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1 ***HNF4g* invalidation prevents diet-induced obesity via intestinal lipid malabsorption**

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26 fructose diet.

28 **Abstract**

29 Changes in dietary habits have occurred concomitantly with a rise of type 2 diabetes (T2D) and
30 obesity. Intestine is the first organ facing nutrient ingestion and has to adapt its metabolism with
31 these dietary changes. HNF-4 γ , a transcription factor member of the nuclear receptor
32 superfamily and mainly expressed in intestine has been suggested involved in susceptibility to
33 T2D. Our aim was to investigate the role of HNF-4 γ in metabolic disorders and related
34 mechanisms. *Hnf4g*^{-/-} mice were fed high-fat/high-fructose (HF-HF) diet for 6 weeks to induce
35 obesity and T2D. Glucose homeostasis, energy homeostasis in metabolic cages, body
36 composition and stool energy composition, as well as gene expression analysis in jejunum were
37 analyzed. Despite an absence of decrease in calorie intake, of increase in locomotor activity or
38 energy expenditure, *Hnf4g*^{-/-} mice fed HF-HF are protected against weight gain after 6 weeks
39 of HF-HF diet. We showed that *Hnf4g*^{-/-} mice fed HF-HF display an increase in fecal calorie
40 loss, mainly due to intestinal lipid malabsorption. Gene expression of lipid transporters, *Fatp4*
41 and *Scarb1* and of triglyceride-rich lipoprotein secretion proteins, *Mttp* and *ApoB* are decreased
42 in gut epithelium of *Hnf4g*^{-/-} mice fed HF-HF, showing the HNF-4 γ role in intestine lipid
43 absorption. Furthermore, plasma GLP-1 and jejunal GLP-1 content are increased in *Hnf4g*^{-/-}
44 mice fed HF-HF, which could contribute to the glucose intolerance protection. The loss of HNF-
45 4 γ leads to a protection against a diet-induced weight gain and to a deregulated glucose
46 homeostasis, associated with lipid malabsorption.

47

49 **Introduction**

50 HNF-4 belongs to the nuclear receptor superfamily and in mammals, two paralog genes encode
51 the HNF-4 α and HNF-4 γ forms. HNF-4 α is expressed in liver, kidney, pancreas and intestine
52 (Benoit et al., 2006). Numerous studies *in vivo* and *in vitro* have shown that HNF-4 α plays
53 pleiotropic roles in liver functions and is a central transcription factor at the crossroads between
54 epithelial morphogenesis and functions (Battle et al., 2006; Hwang-Verslues and Sladek, 2010;
55 Ribeiro et al., 2007). Intestinal mice *Hnf4a* gene invalidation induces impairment of intestinal
56 epithelium homeostasis, regeneration, cell architecture and fatty acid uptake (Cattin et al., 2009;
57 Frochot et al., 2012; Montenegro-Miranda et al., 2020; Saandi et al., 2013).

58 HNF-4 γ is expressed mainly in intestine and colon, in kidney and to a lesser extent in pancreas
59 (Bookout et al., 2006), being almost absent from liver (Plengvidhya et al., 1999; Taraviras et
60 al., 2000). HNF-4 γ is highly expressed during intestine specification (Li et al., 2009). Recently,
61 a novel variant of HNF-4 γ , designated HNF-4 γ 2 was found to promote transactivation capacity
62 and hepatic function of dedifferentiated hepatoma cells better than HNF-4 α (Sasaki et al.,
63 2018). Furthermore, an integrative multi-omics analysis in intestinal organoids highlighted
64 HNF-4 γ as a major driver of enterocyte differentiation (Lindeboom et al., 2018). It is
65 noteworthy that the physiological role of HNF-4 γ was much less studied than that of HNF-4 α
66 and there is a lack of information on HNF-4 γ . Using constitutive *Hnf4g* gene invalidation model
67 fed control diet, we demonstrated that loss of HNF-4 γ leads to an overproduction of GLP-1,
68 leading to an exaggerated glucose-induced insulin secretion that improves glucose tolerance of
69 *Hnf4g*^{-/-} mice through an increase in GLP-1 incretin effect and a trophic impact on pancreatic
70 β -cell mass (Baraille et al., 2015). HNF-4 γ loss impacts the abundance of β -cells but not on
71 their insulin secretory capacity and led to a resistance to streptozotocin, a β -cell cytotoxic drug
72 (Baraille et al., 2015). The role of HNF-4 γ deserves thus further attention in the susceptibility
73 to type 2 diabetes (T2D) using appropriate mouse model.

74 T2D is one of numerous co-morbidities associated with obesity as well as cardiovascular
75 diseases (Stahel et al., 2020). The duration and the amplitude of the post-prandial peak of
76 circulating triglyceride rich lipoproteins (TRL) from intestinal origin are risk of cardiovascular
77 diseases (Bansal et al., 2007; Duez et al., 2008; Hsieh et al., 2008; Nordestgaard et al., 2007).
78 Furthermore, changes of intestinal TRL secretion have been reported in the context of insulin
79 resistance or diabetes, in animal models (Haidari et al., 2002; Vine et al., 2007) and in humans
80 (Duez et al., 2006). There is evidence for an increased basal rate of intestine-specific
81 apolipoprotein (apo)B-48-containing lipoprotein secretion in insulin resistance and T2D (Adeli
82 and Lewis, 2008). Inversely, insulin reduces TRL and apoB-48 secretion (Levy et al., 1996). In
83 insulin resistant fructose-fed hamsters, *de novo* lipogenesis is enhanced (Haidari et al., 2002;
84 Lewis et al., 2005). Hyperinsulinemic insulin-resistant human subjects display increased
85 production rates of intestinal apoB48-containing lipoproteins (Duez et al., 2006), and in
86 individuals with type 2 diabetes, intestinal chylomicron production is resistant to insulin's acute
87 suppressive effects (Nogueira et al., 2012). The relationships between T2D and lipid
88 metabolism in the context of *hmf4g* invalidation deserve further investigation.

89 Changes in dietary habits, including increments in calorie and saturated fatty acid intakes, have
90 occurred concomitantly with a rise of T2D and obesity (Shikany and White, 2000). Intestine
91 has to adapt its metabolism to accommodate the increased lipid intake.

92 The intestine ensures the transport of alimentary fat, which is the most calorie-dense nutrient.
93 Enterocytes ensure the transfer of dietary lipids to the organism through complex processes
94 (Williams, 2008). Triglycerides (TG) are hydrolyzed mainly by pancreatic enzymes into fatty
95 acids and monoglycerides. The uptake of fatty acids occurs by passive diffusion and by a
96 saturable/ protein-mediated mechanism comprising the fatty acid translocase (CD36), the fatty
97 acid-binding protein from the plasma membrane (FABPpm), as well as the fatty acid transport
98 protein (FATP) family (Gimeno et al., 2003; Nordestgaard et al., 2007; Stahl et al., 1999). After

99 resynthesis within the endoplasmic reticulum membrane, TG are used to form chylomicrons,
100 the intestine-specific postprandial form of TRL, which will be secreted into the lymph and then
101 directed toward circulation. The assembly of one TRL results from the fusion between one
102 apoB molecule, which is necessary for their formation, and one independently formed TG
103 droplet (Davidson and Shelness, 2000). The microsomal TG transfer protein (MTP) has a
104 prominent role in chylomicron assembly, ensuring the lipidation-dependent stabilization of
105 apoB and the transfer of lipids to the TG droplet in the endoplasmic reticulum lumen (Iqbal and
106 Hussain, 2009). During the postprandial period, TG are also transiently stored in enterocytes,
107 as cytosolic lipid droplets surrounded by proteins such as ADRP, which can be subsequently
108 hydrolyzed to reenter the secretory pathway (Robertson et al., 2003). Thus perturbations of their
109 basal level of expression and/or their nutrient-dependent modulation should interfere with the
110 enterocyte function of dietary lipid absorption and would reveal functional role of HNF-4 γ in
111 intestinal absorption of lipids.

112 To test the hypothesis of a role of HNF-4 γ in metabolic disorders related to intestine, we
113 submitted the constitutive *Hnf4g* gene invalidation model to a high-fat/high-fructose (HF-HF)
114 diet for 6 weeks, in order to induce obesity and T2D.

115

116 **Materials and methods**

117 **Animals and treatments**

118 Total and constitutive *Hnf4g* gene invalidation was as previously described (Baraille et al.,
119 2015; Gerdin et al., 2006). Heterozygote *Hnf4g* knockout mice (*Hnf4g*^{+/-}) were obtained from
120 Deltagen (San Carlos CA, USA). Briefly, *Hnf4g* knockout mice were generated by homologous
121 recombination using ES cells derived from 129/OlaHsd mouse substrain. F1 mice were
122 generated by breeding chimeras carrying a disrupted *Hnf4g* gene with C57BL/6 females
123 resulting in F1 heterozygote offspring. *Hnf4g*^{+/-} mice on a C57BL/6J genetic background, were

124 mated to obtain *Hnf4g^{-/-}* mice on the same genetic background. *Hnf4g^{-/-}* male mice were compared
125 with C57Bl/6J wild-type male WT mice, matched in age and housed in the same room.
126 Mice were housed in groups and maintained on a 12-hour light-dark cycle with *ad libitum*
127 access to water and diet: chow diet (CD: 5% Kcal fat - reference A03/R03, Safe-Diets) or high
128 fat diet (60% Kcal fat - D12492, Research Diets) with 30% fructose (Sigma) in drinking water
129 (HF-HF). Diets detail composition are described in Table 1. Mice were 2 month-old when HF-
130 HF diet started for 6 weeks. Mice were euthanized by cervical dislocation. Experimental
131 procedures agreed with the French ethical guidelines for animal studies and were approved by
132 the Regional Animal Care and Use Ethic Committee Charles Darwin C2EA – 05, agreement
133 number (#4132 – 2016021710083817 v3).

