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HAL Authorization

1 **Cardiac remodeling and higher sensitivity to ischemia-reperfusion injury in**
2 **female rats submitted to high-fat high-sucrose diet: an *in vivo* / *ex vivo***
3 **longitudinal follow-up.**

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10 **Running title:** Prediabetes and female rat heart

11

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25

26 **Key words:** Prediabetes; High-fat high-sucrose diet; Female gender; Cardiac MRI; Ischemia-
27 reperfusion; Oxidative stress.

28 **Abstract**

29 Prediabetes is an important risk factor for type 2 diabetes and cardiovascular complications,
30 such as myocardial infarction. However, few studies explore female sex in this context. Here,
31 we aim to investigate the effects of high-fat high-sucrose diet on cardiac parameters and
32 sensitivity to ischemia-reperfusion injury of female Wistar rats. Female Wistar rats received
33 for 5 months normal diet (CTRL group) or high-fat high-sucrose diet (HFS group). Every
34 month MRI was performed to follow myocardial morphology, function and perfusion; cardiac
35 and hepatic triglyceride content; and amount of sub-cutaneous and visceral adipose tissues.
36 Then, *ex vivo* experiments were performed on isolated perfused hearts to evaluate tolerance to
37 ischemia-reperfusion, with simultaneous measurement of energy metabolism by ³¹P MRS and
38 contractile function. Coronary flow was measured before and after ischemia. At the end of the
39 experiments, hearts were freeze-clamped for biochemical assays. Five months of high-fat
40 high-sucrose diet induced a prediabetic condition in female Wistar rats, associated with an
41 increase in myocardial perfusion, systolic and diastolic wall thickness. HFS rats also exhibited
42 higher sensitivity to ischemia-reperfusion injury in comparison to controls, characterized by
43 impaired cardiac function, energy metabolism and endothelial function. Biochemical analyses
44 in hearts highlighted eNOS uncoupling, higher malondialdehyde level and lower S-
45 Glutathionylation of proteins in HFS rats, indicating higher oxidative stress. Prediabetes
46 induced by energy-dense diet was associated with modification of cardiac morphology and
47 higher myocardial sensitivity to ischemia-reperfusion injury. These results may be related to
48 the high risk of cardiovascular complications among type 2 diabetic women.

49 **Key words:** Prediabetes; High-fat high-sucrose diet; Female gender; Cardiac MRI; Ischemia-
50 reperfusion; Oxidative stress.

51

52 **1. Introduction**

53 Type 2 diabetes is worldwide recognized as a public healthcare issue affecting 425 million of
54 people [1]. The leading cause of mortality and morbidity in type 2 diabetic patients remains
55 cardiovascular (CV) complications. Interestingly, type 2 diabetes leads to CV complications
56 [2] and mortality [3] more frequently in women than men. The risk of myocardial infarction is
57 indeed 5 times higher in diabetic women than in non-diabetic women, whereas this risk
58 increases only twice in men [2]. However, until now, only a small number of studies focus on
59 female sex.

60 Prediabetes, a condition characterized by increased fasting plasma glucose and/or intolerance
61 to glucose, is also related to an increase of type 2 diabetes and CV risk [4]. Recently, the
62 Maastricht study showed that there are already sex differences in CV risk before the onset of
63 type 2 diabetes, with more adverse cardiometabolic profile in prediabetic women [5]. During
64 the transition from normoglycemia to impaired fasting glucose and type 2 diabetes, women
65 present higher levels of endothelial factors and fibrinolytic / thrombotic factors than men,
66 leading to greater endothelial dysfunction and insulin resistance [6]. The risk of heart and
67 coronary disease is also higher at lower glucose levels in women [7]. Factors such as
68 oxidative stress, inflammation, dyslipidemia and mitochondrial dysfunction can contribute to
69 endothelial dysfunction, to microvascular and macrovascular damage, leading to CV
70 complications [8]. Prediabetes is often associated with other risk factors of type 2 diabetes and
71 CV diseases, such as abdominal obesity, hypertriglyceridemia, low HDL-cholesterol and
72 raised blood pressure, clustered as metabolic syndrome (MetS) [9]. MetS is a strong predictor
73 of the onset of type 2 diabetes with a higher risk of 52%. MetS could be responsible for 17%
74 of CV events and 7 % of death by CV disease [10] and contributes to the increase by 78% of
75 CV risk and death in MetS patients [11]. Therefore, more studies are needed to better
76 understand prediabetic and metabolic syndrome conditions leading to higher CV risk in type 2

77 diabetes, particularly in women. As prediabetes and metabolic syndrome are strongly
78 associated with obesity [12], it becomes clear that our environment and life habits play an
79 important role in the disease pathogenesis. The increased intake of western diet and the
80 decrease of physical activity combined with genetic predisposition have strongly contributed
81 to appearance and spreading of MetS [13]. Thus, energy-dense-diet-induced animal model
82 could help us explore CV complications of prediabetes and MetS conditions.

83 The first objective of our study was to investigate the effect of a high-fat high-sucrose diet on
84 cardiac morphology and function in parallel to the development of prediabetes in female rats,
85 with a longitudinal follow-up *in vivo* using magnetic resonance imaging (MRI) and
86 spectroscopy (MRS). The second objective was to assess the impact of high-fat high-sucrose
87 diet on the sensitivity of heart during an ischemia-reperfusion injury, in which we combined
88 measurement of myocardial and endothelial function with simultaneous evaluation of energy
89 metabolism using ³¹P MRS. Finally, at the end of the experiments, we have explored cellular
90 integrity, nitric oxide (NO) pathway, apoptosis and oxidative stress in the myocardial tissue to
91 probe further into the molecular mechanisms induced by high-fat high-sucrose diet. To our
92 knowledge, this is the first study to consider cardiac longitudinal follow-up and tolerance to
93 ischemic insult in prediabetic female rats under high-fat high-sucrose diet, using MRI and
94 MRS combined with biochemical analyses.

95 **2. Materials and Methods**

96 For details, see Supplementary Material.

97 **2.1 Animal model**

98 Seven-week-old female Wistar rats (around 170 g, Charles River, France) (n=20) were housed
99 in a ventilated rack cabinet, with controlled temperature (22-24°C) and multiple
100 environmental enrichments. All procedures involving animals were approved by the Animal

101 experiment ethic committee of the University (APAFIS#10547-2017071009112930) and were
102 performed in conformity with the Directive 2010/63/EU of the European Parliament on the
103 protection of animals used for scientific purposes. Animals had access to food and water *ad*
104 *libitum*. Female Wistar rats were divided randomly in two groups of 10: The control group
105 (CTRL) was fed with standard diet (SAFE, A04C-10); and the HFS group was fed with high-
106 fat high-sucrose diet (SAFE, U8978 v19; composition detailed in Table 1). Rats received diets
107 for five months, and weight and food intake were measured every week. Food efficiency was
108 calculated as the ratio of weight gain on food intake over the 5 months of diet.

109 **2.2 Study design**

110 The experimental protocol is summarized in Figure 1. At 7 weeks of age, twenty female rats
111 underwent an initial MRI/MRS investigation (T0). They were randomized into two groups
112 (CTRL and HFS), and an MRI/MRS exam was performed on all rats every month. After 5
113 months of diet, they underwent an intraperitoneal glucose tolerance test (IPGTT) to evaluate
114 their metabolic status, and arterial blood pressure (BP) was measured. After sacrificing the
115 animals, *ex vivo* experiments on isolated perfused heart were performed to assess myocardial
116 function and tolerance to ischemia-reperfusion injury. At the end of the experiments, hearts
117 were freeze-clamped for biochemical analyses.

118 **2.3 Magnetic Resonance Imaging (MRI) and Spectroscopy (MRS) investigations**

119 The MRI and MRS examinations were performed to evaluate *in vivo* cardiac morphology,
120 cardiac function, myocardial perfusion, cardiac and hepatic triglycerides (TG) content and the
121 amount of visceral (VAT) and subcutaneous (SCAT) adipose tissue in the body. All
122 acquisitions were performed using a Bruker Biospec Avance MR system equipped with a 4.7
123 Tesla magnet (Bruker, Ettlingen, Germany) and a proton volume resonator (diameter 60 mm;
124 homogeneous length 80 mm).

125 Before the experiments, rats were sedated by inhalation of 3.5% isoflurane. During MRI/MRS
126 investigations, anesthesia was maintained at 1.5-2.5% isoflurane in 1 L/min pure oxygen-flow
127 to obtain regular breathing at a rate of around 70 breaths per minute. Respiration, ECG signal
128 and temperature were monitored during the whole procedure.

129 Every month, an ECG-gated gradient-echo cine-MRI sequence (cine-FLASH) was used for
130 the assessment of myocardial mass and function [14]. *In vivo* myocardial perfusion was
131 quantified using the Arterial Spin Labeling (ASL) technique [15]. At 5 months, cardiac and
132 hepatic ¹H MR spectra were acquired for TG assessment using an ECG- and respiratory-gated
133 Point-Resolved Spectroscopy (PRESS) sequence [16]. The TG/water signal ratio was
134 calculated to obtain TG concentration. For a quantitative map of adipose tissue distribution,
135 whole-body scanning was performed. Sixty-four contiguous axial imaging slices were
136 selected across the animal body length excluding the tail [17, 18]. After each experiment,
137 animals were kept alone under infrared light during wakefulness.

