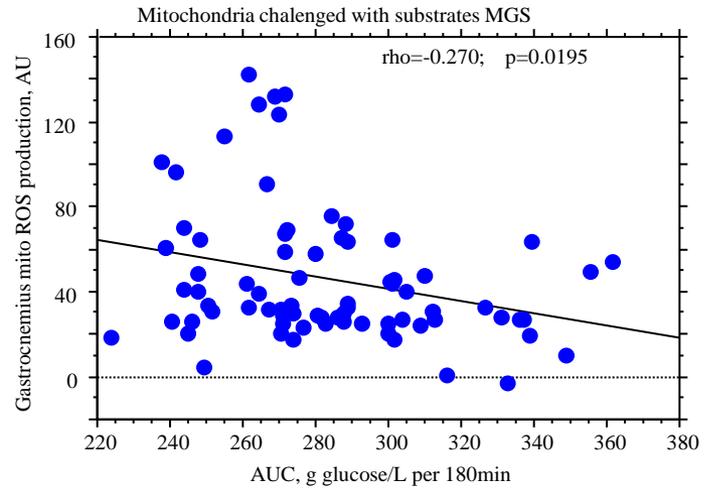
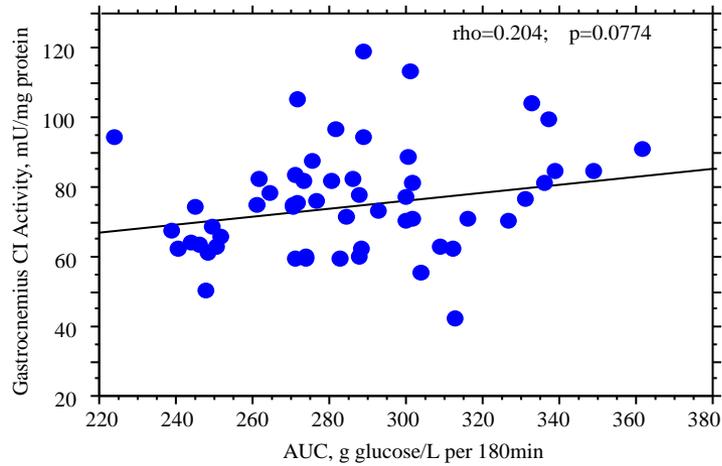


Table 1: Food consumption and rat body and organ weights.

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

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: * $p<0.05$, ** $p<0.01$. 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 | | | |
| | 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 | 0.0023 | <0.0001 | NS |
| 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 | | | |
| | HFHFr | 0.07 \pm 0.03 | 0.05 \pm 0.02 | 0.07 \pm 0.02** | 0.05 \pm 0.02 | 0.07 0.14 | 0.0003 | NS | NS |
| 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 | | | |
| | 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 | NS | 0.0186 | NS |
| 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 | | | |
| | 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 | NS | NS | NS |
| 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 | | | |
| | 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 | 0.0012 | 0.0002 | NS |
| 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 | | | |
| | 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* | <0.0001 | 0.0131 | NS |
| 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 | | | |
| | 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 | NS | 0.0210 | NS |
| 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 | | | |
| | 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 | NS | NS | NS |
| 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 | | | |
| | 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 | NS | NS | NS |

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 |
| Δ9-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 |
| Δ6-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 |
| Δ5-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 | | | |
| | HFHFr | 1.26±0.65 | 0.82±0.28 | 0.96±0.38 | 0.84±0.20 | 1.06±0.39* | 0.0419 | NS | NS |
| PPAR α /RPS9 | Control | 1.00±0.27 | 0.71±0.37 | 0.97±0.31 | 1.00±0.31 | 0.91±0.27 | | | |
| | HFHFr | 1.22±0.27 | 0.79±0.21 | 0.88±0.29 | 0.89±0.19 | 0.86±0.22 | NS | 0.0167 | NS |
| PPAR γ /RPS9 | Control | 1.00±0.48 | 0.60±0.35 | 0.75±0.30 | 0.80±0.17 | 0.64±0.17 | | | |
| | HFHFr | 1.43±0.65 | 0.80±0.32 | 0.85±0.40 | 0.74±0.29 | 0.76±0.33 | NS | 0.0017 | NS |
| 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 | | | |
| | HFHFr | 1.08±0.33 | 1.33±0.32 | 0.95±0.32 | 0.86±0.19 | 1.10±0.51 | 0.0145 | 0.0040 | NS |
| Srebp-1c/RPS9 | Control | 1.00±0.45 | 0.74±0.36 | 1.46±0.52 | 1.52±0.78 | 1.60±0.91 | | | |
| | HFHFr | 1.22±0.34 | 1.40±0.83 | 1.52±0.47 | 1.59±0.46 | 1.33±0.38 | NS | NS | 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. 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 g 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 |
| | HFHFr | 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 |
| | HFHFr | 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 |
| | HFHFr | 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 |
| | HFHFr | 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 |
| | HFHFr | 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 |
| | HFHFr | 0.79±0.22 | 1.15±0.42 | 0.94±0.44 | 11.26±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 |
| | HFHFr | 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 |
| | HFHFr | 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: arbitrary 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 |
|----------------------------|---------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|----------------|
| Mito ROS production | | | | | | | | | |
| 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 |
| Oxidative stress | | | | | | | | | |
| 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.