134 **Glucose tolerance test**

135 Glucose tolerance tests were performed after 6 weeks of HF-HF or control diets. After overnight
136 fasting, mice received a 3.6g/kg glucose load for oral glucose tolerance tests (OGTT). Blood
137 glucose concentrations were measured with a glucometer (Accu-checkGo, Roche). Blood
138 samples (70µl at t0 and 10 min after glucose challenge) were collected from the tail into EDTA
139 pre-coated microvette (Sarstedt). Plasma insulin (Alpco) and total glucagon-like peptide-1
140 (GLP-1) (Millipore) were measured by ELISA.

141 **Plasma triglyceride levels after an olive oil bolus**

142 The plasma triglyceride levels after an olive oil bolus were measured after 6 weeks of HF-HF
143 or control diets. After overnight fasting, mice received a 200 microL olive oil load. Blood
144 samples were collected as described in paragraph 2.2 at 0, 30, 60, 90 and 120 min after the olive
145 oil challenge. Plasma triglyceride concentrations were measured with the kit Triglycerides FS
146 (DiaSys).

147 **Metabolic parameters, body composition and stool analysis**

148 After 4 weeks of HF-HF or control diets, mice were housed individually in metabolic cages
149 (Phenomaster, TSE Systems) 1 week for habituation and 1 week for measurement and were fed
150 *ad libitum* with control or HF-HF diet. Food and water intake as well as O₂ consumption, CO₂
151 production, respiratory quotient, whole energy expenditure were automated measured. The
152 tridimensional locomotor activity is measured by the spontaneous (voluntary) activity on the
153 cage surface (XY axes) and in height (XZ axes). A count is register every time an infrared beam
154 is broken in the horizontal plane or in the vertical plane. The locomotor activity was recorded
155 for 5 consecutive days during nights and days and the mean of records was calculated. The
156 tridimensional locomotor activity is expressed as the sum of the XY activity mean (day and
157 night) and of the XZ activity mean (day and night) as counts in 24h / mouse. The whole body
158 composition was analyzed with the Bruker's minispec Whole Body Composition Analyzer. This
159 analyzer for measurement of lean tissue, fat and fluid in living mice is based on Time Domain
160 (TD)-Nuclear magnetic resonance (NMR). Stools were daily collected and stored at -20°C.
161 After homogenization, total and, lipid and nitrogen energy contents were as-determined as
162 previously described in (Layec et al., 2013).

163 **Intestinal GLP-1 protein content**

164 Proximal jejunum, distal ileum, and whole colon were sliced into small pieces, homogenized
165 in ethanol/acid (100% ethanol/sterile water/12N HCl 74:25:1 v/v/v) solution (5 ml/g tissue) and
166 incubated overnight at 4°C. Homogenates were centrifuged and supernatants were collected for
167 total GLP-1 content measurement using ELISA kit (Millipore).

168 **Intestinal epithelial cell isolation and protein concentration measurement**

169 After flushing with PBS, jejunum was cut into small pieces and incubated 4h (4°C) in Cell
170 recovery solution (BD Biosciences) containing 2% protease inhibitors (Sigma). Epithelial cells
171 were filtered, centrifuged, and washed with PBS, to obtain epithelial cell suspension (Archer et
172 al., 2005). Proteins were extracted from an aliquot of epithelial cells with a lysis buffer (Tris

173 HCl 20 mM pH 7.4, NaCl 150 mM, EDTA 5 mM, Triton 1%, DOC 0.5 %, protease and
174 phosphatase inhibitors). Protein concentration was determined with the BCA protein assay kit
175 (Pierce).

176 **Triglyceride levels in epithelial cells**

177 Lipids were extracted from an aliquot of epithelial cells with five volumes of chloroform-
178 methanol (2:1 vol/vol), with vigorous shaking for 5 min. After centrifugation for 20 min at
179 1,000 g, the lower organic phase was collected and dried at 45°C overnight. The triglyceride
180 levels were measured with the TG PAP 150 kit (Biomérieux).

181 **RNA extraction and gene expression analysis**

182 Total RNA were isolated from jejunum epithelial cells with Trizol reagent (MRC). Reverse
183 transcription (RT) was performed with 5µg of RNA. Semi quantitative real-time PCR was
184 performed with SYBR green (Applied) in a Stratagene system. Primer sequences are reported
185 in Table 2.

186 **Statistical Tests**

187 Results are expressed as means ± SEM. Statistical analyses were performed using GraphPad
188 Prism (GraphPad Software, La Jolla, CA). Identified outliers with the method « ROUT Q=1% »
189 (Graphpad prism software) were removed. The significance was evaluated by 1-way ANOVA
190 or 2-way ANOVA and followed by four Tukey's multiple comparisons tests: WT vs *Hnf4g*^{-/-}
191 on CD, WT vs *Hnf4g*^{-/-} on HF-HF, WT CD vs WT HF-HF, *Hnf4g*^{-/-} CD vs *Hnf4g*^{-/-} HFHF. A *P*
192 value <0.05 was considered statistically significant.

193

194 **Results**

195

196 **Impact of *Hnf4g* gene invalidation on weight gain induced by a high-fat/high-fructose.**

197

198 Metabolic characteristics of *Hnf4g*^{-/-} mice were compared with those of WT mice, both groups
199 being fed *ad libitum* either with control diet or high-fat/high fructose (HF-HF) diet. The body
200 weight gain curves were comparable between WT and *Hnf4g*^{-/-} mice fed control diet. There was
201 no weight gain after 6 weeks (99% for WT vs 94% for *Hnf4g*^{-/-}) (Fig. 1A). As expected, 6 weeks
202 of HF-HF diet promoted weight gain by 35% in WT mice but surprisingly, by only 10% in
203 *Hnf4g*^{-/-} mice (Fig. 1A). We then performed a body composition analysis by NMR. On control
204 diet, *Hnf4g*^{-/-} mice showed a non-significant 1.5-fold higher fat mass than WT mice (Fig. 1B).
205 As expected, on HF-HF diet WT mice showed a 2.4-fold increase in fat mass whereas *Hnf4g*^{-/-}
206 mice only 1.5-fold (Fig. 1B). However, on HF-HF diet, the increase in fat mass was 1.6-fold
207 lower in *Hnf4g*^{-/-} mice than in WT mice (Fig. 1B). In parallel, lean mass of *Hnf4g*^{-/-} mice was
208 similar to that of WT mice regardless the diet (data not shown). Thus *Hnf4g* gene invalidation
209 led to a partial protection against diet-induced weight gain characterized by less fat-mass gain.
210 In order to explain such a resistance to weight gain, we analyzed food intake, locomotor activity
211 and energy expenditure.

212

213 **Impact of high-fat/high-fructose diet on *Hnf4g*^{-/-} mouse energy homeostasis**

214

215 We next analyzed the energy homeostasis of WT and *Hnf4g*^{-/-} mice fed control or HF-HF diets
216 in metabolic cages after 5 weeks of diet. We showed that total calorie intake (high-fat pellets
217 and fructose in the drinking water) normalized by mouse body weight was not affected by
218 *Hnf4g* gene invalidation regardless the diet (Fig. 1C). The XY and XZ axis locomotor activity
219 was next recorded. There was no significant difference between WT and *Hnf4g*^{-/-} mice for their
220 XYZ axis locomotor activity regardless the diet but HF-HF diet induced a 1.4-fold decrease in
221 the locomotor activity regardless the mouse genotype (Fig. 1D). The calculated energy
222 expenditure (normalized to mouse body weight) of *Hnf4g*^{-/-} mice was similar to that of WT

223 mice fed control diet (Fig. 1E). As expected, the energy expenditure of WT fed HF-HF diet is
224 10% decreased compared to WT mice fed control diet (Fig. 1E). However, the energy
225 expenditure of *Hnf4g*^{-/-} mice fed control diet is similar to that of fed HF-HF diet (Fig. 1E).
226 Thus, the WT mice weight gain, induced by HF-HF diet, is explained by an increase in fat mass,
227 a decrease in locomotor activity and in energy expenditure. However, the partial protection to
228 diet-induced weight gain of *Hnf4g*^{-/-} mice was not due to a decrease in calorie intake, an increase
229 in locomotor activity nor an energy expenditure. Then, we made the hypothesis that *Hnf4g* gene
230 invalidation leads to nutrient malabsorption revealed by HF-HF diet.

231

232 **Impact of *Hnf-4γ* gene invalidation on nutrient absorption**

233

234 For assessment of intestinal absorption total calories were measured in feces. We showed a 25%
235 increase in fecal calorie loss in *Hnf4g*^{-/-} mice fed HF-HF diet compared to WT mice (Fig. 2A).
236 More strikingly, this calorie loss is mainly due to a 4-fold increase of lipids in feces (Fig. 2B)
237 whereas the protein and carbohydrate amounts were similar regardless genotypes and diets
238 (*data not shown*). The higher calorie loss in *Hnf4g*^{-/-} mice could be responsible of for the
239 protection against weight gain induced by HF-HF diet. We made the hypothesis that the *Hnf4g*
240 gene invalidation leads to a lipid malabsorption.