138 **2.4 Intra-peritoneal Glucose Tolerance Test (IPGTT) and Blood Pressure assessment at** 139 **T5 months**

140 An IPGTT was performed after 6 hours of fasting to evaluate glucose homeostasis. Glycemia
141 was measured before and 15, 30, 60, 90, 120 minutes after injection of a bolus of glucose,
142 using ACCU-CHEK Performa Nano Glucometer (Roche, Switzerland).

143 BP was recorded non-invasively by the tail cuff method (Bioseb, Chaville, France) [19].

144 **2.5 Isolated perfused heart preparation and tolerance to ischemia-reperfusion injury**

145 Rats were anesthetized by intraperitoneal injection of 90mg/kg pentobarbital sodium. Hearts
146 were cannulated and perfused in the Langendorff mode at constant pressure as described
147 previously [20].

148 **2.5.1 Experimental protocol**

149 After stabilization, hearts were perfused for 24 minutes with a physiological Krebs-Henseleit
150 buffer containing 0.4 mM palmitate, 3% albumin, 11 mM glucose, 3U/L insulin, 0.8 mM
151 lactate and 0.2 mM pyruvate. Before low-flow ischemia, they were perfused with
152 physiological Krebs-Henseleit buffer containing 1.2 mM palmitate and then underwent a low-
153 flow ischemia (0.5 mL/min/g wet wt) of 32 minutes with the same buffer. A higher
154 concentration of palmitate (1.2 mM) was provided during ischemia to cause maximal damage
155 during ischemia and reperfusion [21]. Finally, flow was restored entirely for 32 minutes with
156 the physiological Krebs-Henseleit buffer containing 0.4 mM palmitate. This protocol was
157 previously reported to provide sufficient level of ischemia in a type 2 diabetic animal model
158 [20].

159 **2.5.2 Evaluation of myocardial function and energy metabolism**

160 A water-filled latex balloon was inserted in the left ventricle to record developed left
161 ventricular pressure (DP) and heart rate (HR) [22]. The product of DP and HR (in
162 mmHg/min) was used as an index of myocardial function. Coronary flow (CF) was measured
163 by collection of coronary effluent before and after ischemia (at 20 minutes and 80 minutes of
164 protocol). CF is expressed in mL/min/g wet weight.

165 Energy metabolism was evaluated as previously described [23]. Briefly, perfused rat hearts
166 were placed in a 20-mm magnetic resonance sample tube and inserted in a 4.7 Tesla magnet
167 (Oxford instruments, Oxford, UK) interfaced with a Bruker-Nicolet Avance WP-200
168 spectrometer (Bruker, Karlsruhe, Germany). A series of eight ³¹P NMR spectra were recorded
169 during every period of the experimental protocol to quantify phosphorus metabolite signals
170 (ATP, PCr and Pi) and intracellular pH.

171 **2.6 Dissection and collection of samples**

172 VAT, SCAT, tibia and blood were collected for evaluation of physiological parameters. At
173 the end of the experiments, hearts were freeze-clamped in liquid nitrogen with a Wollenberger
174 clamp and kept at -80°C for subsequent analysis.

175 **2.7 Biochemical analyses**

176 In plasma, assay kits were used to determine glucose (Randox Laboratories, Crumlin, Antrim
177 UK), Free Fatty Acids (FFAs) (NEFA kit; Roche Diagnostics, Roche Applied Science,
178 Mannheim, Germany), TG (Abcam, ab 65336), HDL- and LDL-Cholesterol (Abcam, ab
179 65390).

180 In freeze-clamped hearts, cellular integrity was evaluated by measurement of creatine kinase
181 (CK) (CK-NAC, Randox Laboratories, Crumlin, UK) and lactate dehydrogenase (LDH)
182 activities [23]; apoptosis with expression of cleaved caspase-3 by Western Blot [24]; NO
183 pathway with the expression of total eNOS (endothelial NO synthase), P-eNOS
184 (phosphorylated on Ser1177) and eNOS dimer-to-monomer ratio (eNOS d/m) by Western
185 Blot [25, 26]; proteins post-translational modification with evaluation of S-Glutathionylation
186 by Western Blot; and oxidative stress with assessment of Malondialdehyde (MDA), an index
187 of lipid peroxidation, with the lipid peroxidation assay kit (MAK085, Sigma-Aldrich, St
188 Louis, USA).

189 **2.8 Expression of results and statistical analyses**

190 Data are graphically provided as mean \pm SEM of absolute values. Graph Pad Prism software
191 5.0 (La Jolla, CA) was used for all statistical processing. Significant differences between
192 groups were determined using two-way analysis of variance (ANOVA) with repeated
193 measures over time for the time-dependent variables followed by Bonferroni post-hoc test. An
194 unpaired Student's *t*-test was used for the other parameters. Correlation analyses were also

195 performed between imaging and other parameters. A p value of less than or equal to 0.05 was
196 considered to indicate significant difference.

197 **3. Results**

198 **3.1 Effect of high-fat high-sucrose diet on physiological parameters**

199 Physiological parameters are summarized in Table 1. Food intake was significantly lower in
200 HFS versus CTRL during the whole diet ($p < 0.0001$), while calorie intake and food efficiency
201 were significantly increased (respectively $p < 0.001$ and $p < 0.01$). After 5 months of diet, the
202 weight and the lean mass of animals was not different between groups, and neither systolic or
203 diastolic arterial blood pressure. However, fat mass percentage was significantly higher in
204 HFS vs. CTRL. Amount of VAT and SCAT measured by MRI was significantly higher at T5
205 ($p = 0.014$ and $p = 0.01$, respectively), as well as VAT measured by dissection ($p < 0.05$ vs.
206 CTRL; $p = 0.08$ for SCAT) in HFS vs. CTRL. Measurement by MRI and dissection were
207 strongly correlated (VAT, $r = 0.98$; SCAT, $r = 0.90$). The percentage of cardiac TG was not
208 different between groups, but the percentage of hepatic TG was significantly higher in HFS
209 vs. CTRL at T5 ($p < 0.001$). Heart weight-to-tibia-length ratio was similar in both groups.
210 Plasma FFAs and fasting plasma glucose were significantly higher in HFS compared with
211 CTRL ($p < 0.05$ and $p < 0.01$ respectively). No difference was found in plasma TG and HDL-
212 Cholesterol, but LDL-Cholesterol was significantly higher in HFS compared with CTRL
213 ($p < 0.05$). The IPGTT highlighted a significant glucose intolerance in HFS compared with
214 CTRL ($p < 0.01$).

215 **3.2 Effect of high-fat high-sucrose diet on cardiac morphology, perfusion and function**

216 The effects of high-fat high-sucrose diet on cardiac parameters are shown in Figure 2. The
217 high-fat high-sucrose diet led to thickening of the myocardial wall in both diastole ($p < 0.01$,
218 Figure 2A) and systole ($p < 0.01$, Figure 2B), and to increased left ventricular mass ($p < 0.05$,

219 Figure 2C) starting at the 3rd month of diet in HFS vs. CTRL. Myocardial perfusion (Figure
220 2D) was significantly increased in HFS ($p < 0.01$) from T2 month vs. CTRL. However, the
221 high-fat high-sucrose diet had no effect on cardiac function parameters (supplemental
222 material) compared to CTRL.

223 **3.3 Effect of high-fat high-sucrose diet on myocardial tolerance to ischemia-reperfusion** 224 **injury**

225 **3.3.1 *Ex vivo* myocardial function and coronary flow**

226 Myocardial function (Figure 3A) evaluated by the product of DP and HR was significantly
227 lower in baseline conditions ($p < 0.05$) and during reperfusion ($p < 0.01$) in HFS vs. CTRL. The
228 percentage of recovery, expressed as percentage of control values, was significantly lower in
229 HFS compared with CTRL ($53 \% \pm 6 \%$ vs. $29 \% \pm 6 \%$; $p < 0.05$) during reperfusion. CF was
230 not different between groups in baseline conditions (Figure 3B). However, CF was
231 significantly lower in HFS vs. CTRL during reperfusion ($p < 0.05$) (Figure 3C).

232 **3.3.2 *Ex vivo* energy metabolism and intracellular pH (pHi)**

233 Figure 4 shows kinetics of PCr (A), ATP (B), Pi (C) and pHi (D) during the ischemia-
234 reperfusion protocol. During the control period, no difference in PCr, ATP and Pi content was
235 found between the two groups. During ischemia, PCr and ATP were significantly decreased in
236 HFS compared with CTRL ($p < 0.05$) and Pi was similar in both groups. During reperfusion,
237 PCr and ATP were significantly decreased, and Pi was significantly increased in HFS vs.
238 CTRL (respectively $p < 0.01$, $p < 0.05$ and $p < 0.05$). pHi was similar in both groups during the
239 whole protocol.

240 **3.3.3 Biochemical analyses in freeze-clamped hearts**

241 At the end of the *ex vivo* experiments, we studied NO pathway involved in the endothelial
242 function. The expression of total eNOS and P-eNOS (Figures 5A and 5B) was similar
243 between CTRL and HFS. However, we found an increase of uncoupled eNOS (inactive form)
244 in HFS, characterized by significantly lower eNOS dimer-to-monomer ratio (Figure 5C) in
245 comparison to CTRL ($p < 0.01$), indicating an impaired endothelial function. Total S-
246 Glutathionylation (Figure 5D) of proteins was decreased ($p = 0.052$) in HFS compared with
247 CTRL, indicating a potentially diminished protection against oxidative stress. S-
248 Glutathionylation of eNOS (Figure 5E) was similar between groups. Malondialdehyde, a lipid
249 peroxidation marker, was significantly increased in HFS vs. CTRL ($p < 0.05$; 90.4 ± 6.3 vs.
250 73.4 ± 4.7 nmol/g).