241 We measured the triglyceride level in jejunal epithelial cells and showed that in *Hnf4g*^{-/-} mice
242 fed control diet, intra-epithelial triglyceride amount is increased 2-fold (not significant)
243 compared to WT mice. More strikingly, in WT mice fed HF-HF, the intra-epithelial
244 triglycerides are increased 10-fold compared to that of control diet, whereas in *Hnf4g*^{-/-} mice,
245 the 2.6-fold increase is not-significant (Fig. 2C).

246 We then analyzed the kinetic of plasma triglyceride concentrations after an olive oil bolus, as
247 reflect of enterocyte triglyceride secretion. The plasma triglyceride concentrations after an olive

248 oil bolus are similar in WT and *Hnf4g*^{-/-} mice fed control diet. As expected under HF-HF, the
249 concentration of plasma triglycerides in WT mice is increased 3- fold at 90min compared to
250 that of mice on control diet whereas the concentration of plasma triglycerides in *Hnf4g*^{-/-} mice
251 remains similar to that of on control diet (Fig. 2D).

252 The partial protection to diet-induced weight gain of *Hnf4g*^{-/-} mice could be due to an intestinal
253 lipid malabsorption at uptake step (showed by increased lipids in feces and decreased in jejunal
254 epithelial cells) and at secretion step (showed by plasma concentrations).

255

256 **Impact of high-fat/high-fructose diet on gene expression involved in lipid absorption in** 257 ***Hnf4g*^{-/-} mice gut epithelium**

258

259 We next analyzed gene expression of lipid membrane transporters in jejunum from *Hnf4g*^{-/-} and
260 WT mice fed control or HF-HF diet (Fig. 3A). The HF-HF diet increased the fatty acid
261 transporter gene expression *Fabpm* and *Cd36*, regardless the mouse genotype, *Hnf4g*^{-/-} or WT
262 (Fig. 3A, upper panels). The gene expression of the fatty acid transporter *Fatp4* was reduced
263 by 1.8-fold in *Hnf4g*^{-/-} mice compared to WT mice, fed control diet and by 1.3-fold on mice fed
264 HF-HF diet (Fig. 3A, lower left panel). The gene expression of the cholesterol transporter
265 *Scarb1* (encoding SR-B1) was reduced in *Hnf4g*^{-/-} mice compared to WT mice, regardless the
266 diet, CD or HF-HF. However the HF-HF diet increased the *Scarb1* gene expression whatever
267 the mouse genotype, *Hnf4g*^{-/-} or WT (Fig. 3A, lower right panel).

268 Then, we analyzed expression of genes involved in lipid storage (*Plin2*, encoding Perilipin 2 or
269 ADRP) and secretion (*Mttp* and *ApoB*) (Fig. 3B). The HF-HF diet induced an increased gene
270 expression of *Plin2* (2.4-fold) and of *Mttp* (1.8-fold) in WT mice but not in *Hnf4g*^{-/-} mice.
271 However, the expression of *Plin2*, *Mttp* and *apoB* is lowered in *Hnf4g*^{-/-} mice fed HF-HF diet
272 by 1.5-, 3.9- and 1.8-fold, respectively compared to WT mice fed HF-HF diet (Fig. 3B).

273 This decrease in gene expression of lipid transporters, lipid storage and secretion proteins could
274 explain in part the lipids fecal calorie loss suggesting a lipid malabsorption due to lipid transfer
275 failing toward blood circulation.

276 Note that gene expression of *Hnf4a* was 1.6- and 2.4-fold increase in *Hnf4g*^{-/-} mice than in WT
277 mice on control and HF-HF diets, respectively (Fig. 3C).

278

279 **Impact of high-fat/high-fructose diet on *Hnf4g*^{-/-} mice glucose homeostasis**

280

281 The lipid malabsorption that could protect *Hnf4g*^{-/-} mice against weight gain induced by an HF-
282 HF diet, could also allow a preferential use of glucose and thus protects from a deregulation of
283 glucose homeostasis. We challenged glucose tolerance by an OGTT and as expected, WT mice
284 fed HF-HF present a glucose intolerance compared to WT mice fed control diet (Fig. 4A). The
285 area under the curve (AUC) was 1.8- fold higher in WT mice fed HF-HF diet than in WT mice
286 fed control diet (Fig. 4B). We also confirmed as previously described in Baraille & al, that
287 *Hnf4g* gene invalidation led to improvement of glucose tolerance in mice fed control diet (Fig.
288 4A) (Baraille et al., 2015). Indeed, the AUC was 1.4- fold lower in *Hnf4g*^{-/-} mice fed control
289 diet than in WT mice (Fig. 4B). This feature was further exacerbated in *Hnf4g*^{-/-} mice fed HF-
290 HF diet compared to WT mice (Fig. 4A) since AUC was 1.8-fold lower in *Hnf4g*^{-/-} HF-HF fed
291 mice than in WT mice (Fig. 4A). Importantly, AUC of oral glucose tolerance test of *Hnf4g*^{-/-}
292 HF-HF fed mice was similar to that of WT control diet fed mice (Fig. 4B). As expected, fasting
293 blood glucose and insulin were increased 1.18- and 2.74- fold respectively, in WT mice fed HF-
294 HF compared to control diet (Fig. 4C, D) whereas fasting blood glucose and insulin remained
295 unaffected in *Hnf4g*^{-/-} mice fed HF-HF compared to control diet (Fig. 4C and D). Furthermore,
296 the HF-HF diet induced a 3.3-fold increase of HOMA-IR in WT mice without significant effect

297 on HOMA-IR in *Hnf4g*^{-/-} mice (Fig. 4E). These results indicate that *Hnf4g* gene invalidation
298 protects mice against glucose intolerance induced by the HF-HF diet.

299

300 **Impact of high-fat/high-fructose diet on *Hnf4g*^{-/-} mice GLP-1 intestinal homeostasis**

301

302 We then measured the total plasma GLP-1 concentration 10 min after a glucose challenge. As
303 previously described in Baraille & al, we confirmed that plasma total GLP-1 concentration was
304 3.3-fold increase in *Hnf4g*^{-/-} than in WT mice fed control diet, (Fig. 5A) (Baraille et al., 2015).
305 Although the HF-HF diet induced a 3-fold increase in plasma total GLP-1 concentration in WT
306 mice and a 1.4-fold non-significant increase in *Hnf4g*^{-/-} mice, the plasma total GLP-1
307 concentration in *Hnf4g*^{-/-} mice fed HF-HF remained 1.53 fold higher than in WT mice fed HF-
308 HF (Fig. 5A). We next measured GLP-1 content in mouse jejunum and we showed that GLP-1
309 content in jejunum of *Hnf4g*^{-/-} mice is 2- and 1.7-fold increase in control and HF-HF diet,
310 respectively (Fig. 5B). In jejunum, the level of *Gcg* mRNA, encoding proglucagon, is increased
311 by HF-HF diet 1.95- and 1.6-fold in WT and *Hnf4g*^{-/-} mice, respectively (Fig. 5C). These results
312 showed that the *Hnf4g* gene invalidation leads to an increase in GLP-1 jejunum content that
313 could explain the increase in plasma GLP-1 in response to glucose challenge in mice fed control
314 and HF-HF diet.

315

316 **Discussion**

317

318 Our results demonstrate that the gene invalidation of the nuclear receptor HNF-4 γ induces a
319 protection against the weight gain induced by a high-fat/high-fructose diet. The weight gain
320 protection is mainly due to intestinal lipid malabsorption leading to a calorie loss in feces.
321 *Hnf4g*^{-/-} mice were also protected against glucose intolerance induced by the HF-HF diet. An

322 increase in jejunal GLP-1 content could participate to this protection *via* a possible incretin
323 effect.

324 Mice invalidated for *Hnf4g* gene were reported to present a lower food intake, associated with
325 lower night energy expenditure, than wild type mice (Gerdin et al., 2006). Our previous results
326 did not show difference in food intake in *Hnf4g*^{-/-} and *WT* mice fed control diet but a strong
327 improvement of glucose tolerance of *Hnf4g*^{-/-} mice (Baraille et al., 2015), suggesting that HNF-
328 4 γ could be involved in susceptibility to type 2 diabetes. In order to generate rapid glucose
329 intolerance and insulin resistance, we challenged mice with a high fat and high fructose diet.
330 This long term diet (16 weeks) is widely used to induce NASH but a glucose intolerance along
331 with insulin resistance appear earlier after 4 weeks of diet along with weight gain (Charlton et
332 al., 2011; Dissard et al., 2013; Tsuchiya et al., 2013). As expected we observed in *WT* mice fed
333 HF-HF a weight gain from one week and a glucose intolerance at 6 weeks that are mainly due
334 to an increase in fat mass and a decrease in locomotor activity and in energy expenditure. One
335 of the most striking effect of *Hnf4g* gene invalidation is a weight gain and a glucose intolerance
336 protection against HF-HF diet. We could expect an increase in energy expenditure or in
337 locomotor activity to explain this protection. However, energy homeostasis analysis in
338 metabolic cages does not show differences between *WT* and *Hnf4g*^{-/-} mice fed HF-HF. We
339 hypothesized that *Hnf4g* gene invalidation induces a loss of ingested calories and we showed
340 increased calories in feces. We made the hypothesis of a lipid failing absorption revealed by
341 the HF-HF diet challenge.