251 Myocardial CK and LDH activities as markers of cellular integrity were similar in CTRL
252 (11.03 ± 0.44 and 3.97 ± 0.28 U/mg protein) and HFS (10.65 ± 0.55 and 3.97 ± 0.25 U/mg
253 protein) groups after ischemia-reperfusion injury. In both groups, the active form of caspase-3
254 protein, was not expressed in hearts (data not shown) suggesting absence of apoptosis.

255 **3.4 Correlation analyses**

256 Correlation analyses are shown in Figure 6. FFAs, intrahepatic TG and percentage of
257 functional recovery during reperfusion were correlated with the amount of VAT measured by
258 MRI (respectively $r^2 = 0.68$; $r^2 = 0.88$ and $r^2 = 0.46$).

259 **4. Discussion**

260 The aim of this study was to investigate the effects of long-term high-fat high-sucrose diet-
261 feeding i) on the development of prediabetes and/or metabolic syndrome (MetS), ii) on
262 cardiac parameters and iii) on myocardial tolerance to ischemia-reperfusion injury in female
263 Wistar rats. Here, the emergence of a prediabetic state combined with metabolic disturbances
264 has led to cardiac remodeling characterized by thickening of the myocardial wall, and to

265 increased myocardial perfusion. High-fat high-sucrose diet also decreased the sensitivity of
266 the heart to ischemia-reperfusion injury characterized by an impairment of cardiac function,
267 energy metabolism and endothelial function. Finally, biochemical analyses in heart tissue
268 highlighted eNOS uncoupling, decreased S-Glutathionylation of proteins and increased MDA
269 in HFS compared with CTRL, indicating exacerbated oxidative stress.

270 Diet-induced models appear to be one of the best strategies to study prediabetic and MetS
271 conditions associated with CV complications. In 2016, Wong et al. reviewed the different
272 existing types of diet [27]. To get closer to human disease, Panchal et al. suggested to use a
273 combination of fat- and carbohydrate-enriched diet, which groups a maximum of criteria and
274 leads to cardiac dysfunction and hypertrophy in rats after 16 weeks of diet [28]. Here we used
275 high-fat high-sucrose diet containing 19.4% of protein, 35.8% of fat and 32.1% of
276 carbohydrate. After five months of diet, animals displayed the typical characteristics for
277 diagnosis of prediabetes (higher fasting glucose and lower glucose tolerance), which were
278 associated with metabolic disturbances that could lead to MetS and type 2 diabetes. While
279 many studies [29] showed increased body weight in animals under various high-fat diets, we
280 found that the weight of female HFS rats was not significantly increased in comparison with
281 the control group. Aubin et al. reported the same observation in their study on female Sprague
282 Dawley rats fed with HFD for 8 weeks [30]. Nishikawa et al. suggested that the female sex
283 could be more resistant to high-fat diet-induced obesity, which could explain our results [31].
284 Also, food intake was lower in HFS rats, possibly because the high-fat high-sucrose diet was
285 more caloric. Consistent with this explanation, an increase of serum leptin, the hormone
286 regulating satiety, has been previously reported in rats under high-fat diet [32]. Despite a
287 possible regulatory mechanism via leptin, HFS animals had a higher caloric intake leading to
288 abdominal obesity. Indeed, HFS rats did not display weight gain but MRI and dissection
289 highlighted an abdominal obesity with significant increase of fat mass percentage (of both

290 VAT and SCAT). Multiple human studies have shown that waist circumference, an indicator
291 of abdominal obesity, is more representative of total body fat than BMI [33]. Interestingly, the
292 amount of VAT was correlated with changes in plasma FFAs and hepatic TG content.
293 Moreover, VAT was also correlated to the percentage of functional recovery after ischemia-
294 reperfusion injury. These observations confirm that the anatomical distribution of excess fat is
295 closely related to the occurrence of cardiovascular and metabolic complications [34], even in
296 subject with normal weight [35]. Expansion and dysfunction of visceral adipose tissue both
297 result in the release of fatty acids into the circulation and in ectopic fat deposition in organs
298 like heart, liver or pancreas [36]. Here, we found an increase in plasma free fatty acids and
299 LDL-cholesterol associated with an increase in intrahepatic TG content. The increase in
300 myocardial TG content seems to occur later than the increase of hepatic TG content, as shown
301 in male mice under high-fat high-sucrose diet [17]. This may explain why we found no
302 difference between groups regarding cardiac TG content in this study. Finally, in this model
303 we would expect an increase in arterial blood pressure, as described by Aubin et al. in female
304 high-fat fed rats [30]. Here, only a non-significant tendency to elevated systolic and diastolic
305 blood pressures was observed in the HFS group, warranting further investigation on a larger
306 animal group.

307 The combination of these risk factors predisposes women to CV complications. As shown by
308 Levitzky et al. the risk of CV disease at lower glucose levels is higher in women than in men
309 [7]. However, the mechanisms involved are not yet fully understood, and literature studies on
310 female sex are scarce. Here, we followed female rats over months using cardiac MRI and
311 observed multiple cardiac abnormalities. First, cardiac morphology was modified starting at
312 the third month of diet with an increased myocardial wall thickness in systole and diastole.
313 Left ventricular mass evaluated by MRI was increased at three and four months of high-fat
314 high-sucrose diet. Concentric remodeling may occur, for example, as a result of pressure

315 overload (such as high blood pressure or aortic stenosis) whether or not there is ventricular
316 hypertrophy (defined as an increase in mass) [37]. Cardiomyocyte hypertrophy and cardiac
317 fibrosis [37] could also explain the cardiac remodeling found here. However, we note that
318 heart weight to tibia length ratio obtained *ex vivo* was not significantly higher in HFS
319 compared to CTRL. We attribute this apparent inconsistency to the difference in methodology
320 of measurement *in vivo* and *ex vivo*. MRI evaluation of hypertrophy was based on an ellipsoid
321 model [14] evaluating the left ventricle only. *Ex vivo*, we measured the weight of the entire
322 heart just before cannulation, i.e. without potential biases by blood pressure and circulation.
323 Dedicated histology experiments may help confirm this result in future studies.

324 Left-ventricular cardiac function parameters evaluated by MRI were not different in female
325 rats submitted to high-fat high-sucrose diet for 5 months compared with CTRL, whereas *ex*
326 *vivo* basal cardiac function was significantly weaker. The *in vivo* cardiac function
327 measurements reflect dynamic volume measurements whereas *ex vivo* function reflects
328 pressure developed during contraction. Also, *ex vivo* cardiac function is not submitted to the
329 effective regulations and demands *in vivo*. A similar difference between *in vivo* and *ex vivo*
330 cardiac function was found by Cole et al., who found normal cardiac function *in vivo* in rats
331 submitted to high-fat diet but 21% decreased cardiac efficiency *ex vivo* compared with
332 controls [38]. They related the lower cardiac efficiency to an increase in myocardial oxygen
333 consumption. In our study, along with cardiac function we monitored myocardial perfusion *in*
334 *vivo*, which may be linked to oxygen consumption. Interestingly, myocardial perfusion was
335 significantly higher in HFS vs. CTRL, despite normal *in vivo* cardiac function, consistent with
336 the study by Cole et al. We thus hypothesized that HFS hearts needed higher capillary blood
337 flow to produce the same work indicating decreased cardiac efficiency. Moreover, increased
338 myocardial perfusion might be a transient condition found in prediabetic animals [39]. In fact,
339 a previous study in our laboratory showed decreased myocardial perfusion in type 2 diabetic

340 male mice under high-fat high-sucrose diet [17]. Also, Iltis et al. showed defective myocardial
341 blood flow in 8-13 months type 2 diabetic GK rats [40]. Then, it would be interesting to test
342 this diet over a longer period to evaluate whether myocardial perfusion decreases below
343 normal values at later stages in this model as well. Another study in type 1 diabetic patients
344 reported higher myocardial perfusion at rest than in healthy controls [41]. Finally, it is well
345 known that myocardial perfusion can be modified by isoflurane concentration, temperature or
346 heart rate, and that perfusion values likely reflect partial vasodilation [42]. However, these
347 parameters were monitored during the MRI exam and were not different between the groups
348 (data not shown).

349 In summary, 5 months of high-fat high-sucrose diet induced modification of cardiac
350 morphology and efficiency. Using the ischemia-reperfusion *ex vivo* protocol, we further found
351 higher sensitivity to ischemia-reperfusion injury in HFS compared with CTRL, characterized
352 by a significant decrease in myocardial function and energy metabolism during reperfusion.
353 Under physiological conditions mitochondria play a fundamental role in oxidative catabolism
354 leading to the production of energy in the form of ATP. Multiple mitochondrial impairments
355 could be responsible for lower energy metabolism. Among these are mitochondrial
356 uncoupling [38], decreased expression of mitochondrial respiratory chain complex or
357 increased ROS production. Moreover, a loss of nucleotide precursors limiting ATP repletion
358 during reperfusion could contribute to our observation. Because these alterations may be
359 correlated with oxidative stress, we particularly focused on myocardial MDA content as lipid
360 peroxidation marker, which was significantly higher in HFS vs. CTRL. Pakdeechote et al.
361 have previously reported higher vascular superoxide production as well as higher MDA levels
362 in plasma in metabolic syndrome rats [43]. Poudyal et al. also showed lowered capacity to
363 neutralize free radicals characterized by the decrease of anti-oxidant capacity [29]. Finally,
364 ischemia and mostly reflow are well-known to induce high oxidative stress, which is

365 exacerbated here by the 5-month high-fat high-sucrose diet. We also assessed the S-
366 Glutathionylation of proteins, which is the reversible addition of glutathione to cysteine
367 residues inactivating the target proteins [44], and which could be involved in CV
368 complications [45]. Protein S-Glutathionylation occurs mainly at the beginning of reflow in
369 an ischemia-reperfusion protocol and has been shown to be increased in some studies. For
370 example, De Pascali et al. showed an increase in eNOS S-Glutathionylation during ischemia-
371 reperfusion injury in endothelial cells [46]. However, Belcastro et al. explained that S-
372 Glutathionylation can also be a protective mechanism coping with irreversible oxidation [45].
373 This would explain the lower rate of S-Glutathionylation found here in HFS vs. CTRL. We
374 have shown that S-Glutathionylation of all the proteins was significantly decreased in HFS
375 compared with CTRL, but we found no difference in specific S-Glutathionylation of eNOS.
376 Here, we suggest that S-Glutathionylation was decreased by high-fat high-sucrose diet,
377 leading to higher level of damage by oxidative stress in hearts. This result was consistent with
378 the higher MDA heart content mentioned before.

379 Prediabetes and MetS increase the risk of cardiovascular events and have a deleterious impact
380 on endothelial function with enhanced coronary artery disease prevalence [47]. Altered
381 vasodilatation has been previously described. Poudyal et al. showed lower vascular response
382 to noradrenaline, acetylcholine and sodium nitroprusside in a MetS model [29] while
383 Senaphan et al. found decreased eNOS expression in mesenteric arteries [48]. Here, no
384 significant difference was found in coronary flow before ischemia. During reperfusion,
385 however, coronary flow was significantly impaired in HFS vs. CTRL, indicating higher
386 sensitivity of coronary arteries to ischemia-reperfusion injury. We found no difference
387 between the groups concerning the expression of total eNOS or its phosphorylated form.
388 However, we found higher eNOS uncoupling in HFS compared with CTRL, with a lower
389 dimeric (active) form of eNOS and a higher monomer form of eNOS in hearts, indicating that

390 high-fat high-sucrose diet impaired eNOS activity. eNOS uncoupling is known to be
391 responsible for the increase in superoxide anion $O_2^{\cdot-}$ production instead of NO [49]. $O_2^{\cdot-}$ is
392 highly deleterious for the cells and can also react with NO to form peroxynitrite ($ONOO^-$),
393 making it less available for endothelial function. $ONOO^-$ has been shown to inhibit
394 mitochondrial respiratory chain complex [50]. During reperfusion this phenomenon is
395 amplified because the free electrons couple to oxygen to form $O_2^{\cdot-}$. Moreover, patients with
396 MetS show lower levels of superoxide dismutase able to handle superoxide anion [51].
397 Endothelial function also seems to be a key point in cardiac complications of type 2 diabetes.
398 Desrois et al. showed endothelial impairment in female Goto-Kakizaki rats, with lower
399 coronary flow and reduced up-regulation of the NO pathway [22]. Zhang et al. revealed a
400 predisposition of females to vascular lesions after induction of diabetes, in the mesenteric
401 arteries [52] and the aorta [53]. Thus, endothelial dysfunction found in our model of
402 prediabetes could be related to the higher cardiovascular risk in prediabetic and type 2
403 diabetic women.

404 As a perspective, it would be interesting to measure plasma hormones in order to fully
405 elucidate their role in high-fat high-sucrose diet-induced cardiovascular modifications.
406 Baseline cardiac function differs between sexes in terms of heart rate, systolic output, ejection
407 fraction [54]. Studies on rodents [55] have shown that cardiac myocytes of male rats contract
408 stronger and faster than the myocytes of female rats. Estrogen receptors ($ER\alpha$ and β) are both
409 found in cardiomyocytes and may have a strong impact on cardiovascular function [56].
410 Estrogens have been shown to protect the heart from ischemic injury [57]. The loss of
411 estrogens also leads to aggravation of mitochondrial dysfunction, inflammation and cardiac
412 remodeling [58]. Sex hormones could also explain the differences found in endothelial
413 function, as reported by Khalil et al., who showed that estrogen might directly stimulate NO
414 production in women [59]. Interestingly, Al-Mulla et al reported a decrease in estrogens and

415 an increase in testosterone levels in the GK rat model [60]. However, in a model of high-fat
416 diet, Chakraborty et al. reported an increase in estradiol level with high-fat diet [61]. In
417 addition, assessing the cytokine profiles in HFS rats could help us understand diet-induced
418 metabolic and structural changes that occur before ischemia-reperfusion injury. Indeed,
419 Pakdeechote et al. reported higher plasma TNF α and increased expression of iNOS in aorta of
420 rat with diet-induced metabolic syndrome [43]. In future studies it would also be interesting to
421 have dedicated groups for histology investigations. Previous studies showed that 16 weeks of
422 high-carbohydrate high-fat diet could induce cardiomyocyte hypertrophy, as well as left
423 ventricular interstitial collagen deposition and inflammatory cell infiltration in rat hearts [29,
424 37, 62]. Finally, dietary interventions could prevent cardiac changes observed in prediabetic
425 female rats. Abdurrachim et al. showed that intermittent fasting could delay the progression
426 of left ventricular hypertrophy and prevent cardiac dysfunction in prediabetic obese
427 spontaneous hypertensive heart failure rats, by modulating cardiac substrate metabolism [63].
428 Alam et al. showed for example that chronic L-Arginine treatment improved metabolic,
429 cardiovascular and liver complications in diet-induced obesity in rats [62]. We also believe
430 that an anti-oxidant approach would be of great interest in our context. For example,
431 Resveratrol, a polyphenol, well-known for its anti-oxidant property, can protect the heart of
432 high-fat fed rats [64].

433 **5. Limitation of the study**

434 One limitation of our study might be the absence of male groups, which could help us
435 demonstrate sex-specific response to high-fat high-sucrose diet on cardiovascular alterations.
436 However, we believe this is an original study on female sex which is little explored in the
437 literature. Indeed, most of the studies use male population only, although type 2 diabetic
438 women have a higher cardiovascular risk than type 2 diabetic men. Here, our objective was to
439 study the female population by performing a longitudinal follow-up at the beginning of the

440 disease and to observe the early adaptation of the heart to prediabetes. It brings new insights
441 on cardiovascular complications in female rats during the development of prediabetes, with
442 increased myocardial perfusion as well as high sensitivity to ischemia-reperfusion injury via
443 impaired energy metabolism, S-Glutathionylation and eNOS uncoupling.

444 **6. Conclusion**

445 To our knowledge, this is the first study combining a longitudinal cardiac MRI follow-up with
446 an assessment of tolerance to ischemic insult in prediabetic female rats under high-fat high-
447 sucrose diet. In female rats, prediabetes led to myocardial adaptation over months
448 characterized by thickening of the myocardial wall and increase in myocardial perfusion. It
449 also led to higher cardiac sensitivity to ischemia-reperfusion injury. Oxidative stress seems to
450 be at the intersection of the pathways elucidated here, as shown in Figure 7. Oxidative stress,
451 induced by ischemia-reperfusion, was exacerbated under high-fat high-sucrose diet. Here, it
452 might be responsible for i) cardiac dysfunction via impaired energy metabolism with altered
453 ATP and PCr production during ischemia-reperfusion injury, ii) endothelial dysfunction via
454 eNOS uncoupling and decreased NO bioavailability leading to lower coronary flow, and iii)
455 decrease of protective protein S-Glutathionylation mechanism. This work provides insights
456 that may contribute to understanding the cardiovascular complications related to prediabetes
457 and type 2 diabetes in the female sex.

458 **Declarations**

459 **Author's contribution:** All the authors participated substantially in the investigations
460 reported here as indicated : N.F contributed to design, performed the experiments, data
461 analysis and wrote the paper; C.L contributed to experiments; F.K contributed to
462 interpretation of the overall study and manuscript writing; D.B, C.R and J.B contributed to

463 biochemical analyses; M.B and M.D contributed to design, interpretation of the overall study
464 and manuscript writing. All authors read and approved the final manuscript.

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471 collection, analysis and interpretation of data; in the writing of the report; and in the decision
472 to submit the article for publication.

473 **Competing interests:** The authors declare that they have no competing interests.

474 **List of abbreviations**

475 CF: Coronary Flow, CK: Creatine Kinase, CV: Cardiovascular, DP: Developed Pressure,
476 eNOS: Endothelial NO Synthase, P-eNOS: Phosphorylated form of eNOS, HR: Heart Rate,
477 LDH: Lactate Dehydrogenase, MDA: Malondialdehyde, MetS: Metabolic Syndrome, MRS:
478 Magnetic Resonance Spectroscopy, NO: Nitric Oxide, ONOO: Peroxynitrite, SCAT:
479 Subcutaneous Adipose Tissue, TG: Triglycerides, VAT: Visceral Adipose Tissue.