342 Enterocytes ensure the absorption of dietary lipids to the organism through complex processes
343 that can be summarized into three major steps: uptake, storage and/or secretion (Williams,
344 2008). The increase lipid content in feces, the decrease in intra-epithelium triglyceride content
345 and the decrease of plasma triglyceride concentration after an olive oil bolus indicate that *Hnf4g*
346 gene invalidation in HF-HF diet fed mice leads to a lipid malabsorption at the three major steps

347 uptake, storage and secretion. We expected a down-regulation of lipid transporters gene
348 expression in *Hnf4g*^{-/-} mice fed HF-HF. Although HF-HF diet induces an increase in gene
349 expression of fatty acid transporters, such as the *Cd36* and *Fabpm* in jejunum of both *Hnf4g*^{-/-}
350 and *WT* mice, *Hnf4g* gene invalidation impacts the gene expression of *Fatp4* and *Scarb1*
351 regardless the diet.

352 We could hypothesize that gene expression of lipid transporters *Cd36* and *Fabpm* is up-
353 regulated to compensate a lipid malabsorption in *Hnf4g*^{-/-} mice fed HF-HF. Although there is a
354 gene overexpression of these transporters, we cannot exclude a translational down regulation
355 of these transporters or a membrane mislocalization, both could participate in lipid
356 malabsorption.

357 Although the role of FATP4 as a transporter for fatty acid uptake remains unclear, it has been
358 shown that intestinal FATP4 is exclusively found intracellular instead of on the plasma
359 membrane. FATP4 plays a role in fatty acid uptake through its intrinsic intracellular enzymatic
360 activity, through a process known as “vectorial acylation”, i.e., the obligatory step of acyl-CoA
361 formation for fatty acid transport across the plasma membrane, from intestinal lumen toward
362 enterocytes (Digel et al., 2011; Milger et al., 2006). Thereby, a 25% decrease in *Fatp4*
363 expression could participate to lipid malabsorption in *Hnf4g*^{-/-} mice fed HF-HF.

364 The scavenger receptor is known for its function as a cholesterol transporter SR-B1, however
365 its role in cholesterol and lipid metabolism remains unclear in intestine. It has been shown *in*
366 *vitro* that addition of lipid micelles triggers SR-B1 lipid sensing and a signaling cascade leading
367 to ApoB translocation from the apical membrane to the secretory basolateral domains (Beaslas
368 et al., 2009; Saddar et al., 2013). Some reports show that SR-B1 plays an important role in
369 intestinal chylomicron production (Bura et al., 2013; Hayashi et al., 2011). Thus, through an
370 indirect effect, the decrease in *Scarb1* gene expression prevents the lipid transfer in *Hnf4g*^{-/-}
371 mice fed HF-HF.

372 The chylomicron production loss is amplified by the decrease in gene expression of *Mttp* and
373 *ApoB* that are necessary to the production of the lipoprotein particle.

374 A defect in biliary acid metabolism could also account for lipid malabsorption. When the
375 entero-hepatic biliary acid cycle leading to micelle formation is deficient, dietary lipids are not
376 properly embedded with micelles, precluding adequate absorption by enterocytes (Nordskog et
377 al., 2001). A down regulation of pancreatic lipase expression, enzyme responsible of dietary
378 lipid hydrolysis before micelle embedding, could also be questioned (Alkaade and Vareedayah,
379 2017).

380 In our previous work, we demonstrated that HNF-4 γ plays a critical role in glucose homeostasis
381 and that HNF-4 γ loss leads to an improvement of glucose tolerance through a rise of GLP-1
382 incretin effect (Baraille et al., 2015). Here we show that the glucose tolerance improvement is
383 maintained in *Hnf4g*^{-/-} mice despite HF-HF feeding, in such a way that *Hnf4g* gene invalidation
384 leads to a protection against the dietary glucose intolerance. The HOMA-IR was significantly
385 increased only in WT mice fed HF-HF diet, indicating a possible protection of *Hnf4g*
386 invalidation against insulin resistance too. Accordingly, the jejunum GLP-1 content is increased
387 in *Hnf4g*^{-/-} mice fed HF-HF. The expression of *Gcg* gene, encoding GLP-1 in intestine, are
388 increased in *Hnf4g*^{-/-} mice fed control diet and are maintained in *Hnf4g*^{-/-} mice fed HF-HF. The
389 loss of HNF-4 γ improves the GLP-1 producing cell homeostasis, leading to a protection against
390 a diet-induced deregulated glucose homeostasis.

391 The effects of *Hnf4g* gene invalidation are of two types. The first is an invalidation effect seen
392 regardless of diet, CD or HF-HF. This is the case with glucose tolerance and plasma and
393 intraepithelial concentrations of GLP-1 as well as expression of *Hnf4a*. The second is an effect
394 of invalidation revealed by the HF-HF diet. This is the case with the amount of calories and
395 lipids found in the feces as well as plasma triglyceride concentrations, thus revealing lipid
396 malabsorption. In addition, the expression of genes involved in the uptake, storage and secretion

397 of lipids such as *Fatp4*, *Scarb1*, *Plin2*, *Mttp*, and *ApoB* is also impacted by the invalidation of
398 *Hnf4g* with the HF-HF diet. It should be noted that the expression of the *Fatp4* transporter is
399 also impacted under the CD diet.

400 In absence of HNF-4 γ , there is an overexpression of HNF-4 α regardless the diet. It is difficult
401 to assert that the observed effects were the direct consequence of HNF-4 γ loss or resulted
402 indirectly from HNF-4 α increment in *Hnf4g*^{-/-} mouse intestine. However, we could
403 hypothesized that HNF-4 α is able to compensate the loss of HNF-4 γ for intestinal lipid
404 absorption under control diet but that is overtaken under HF-HF diet. HNF-4 α and HNF-4 γ are
405 encoded by two different genes but share high homology in their DNA and ligand binding
406 domains (Drewes et al., 1996; Taraviras et al., 2000). Indeed, it has been recently shown that
407 HNF-4 α and HNF-4 γ share almost all their binding sites on chromatin (Chen et al., 2019) and
408 regulate the expression of genes involved in fatty-acid oxydation (Chen et al., 2020). However,
409 these two transcription factors have a different spatial distribution along the crypt-villus axis,
410 HNF-4 α being expressed along the crypt to villus axis and HNF-4 γ being restricted to the villus
411 (Sauvaget et al., 2002). Furthermore, 9 isoforms of HNF-4 α raised from differential splicing
412 and from 2 different promoters have been described (Torres-Padilla et al., 2001). These
413 isoforms can have opposite roles in colitis and colitis associated colon cancer (Chellappa et al.,
414 2016). These observations show that the two forms of HNF-4, HNF-4 α and HNF-4 γ play some
415 specific roles but we cannot exclude redundancy for others roles, the balance between the
416 expression of HNF-4 α and HNF-4 γ being finely regulated to maintain gut homeostasis.

417 In recent report, it has been shown that *Hnf4a* and *Hnf4g* are redundantly required to drive
418 intestinal differentiation (Chen et al., 2019). Genes that exhibit a direct binding of HNF-4 α or
419 γ are involved in lipid metabolim (Chen et al., 2019). However, an indirect regulation is also
420 possible since it HNF-4 α and γ function maintain also active enhancer chromatin (Chen et al.,

421 2019). However, the transcriptome analysis reveals that *Hnf4g* invalidation alters specifically
422 the expression of 89 genes in intestine. Thus we cannot exclude a direct binding of HNF-4g on
423 genes involved in lipid metabolism such as *Fatp4* and *Scarb1*, the *Hnf4a* expression being
424 repressed by the antisense transcript of *Hnf4a* previously described (Lindeboom et al., 2018).
425 In our previous article, we showed that proglucagon (*Gcg*) gene transcription was not directly
426 activated or repressed by HNF-4a or HNF-4g but rather the expression of *Foxa1*, *Foxa2*, and
427 *Isl1* was enhanced, suggesting that modifications of the transcription factor network favored
428 the GLP-1-secreting cell lineage (Baraille et al., 2015).

429 In conclusion, we demonstrated that loss of HNF-4 γ in mice prevents obesity and glucose
430 intolerance induced by HF-HF diet. The protection against metabolic deleterious effects of HF-
431 HF diet could be due to intestinal lipid malabsorption and glucose homeostasis improvement.
432 Interestingly in human, *HNF4G* was identified as an obesity-associated locus in a meta-analysis
433 of GWAS study (Berndt et al., 2013) and could also be associated with pediatric obesity
434 (Selvanayagam et al., 2018).

435

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440

441 **Author contribution statement**

442 S.A., C.O., E.G.-I., L.L.G., N.K., H.S. and A.R. designed experiments, acquired and analyzed
443 data. S.A., H.S., F.A., K.C., P.S., A.L. and A.R. contributed to data interpretation and to the
444 discussion. S.A., H.S., P.S., A.L. and A.R. wrote the manuscript. P.S. and A.R. reviewed and
445 edited manuscript. A.R. is the guarantor of this work and, as such, had full access to all data in

446 the study and takes responsibility for the integrity of the data and the accuracy of the data
447 analysis.

448

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454

455 **Declaration of interest**

456 No potential conflicts of interest relevant to this article were reported.

457

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460

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- 643

645 **Figure legends**

646 **Figure 1: Impact of high fat-high fructose diet on energy homeostasis in *Hnf4g*^{-/-} and WT**
647 **mice. (A)** Body weight of mice fed control diet or HF-HF diet during 6 weeks. Results were
648 from 5 independent experiments with $12 \leq n \leq 36$ for each condition. **(B)** Body fat mass
649 evaluated by TD-MNR at 5 weeks of diet. **(C)** Food intake is the sum of pellet quantity and
650 drinking water volume recorded for 5 consecutive days in metabolic cages and is expressed in
651 kcal/day/Kg mouse. Control diet = 3.2kcal/g; High-fat diet = 5.24kcal/g; Fructose 30% =
652 1.2kcal/mL. **(D)** The locomotor activity is the sum of XY and XZ mean locomotor activity from
653 5 consecutive nights and days. **(E)** The energy expenditure, measured by indirect calorimetry,
654 is the mean of the energy expenditure from 5 consecutive nights and days. Results (B-E) were
655 from 2 independent experiments with n= 5 to 6 mice per group. **** $p < 0.0001$; ** $p < 0.01$;
656 * $p < 0.05$; ns not significant.

657

658 **Figure 2: Impact of high fat-high fructose diet on intestinal nutrient absorption in *Hnf4g*^{-/-}**
659 **and WT mice. (A)** Total fecal calories were determined by bomb calorimetry in daily collected
660 feces from 3 to 5 consecutive days. Results are the mean of 4 independent measures during 2
661 experiments (n = 5 or 6 animals in each condition). **(B)** The lipid content into feces from *Hnf4g*^{-/-}
662 and WT mice were measured Results are the mean of 3 independent measures during 2
663 experiments (n = 5 or 6 animals in each condition). **(C)** The amount of triglycerides stored in
664 epithelial cells were quantified in isolated epithelial cells. **(D)** Plasma triglyceride
665 concentrations were measured at 30, 60, 90 and 120 min after a 200 microL olive oil bolus (T0
666 is indicated by an arrow). n= 4 or 5 animals per condition. *** $p < 0.001$; ** $p < 0.01$; * $p <$
667 0.05; ns not significant.

668

669 **Figure 3: Impact of high fat-high fructose diet on jejunal expression of genes involved in**
670 **lipid uptake, storage and secretion in *Hnf4g*^{-/-} and WT mice.** Quantitative RT-PCRs for gene
671 expression of (A) fatty acid and cholesterol membrane transporters, (B) genes involved in
672 storage and secretion of chylomicrons, (C) *Hnf4a* in jejunum of *Hnf4g*^{-/-} and WT mice fed
673 control diet or HF-HF diet. The mRNA levels were normalized by cyclophilin mRNA level.
674 Results were mean of 2 to 6 independent experiments with n= 4 to 12 mice per group. **** p
675 < 0.0001 ; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns not significant.

676 **Figure 4: Impact of high fat-high fructose diet on the glucose homeostasis in *Hnf4g*^{-/-} and**
677 **WT mice.** (A) Oral glucose tolerance test (OGTT, 3.6g glucose/kg) after 15h fasting. (B) Area
678 under the curve of the OGTT. (C) Fasted blood glucose. Results (A-C) were mean of 6
679 independent experiments with n= 5 to 7 mice per group. (D) Fasted blood insulin. (E) The
680 HOMA-IR was calculated from blood glucose and insulin values in (C) and (D) as follow:
681 [fasted blood glucose (mg/dL)] x [fasted blood insulin (mU/L)] / 405. Results (D-E) were mean
682 of 3 independent experiments with n= 3 to 10 mice per group. *** $p < 0.001$; ** $p < 0.01$; * p
683 < 0.05 ; ns not significant.

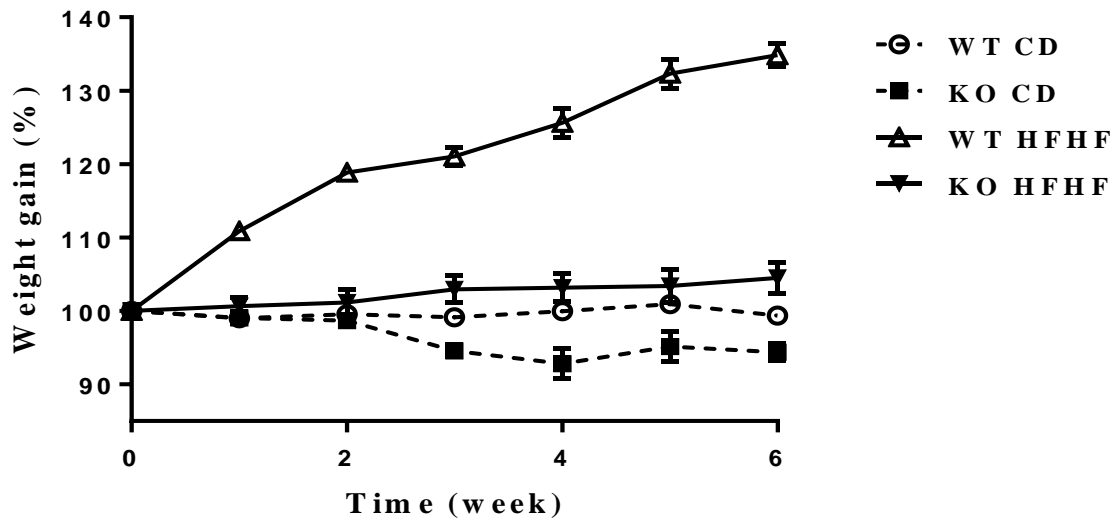
684

685 **Figure 5: Impact of high fat-high fructose diet on the intestinal GLP-1 homeostasis in**
686 ***Hnf4g*^{-/-} and WT mice.** (A) Total plasma total GLP-1 10 min after glucose bolus. Results were
687 mean of 4 independent experiments. (B) Total GLP-1 content in jejunum. Results were mean
688 of 2 independent experiments. (C) Quantitative RT-PCRs for *Gcg* gene expression. The mRNA
689 levels were normalized by cyclophilin mRNA level. Results were mean of 4 independent
690 experiments. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns not significant

691

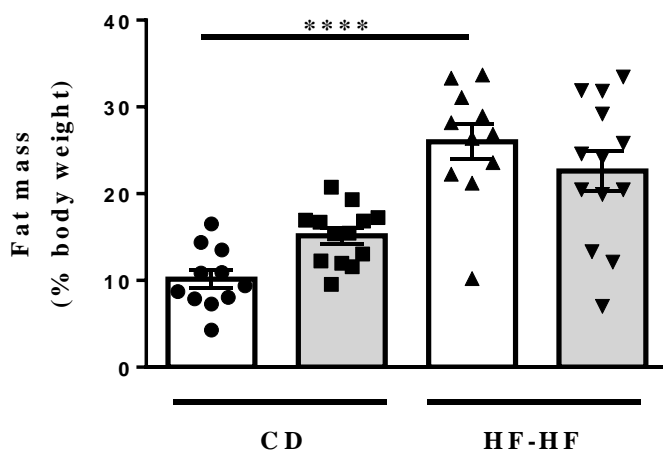
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A

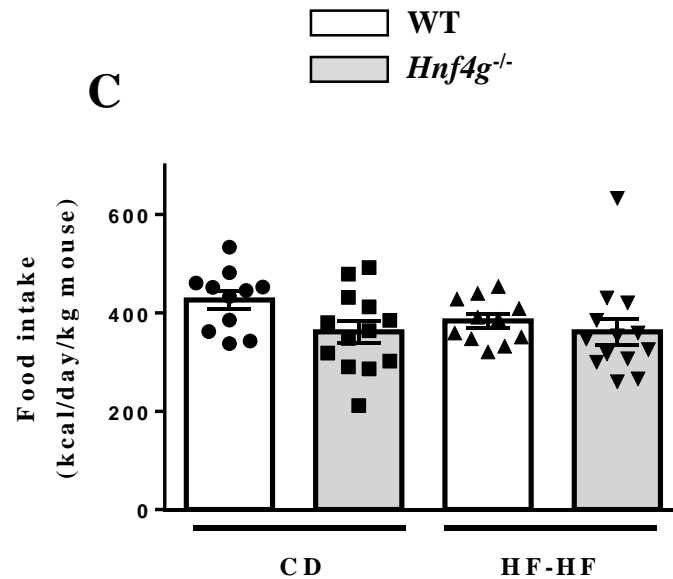


| multiple comparison test | 1 week | 2 week | 3 week | 4 week | 5 week | 6 week |
|--------------------------|--------|--------|--------|--------|--------|--------|
| WT CD vs KO CD | ns | ns | ns | ns | * | ns |
| WT HFHF vs KO HFHF | ns | ** | ** | ** | *** | **** |
| WT CD vs WT HF-HF | ns | ** | *** | *** | *** | **** |
| KO CD vs KO HF-HF | ns | ns | ns | ns | ns | ns |