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Compounds	%
Casein	22.8
DL methionine	0.2
Maltodextrin	17.15
Sucrose	16.64
Anhydrous butter	33.35
Soya oil	2.5
Mineral (AIN 93G-mx)	4.55
Sodium bicarbonate	1.05
Potassium citrate	0.4
Vitamin (AIN 93-vx)	1.3
Choline bitartrate	0.2
Antioxidant	0.002

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Table 1: Composition of the high-fat high-sucrose diet (SAFE, U8978)

	CTRL	HFS	note
Body weight (g)	270.1 ± 11.2	297.8 ± 17.3	
Lean mass (g)	262.8 ± 7.4	271.7 ± 9.1	
Food intake for 5 months (g)	1514 ± 26	1031 ± 10.8 *	
Calories intake for 5 months (kcal)	5054 ± 87	5669 ± 59 *	
AUC Food efficiency (weight gain/food intake)	0.31 ± 0.04	0.50 ± 0.05 †	
Systolic blood pressure (mm Hg)	111.7 ± 3.3	128.7 ± 10.4	
Diastolic blood pressure (mm Hg)	76.1 ± 2.9	90.1 ± 8.1	
VAT MRI (volume mm³/slice)	700 ± 63	1122 ± 128 ‡	Correlation
VAT Dissection (g)	19.3 ± 3.1	38.4 ± 6.7 ‡	r ² =0.90
SCAT MRI (volume mm³/slice)	233 ± 13	402 ± 52 †	Correlation
SCAT Dissection (g)	9.89 ± 1.5	18.9 ± 4.3	r ² =0.98
Cardiac TG (%)	0.28 ± 0.28	0.14 ± 0.14	
Fat mass (%)	9.8 ± 1.2	16.8 ± 2.1 ‡	
Hepatic TG (%)	0.88 ± 0.28	6.10 ± 1.27 *	
Heart weight (g)	0.76 ± 0.02	0.78 ± 0.02	
Tibia length (cm)	3.50 ± 0.04	3.48 ± 0.04	
Heart weight / Tibia length (g/cm)	0.220 ± 0.005	0.230 ± 0.006	
FFAs (mM)	0.09 ± 0.01	0.21 ± 0.04 *	
Fasting glucose (mM)	4.78 ± 0.22	5.72 ± 0.17 †	
TG (mM)	125.2 ± 14.4	90.5 ± 15.2	
HDL-Cholesterol (mM)	1.24 ± 0.07	1.24 ± 0.13	
LDL-Cholesterol (mM)	0.83 ± 0.10	1.19 ± 0.13 ‡	
AUC IPGTT (mM/min)	7.1 ± 0.5	10.4 ± 0.8 †	

704 **Table 2: Physiological characteristics of rats after 5 months of diet.** Data are means ±
705 SEM, two-way ANOVA was used for statistical analysis of IPGTT and t-test was used for all
706 the other parameters. HFS group had lower food intake, higher calorie intake, food efficiency,

707 amount of visceral and subcutaneous adipose tissue, % of fat mass, % of hepatic TG, plasma
708 FFAs, fasting glucose, LDL-Cholesterol and intolerance to glucose. We found no difference
709 between groups for body weight, systolic blood pressure, diastolic blood pressure, cardiac TG
710 content, heart weight-to-tibia length ratio, plasma TG and HDL-Cholesterol. AUC: area
711 under the curve. * $p < 0.001$; † $p < 0.01$ and ‡ $p < 0.05$ vs. CTRL.

712 **Figure 1: Experimental protocol.** At 7 weeks of age, animals (n=20) underwent a first
713 MRI/MRS investigation (T0). Female Wistar rats were randomized in two groups (CTRL and
714 HFS), and every month an MRI/MRS exam was performed on each rat. The following
715 parameters were evaluated: myocardial morphology, function, perfusion, triglyceride content,
716 hepatic triglycerides and visceral and subcutaneous adipose tissue amount. After 5 months of
717 diet, rats underwent an intraperitoneal glucose tolerance test (IPGTT) to evaluate their
718 metabolic status, and arterial blood pressure (BP) was measured. Then, *ex vivo* experiments
719 on isolated perfused heart were performed to evaluate tolerance to ischemia-reperfusion injury
720 with simultaneous measurement of myocardial function, energy metabolism and coronary
721 flow. At the end of the experiments, hearts were freeze-clamped for biochemical analysis.
722 Western blots were performed to analyze expression of eNOS, P-eNOS, eNOS
723 dimer/monomer, S-Glutathionylation, eNOS S-Glutathionylation and Caspase-3 proteins.
724 Colorimetric assays were performed to assess MDA heart content and activity of creatine
725 kinase and lactate dehydrogenase enzymes.

726 **Figure 2: Monitoring of cardiac morphology and perfusion over time by MRI.** Diastolic
727 myocardial wall thickness (Wtdia) (A), Systolic myocardial wall thickness (Wtsys) (B), Left
728 ventricular mass (LVMass) (C), Myocardial perfusion (D). Data are means \pm SEM. Two-way
729 ANOVA was performed to observe the effect of group and time. Wtdia (ANOVA: $p < 0.01$;
730 Bonferroni post-hoc test: T3 $p < 0.001$, T4 $p < 0.001$ and T5 $p < 0.05$) and Wtsys (ANOVA:

731 p<0.01; Bonferroni post-hoc test: T3 p<0.01, T4 p<0.01 and T5 p<0.05) were significantly
732 increased in HFS vs. CTRL from the third month. LVMass was significantly increased in
733 HFS at T3 and T4 months (ANOVA: p<0.05; Bonferroni post-hoc test: T3 p<0.05 and T4
734 p<0.01). Myocardial perfusion was significantly increased from the second month of diet
735 (ANOVA: p<0.01; Bonferroni post-hoc test: T2 p<0.05, T3 p<0.01 and T5 p<0.05). * p<0.01
736 and † p<0.05 vs. CTRL (Two-way ANOVA).

737 **Figure 3:** *Ex vivo* evaluation of myocardial function. Myocardial function (A) was
738 evaluated by the product of developed pressure and heart rate, during the experimental time
739 course in CTRL and HFS rat hearts. Coronary flow was measured during the control period at
740 20 minutes (B) and during reperfusion at 80 minutes (C) of protocol. Results are expressed as
741 means ± SEM. For myocardial function statistical analysis, two-way ANOVA was performed
742 to observe the effect of group and time. T-test was performed for statistical analysis of
743 coronary flow. In HFS, myocardial function was significantly decreased during the control
744 period and reperfusion, % of recovery and coronary flow were significantly impaired
745 compared with CTRL. * p<0.05 and † p<0.01 vs. CTRL.

746 **Figure 4:** *Ex vivo* evaluation of energy metabolism with ³¹P-MRS. Kinetics of
747 phosphocreatine (PCr) (A), ATP (B), Pi (C) and intracellular pH (pHi) (D) during the
748 experimental time course in CTRL and HFS rat hearts. Data are means ± SEM. Two-way
749 ANOVA was performed to observe the effect of group and time. Myocardial PCr and ATP
750 contents were significantly decreased in HFS vs. CTRL during low-flow ischemia and
751 reperfusion. Pi was significantly increased in HFS compared with CTRL only during
752 reperfusion. pHi was similar in both groups during the whole protocol. * p<0.05 and † p<0.01
753 vs. CTRL (Two-way ANOVA).

754 **Figure 5: Biochemical analysis in freeze-clamped hearts at the end of ischemia-**
755 **reperfusion injury.** Determination of protein expression of total eNOS (A), P-eNOS (B),
756 eNOS dimer-to-monomer ratio (C) and determination of S-Glutathionylation of total (D) and
757 eNOS (E) proteins by western blot. Data are means \pm SEM and t-test was used for all the
758 parameters. No difference was found between groups for expression of eNOS, and P-eNOS;
759 eNOS uncoupling was significantly higher in HFS vs. CTRL. No difference was found
760 between groups regarding eNOS, P-eNOS expression and S-Glutathionylation of eNOS. The
761 ratio eNOS d/m was significantly decreased in HFS vs. CTRL, indicating eNOS uncoupling.
762 S-Glutathionylation of total proteins was also decreased in HFS vs. CTRL. * $p < 0.05$ vs.
763 CTRL; † $p < 0.05$ vs. CTRL.

764 **Figure 6: Correlation analysis between imaging and outcomes.** Plasma FFAs (A) and
765 hepatic TG content (B) were correlated to the amount of VAT measured by MRI. Percent of
766 functional recovery during reperfusion was also correlated to the amount of VAT (C).

767 **Figure 7: Potential mechanisms involved in the lower tolerance to ischemia-reperfusion**
768 **in prediabetic female rats.** High-fat high-sucrose diet (HFS) increased sensitivity to
769 ischemia-reperfusion injury *ex vivo*. Oxidative stress was exacerbated, with higher MDA
770 level, eNOS uncoupling and lower protection mechanism by S-Glutathionylation in female rat
771 hearts. It might explain the impaired energy metabolism and endothelial function leading to
772 cardiac dysfunction in female gender. ®: Protein; MDA: Malondialdehyde.