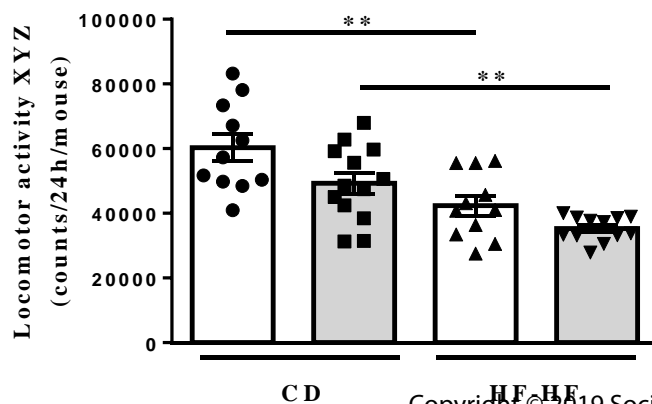
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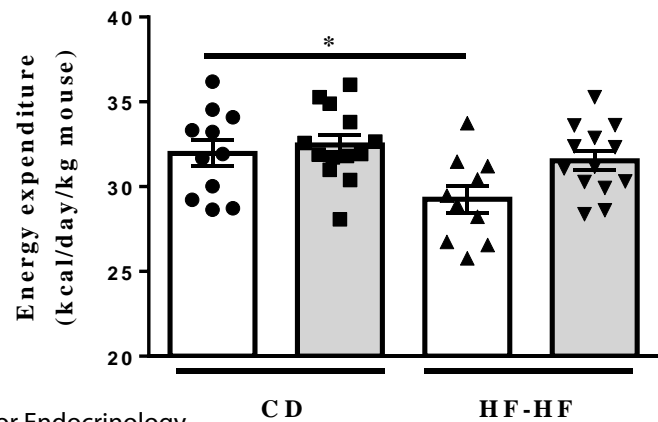
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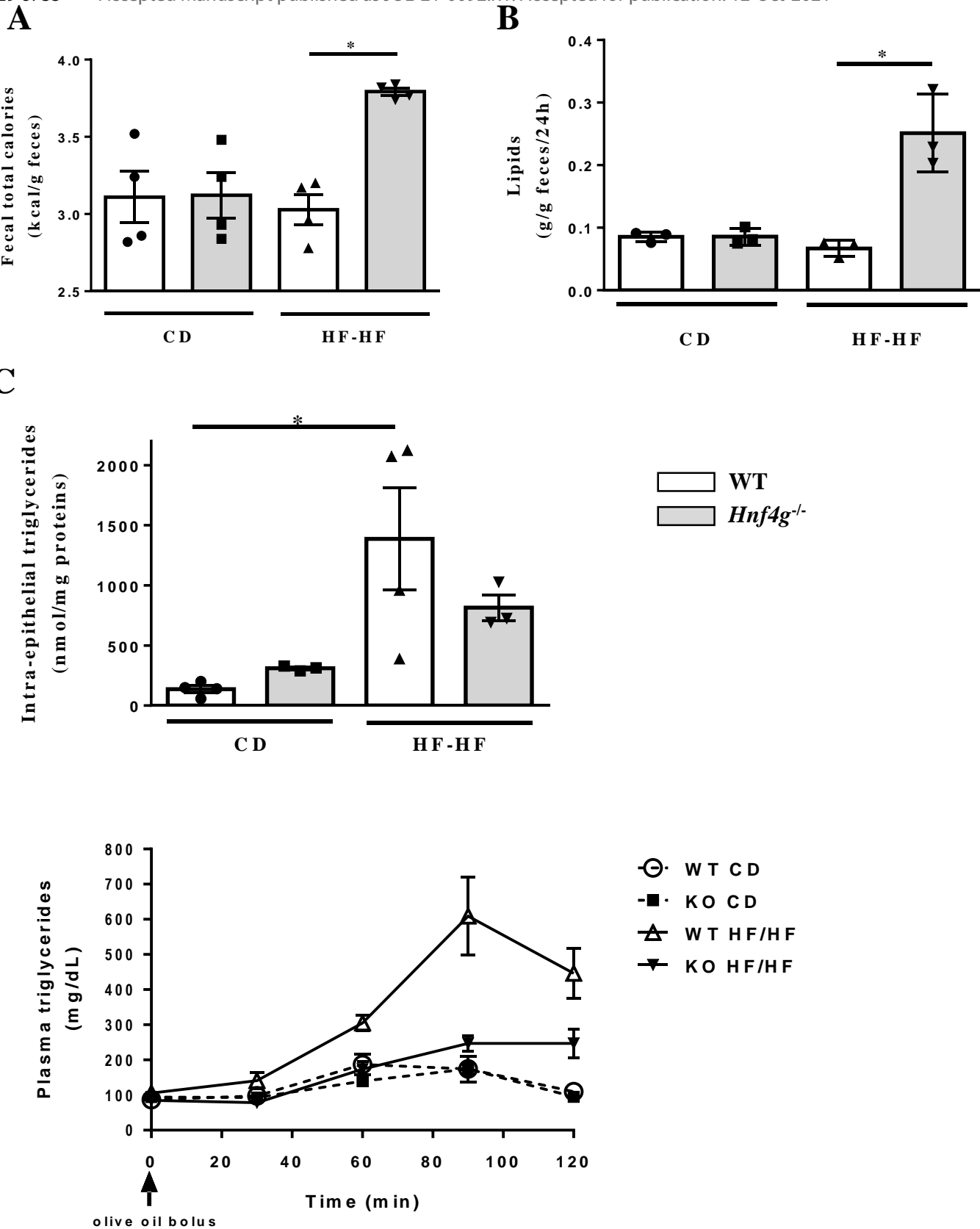