Figure 1

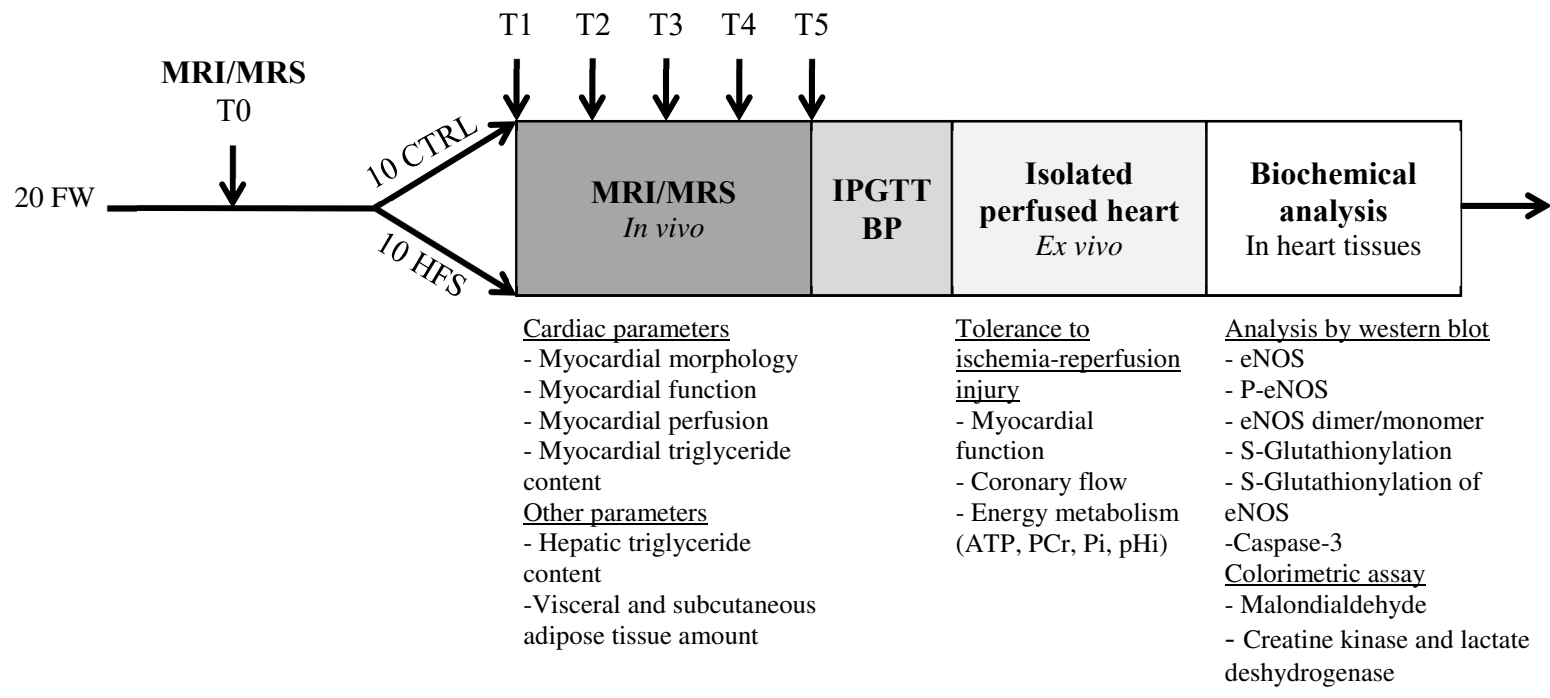
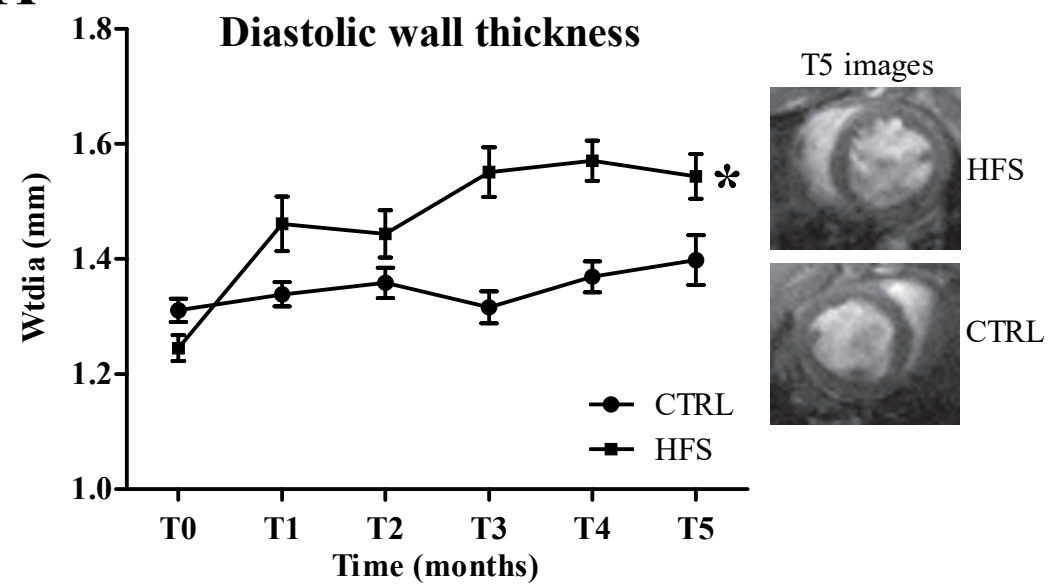
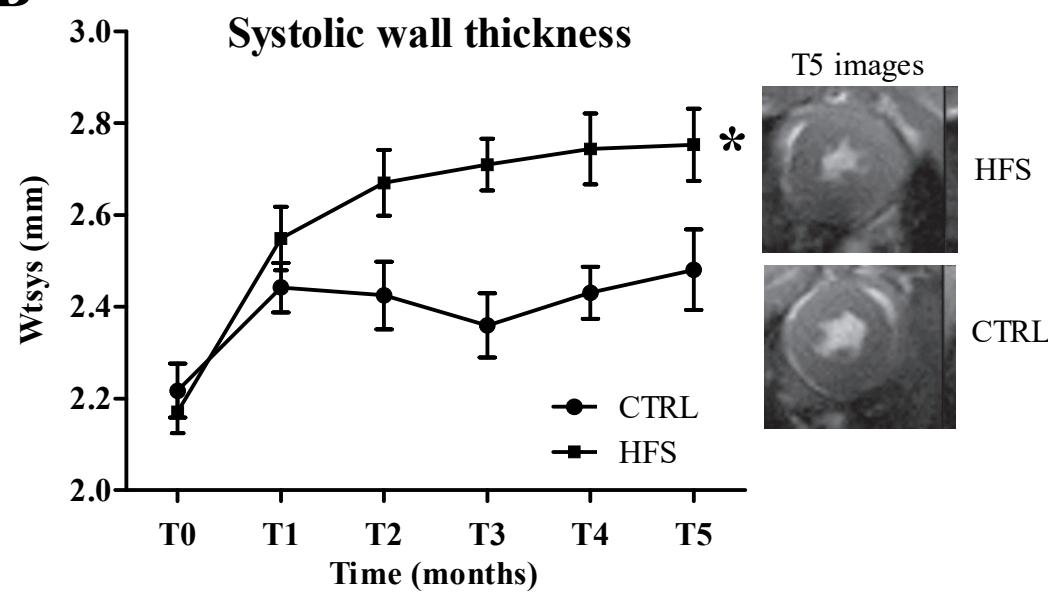


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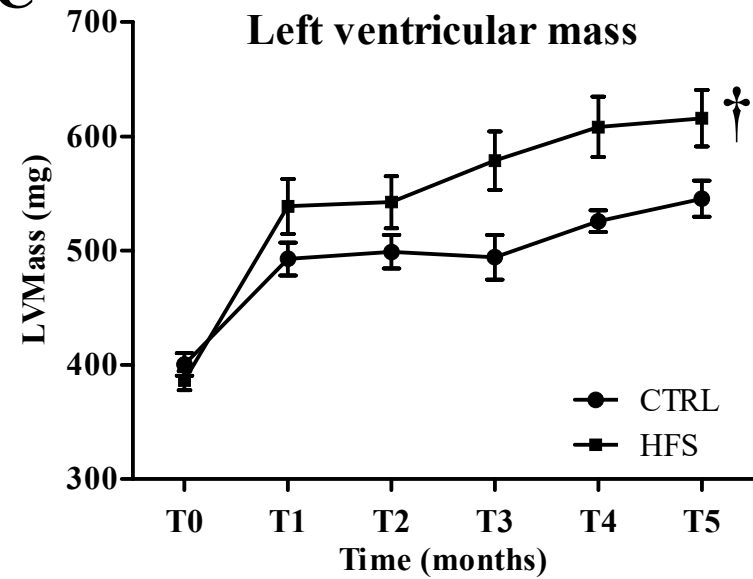
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B



C



D

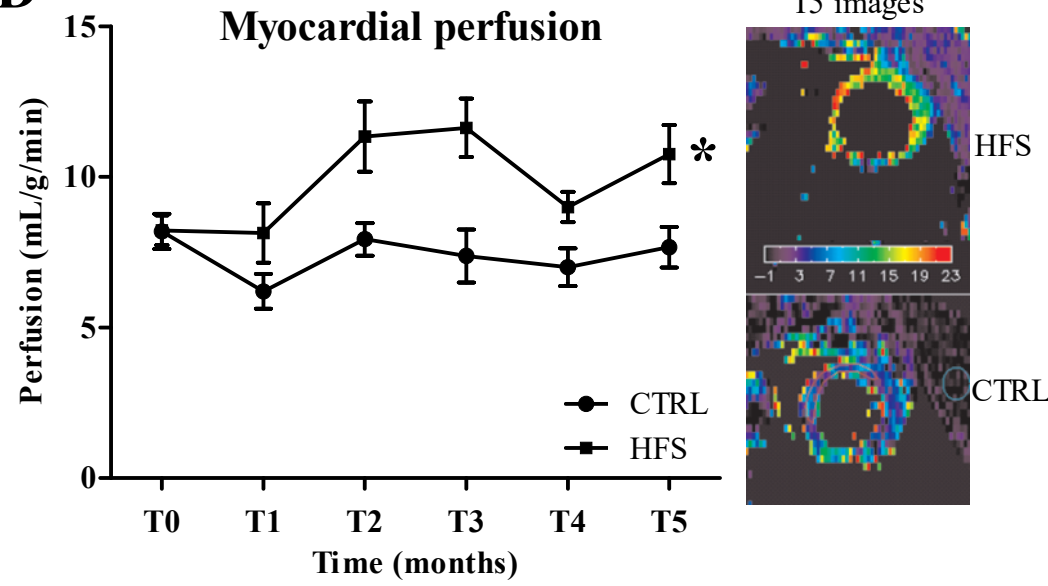


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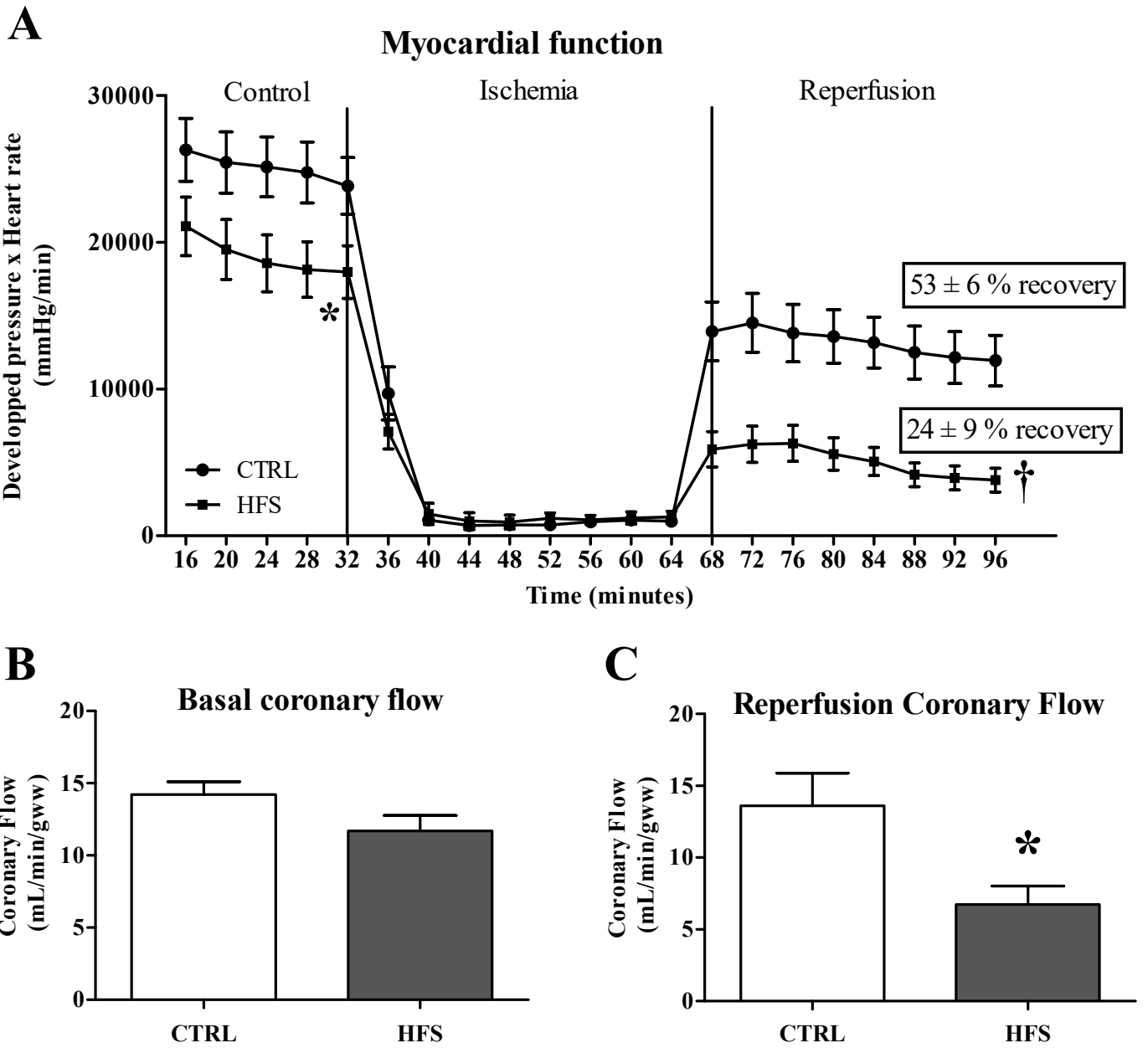
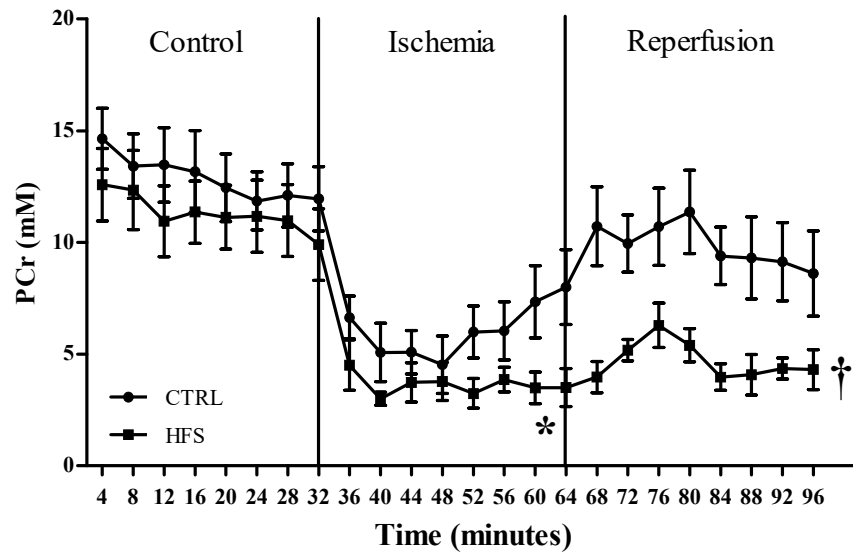
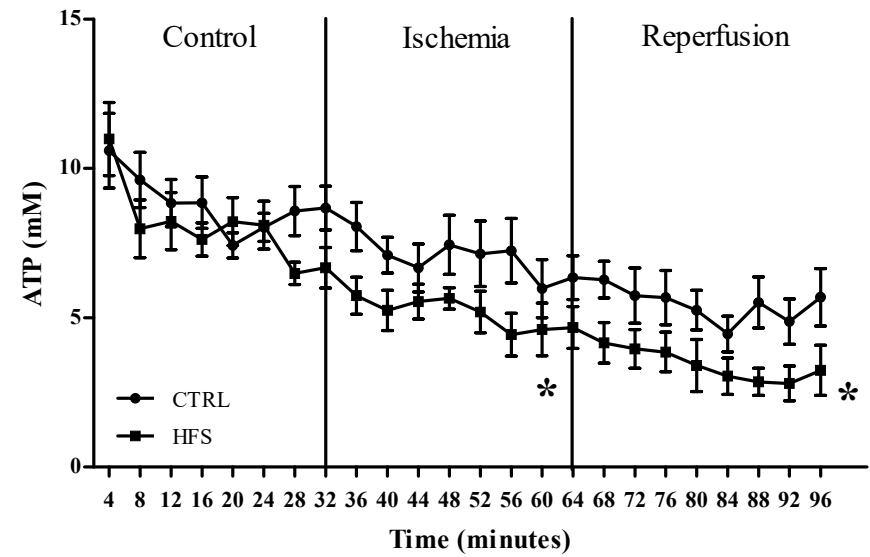