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E

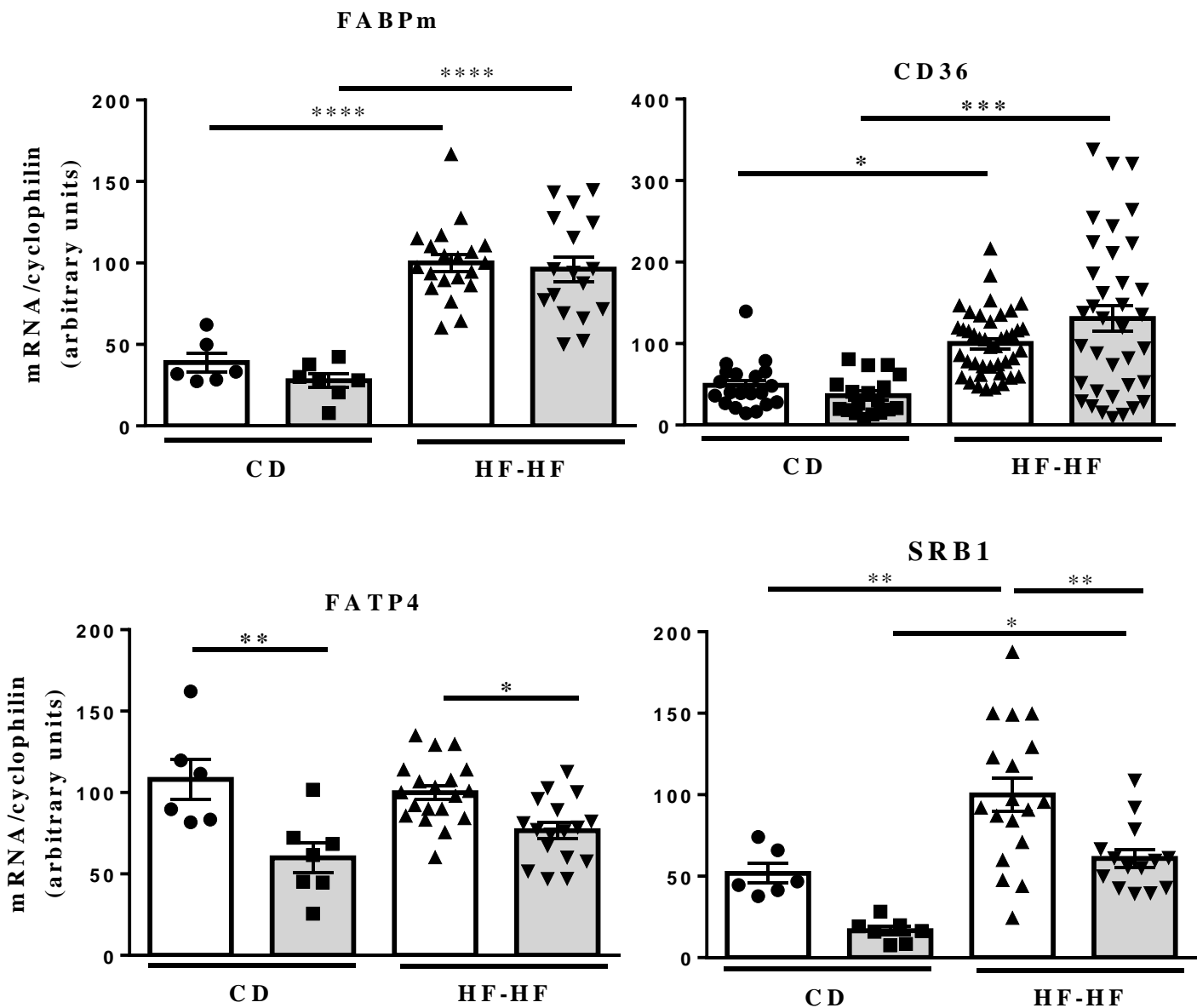


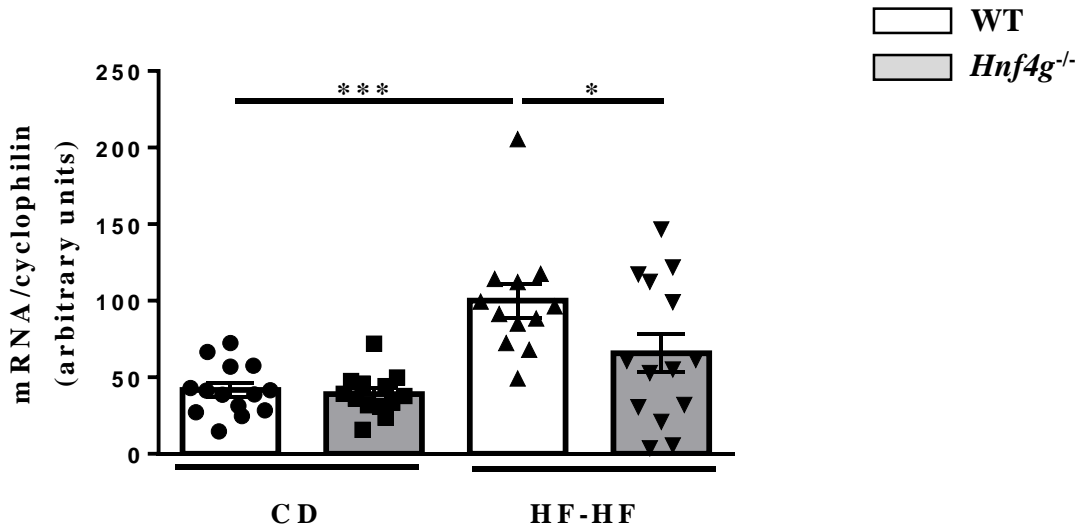
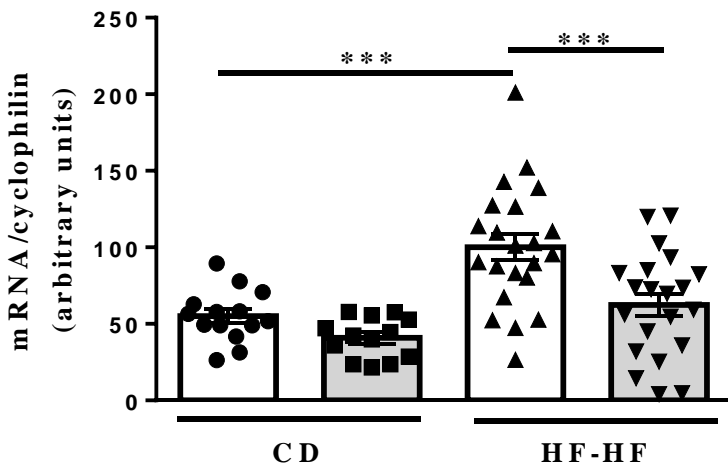
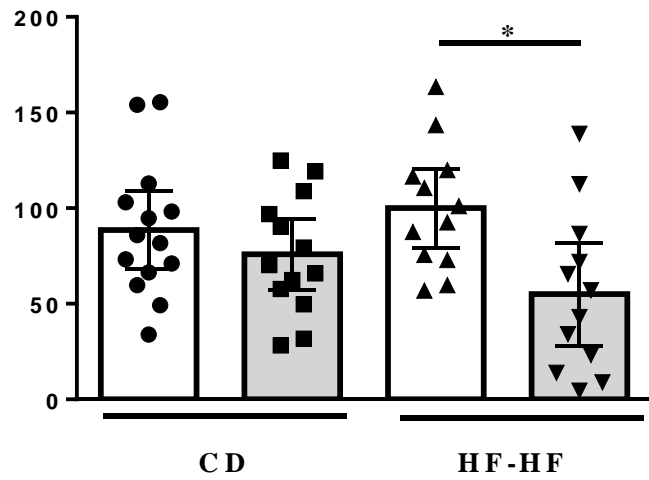
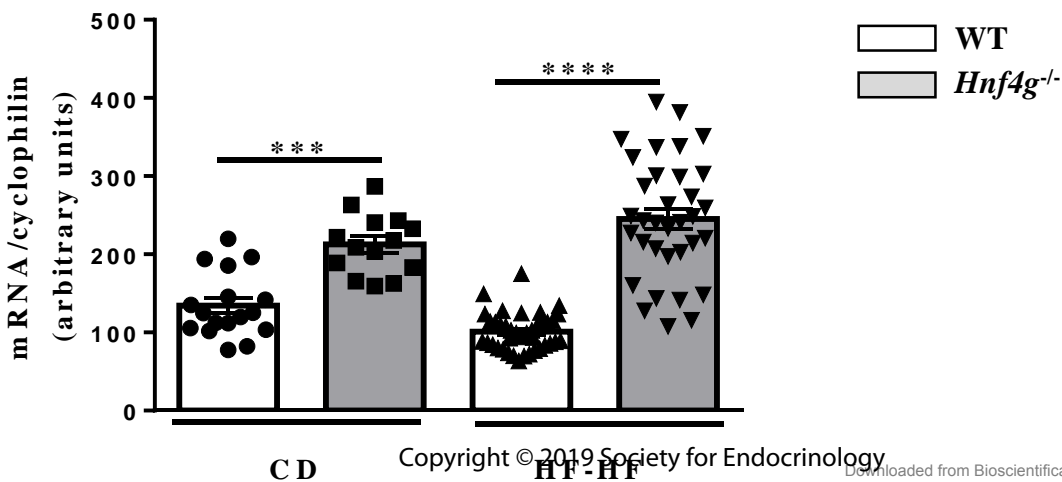


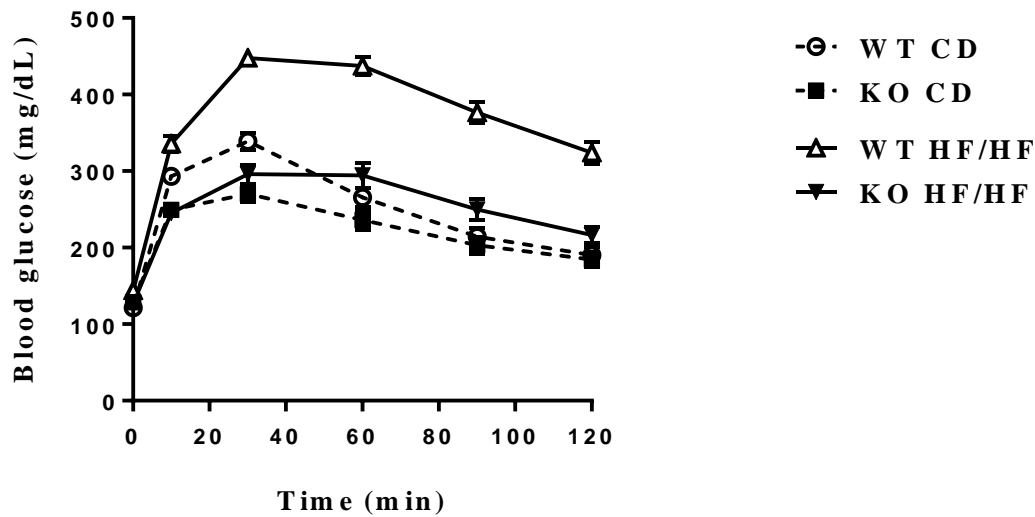
| multiple comparison test | 30 min | 60 min | 90 min | 120 min |
|--------------------------|--------|--------|--------|---------|
| WT CD vs KO CD | ns | ns | ns | ns |
| WT HFHF vs KO HFHF | ns | ns | **** | ** |
| WT CD vs WT HF-HF | ns | ns | **** | **** |
| KO CD vs KO HF-HF | ns | ns | ns | * |

A

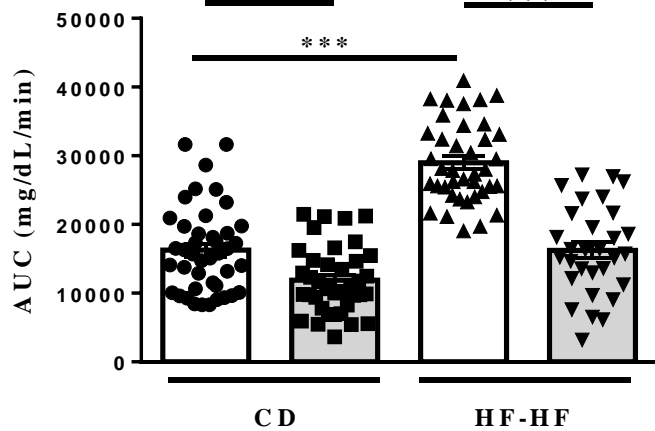
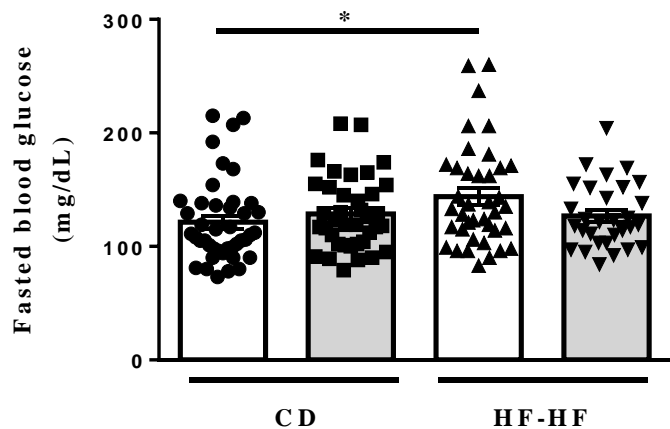
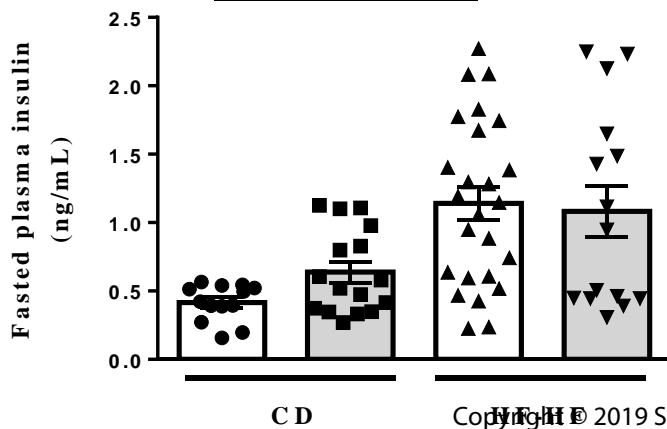
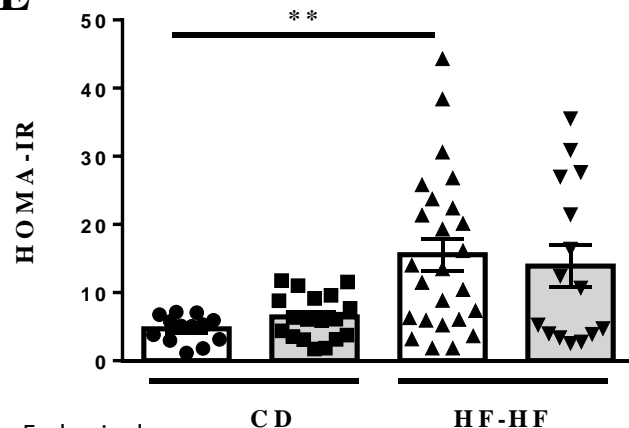
□ WT
 ■ *Hnf4g*^{-/-}