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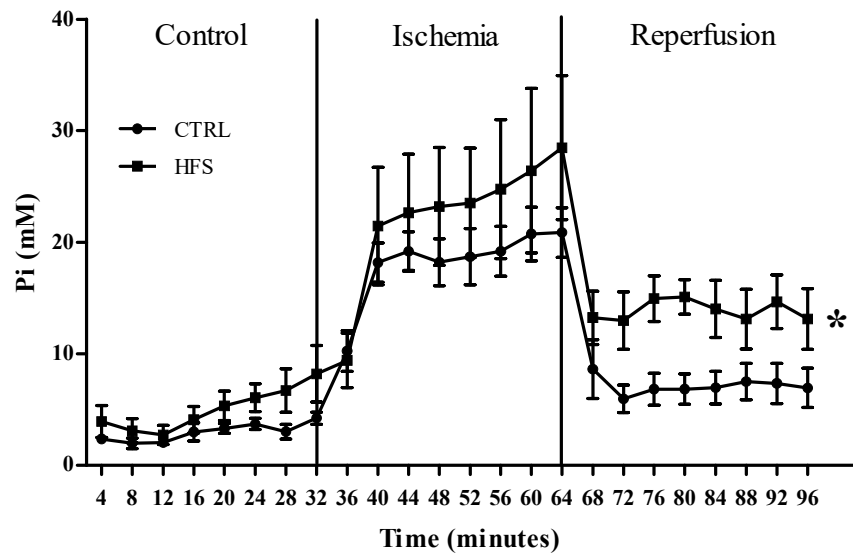
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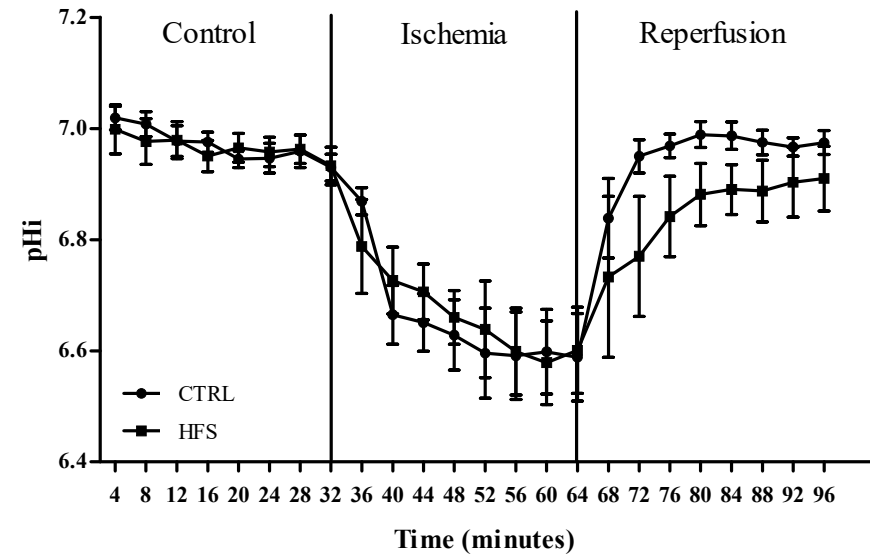
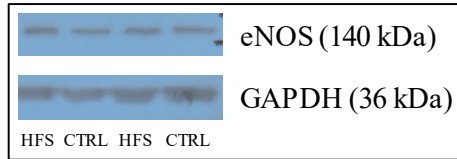
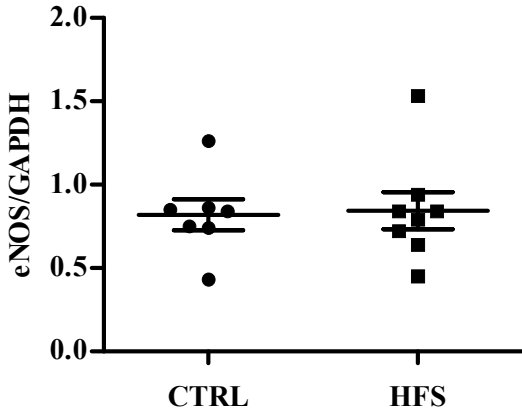
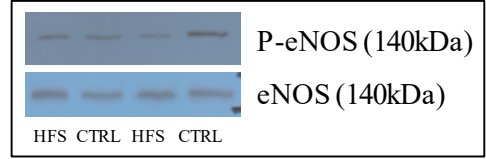
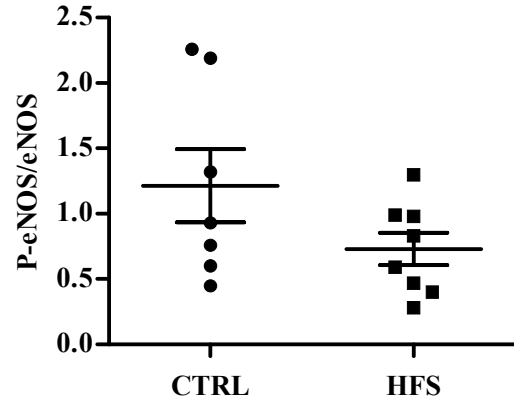


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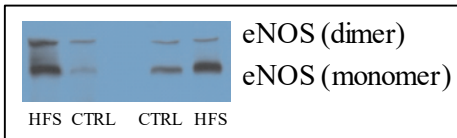
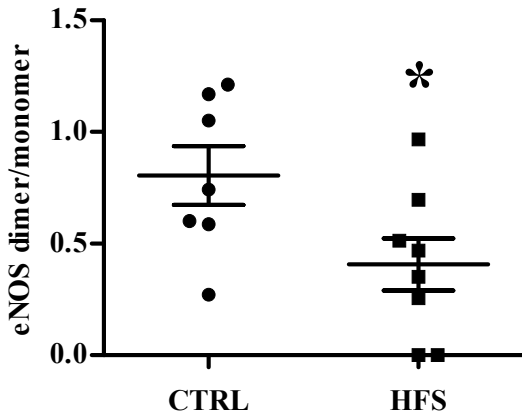
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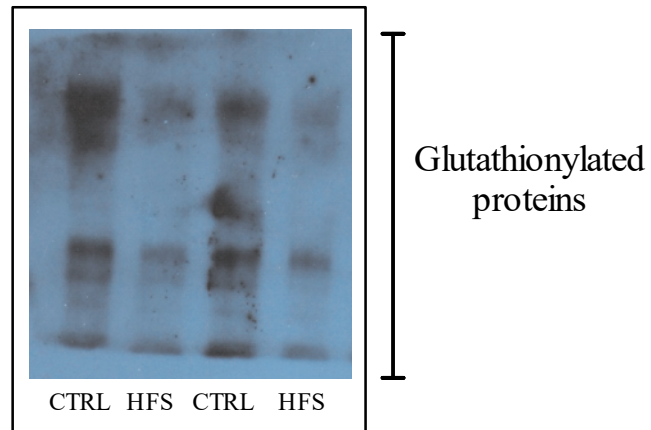
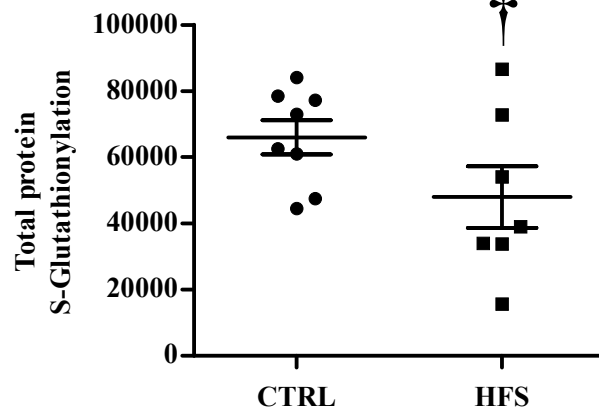
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D



E

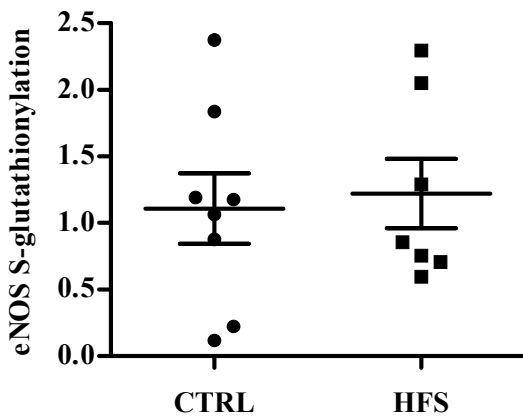
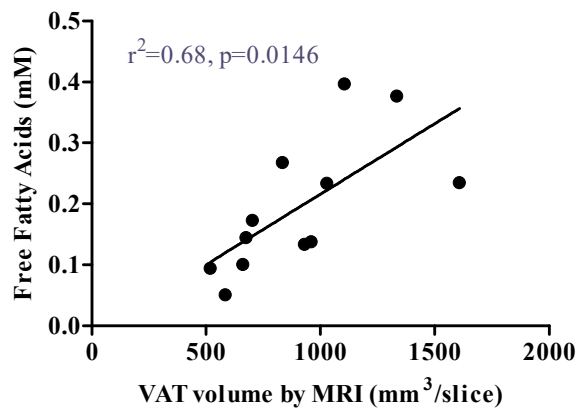
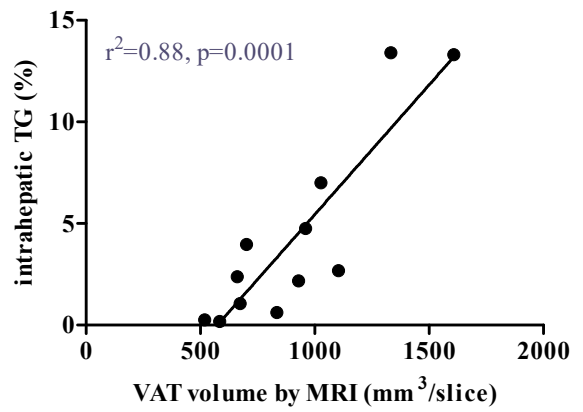


Figure 6

A



B



C

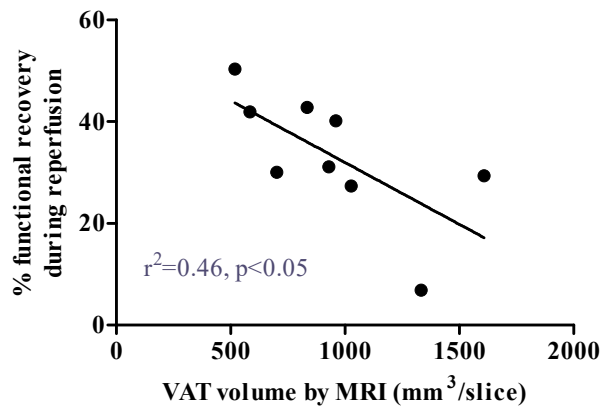


Figure 7