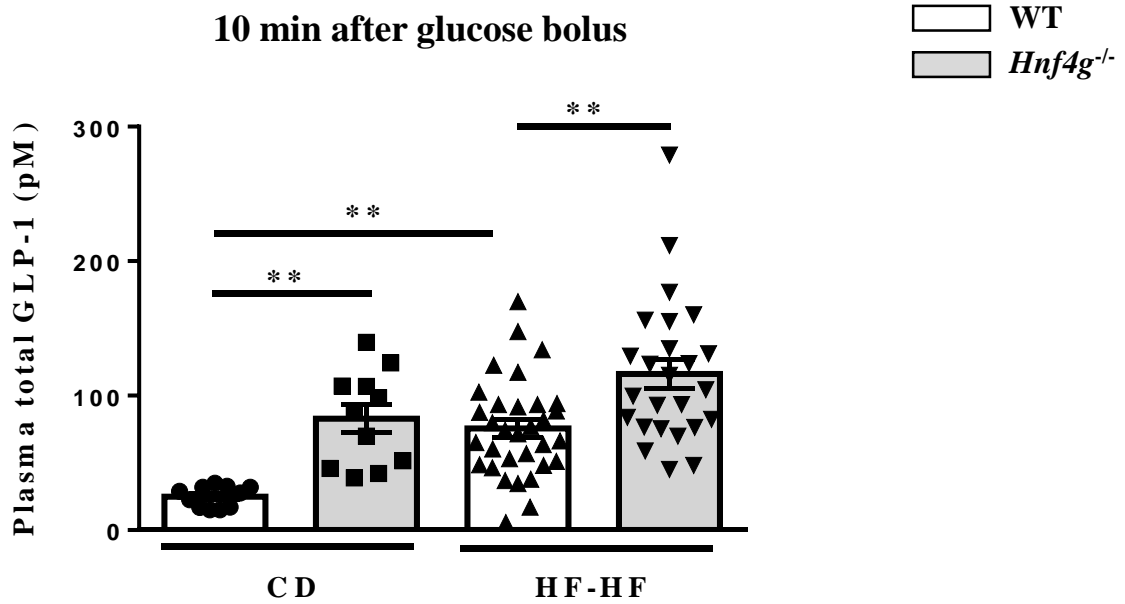
B**A D R P****M T T P****ApoB****C****H N F 4**

A

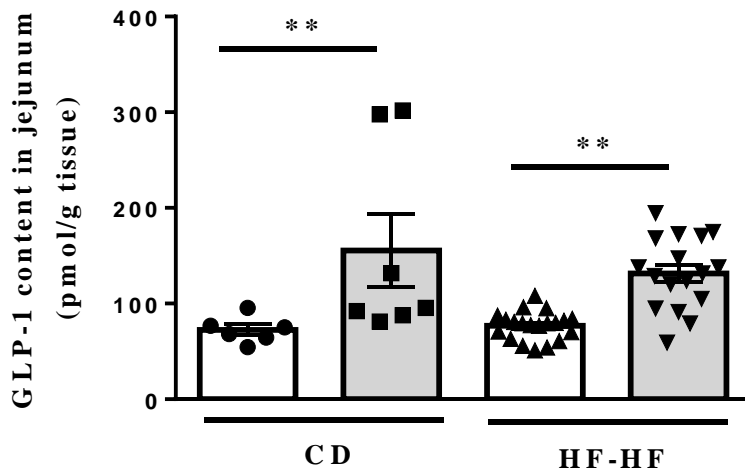
| multiple comparison test | 10 min | 30 min | 60 min | 90 min | 120 min |
|--------------------------|--------|--------|--------|--------|---------|
| WT CD vs KO CD | * | **** | ns | ns | ns |
| WT HFHF vs KO HFHF | **** | **** | **** | **** | **** |
| WT CD vs WT HF-HF | * | **** | **** | **** | **** |
| KO CD vs KO HF-HF | ns | ns | ** | * | ns |

B**C****D****E**

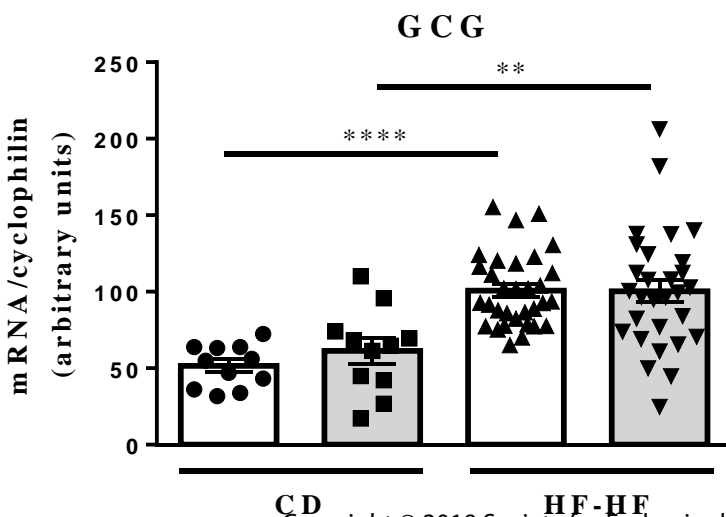
A



B



C



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Figure 5, Ayari S & al

Table 1

Composition of control and high-fat diets

| | Control diet (CD) | High-Fat diet (HFD) |
|------------------------------|------------------------------|--------------------------------|
| Energy composition | | |
| Proteins (Kcal %) | 25.2 | 20 |
| Lipids (Kcal %) | 13.5 | 60 |
| Carbohydrates (Kcal %) | 61.3 | 20 |
| Energy value | | |
| (kcal / g) | 3200 | 5240 |
| Nutrient composition | | |
| Proteins (%) | 24.34 | 26.23 |
| Lipids (%) | 5.8 | 34.89 |
| lard (%) | | 31.66 |
| Carbohydrates (%) | 43.11 | 25.04 |
| starch (%) | 38.1 | |
| maltodextrine (%) | | 16.15 |
| sugars (%) | 4.55 | |
| sucrose (%) | | 8.9 |
| Fibers (%) | 20.6 | 6.46 |
| Minerals and vitamins | 6.15 | 7.37 |

Table 2

Oligonucleotide sequences used for qPCR analysis

| Gene name | Sequence (5' to 3') |
|--------------------|--------------------------------|
| <i>cyclophilin</i> | Fwd: GCCTTAGCTACAGGAGAGAA |
| | Rev: TTCCTCCTGTGCCATCTC |
| <i>fabpm</i> | Fwd: ATGGCTGCTGCCTTTCAC |
| | Rev: GATCTGGAGGTCCCATTCA |
| <i>Srb1</i> | Fwd: GCCCATCATCTGCCAACT |
| | Rev: TCCTGGGAGCCCTTTTTACT |
| <i>Adrp</i> | Fwd: CTCCACTCCACTGTCCACCT |
| | Rev: GCTTATCCTGAGCACCCCTGA |
| <i>Gcg</i> | Fwd: CACGCCCTTCAAGACACAG |
| | Rev: GTCCTCATGCGCTTCTGTC |
| <i>Apo B</i> | Fwd: GCCCATTGTGGACAAGTTGATC |
| | Rev: CCAGGACTTGGAGGTCTTGGA |
| <i>Mttp</i> | Fwd: GGCAGTGCTTTTTCTCTGCT |
| | Rev: TGAGAGGCCAGTTGTGTGAC |
| <i>CD36</i> | Fwd: GCCAAGCTATTGCGACATGA |
| | Rev: ATCTCAATGTCCGAGACTTTTCAAC |
| <i>Fatp4</i> | Fwd: TATGGCTTCCCTGGTGTACTAT |
| | Rev: TTCTTCCGGATCACCACAGTC |
| <i>Hnf4a</i> | Fwd: CGTCCCTCGGCACTGTCC |
| | Rev: TCCTCCAGGCTCACTTGC |