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1 **Moderate chronic ethanol consumption exerts beneficial effects on nonalcoholic fatty liver in**
2 **mice fed a high-fat diet. Possible role of higher formation of triglycerides enriched in**
3 **monounsaturated fatty acids**

4

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17 **Abstract**

18 **Purpose:** Several clinical studies suggested that light-to-moderate alcohol intake could alleviate
19 nonalcoholic fatty liver disease (NAFLD), but the underlying mechanism is still poorly understood.

20 **Methods:** Mice fed a high-fat diet (HFD) were submitted or not to moderate ethanol intake for 3
21 months (ca. 10 g/kg/day) via the drinking water. Biochemical, analytical and transcriptomic
22 analyses were performed in serum and liver.

23 **Results:** Serum ethanol concentrations in ethanol-treated HFD mice were comprised between 0.5
24 and 0.7 g/l throughout the experiment. NAFLD improvement was observed in ethanol-treated HFD
25 mice as assessed by reduced serum transaminase activity. This was associated with less
26 microvesicular and more macrovacuolar steatosis, the absence of apoptotic hepatocytes and a trend
27 towards less fibrosis. Liver lipid analysis showed increased amounts of fatty acids incorporated in
28 triglycerides and phospholipids, reduced proportion of palmitic acid in total lipids and higher
29 desaturation index, thus suggesting enhanced stearoyl-coenzyme A desaturase activity. mRNA
30 expression of several glycolytic and lipogenic enzymes was upregulated. Genome-wide expression
31 profiling and gene set enrichment analysis revealed an overall downregulation of the expression of
32 genes involved in collagen fibril organization and leukocyte chemotaxis and an overall upregulation
33 of the expression of genes involved in oxidative phosphorylation and mitochondrial respiratory
34 chain complex assembly. In addition, mRNA expression of several proteasome subunits was
35 upregulated in ethanol-treated HFD mice.

36 **Conclusions:** Moderate chronic ethanol consumption may alleviate NAFLD by several mechanisms
37 including the generation of non-toxic lipid species, reduced expression of profibrotic and
38 proinflammatory genes, restoration of mitochondrial function and possible stimulation of
39 proteasome activity.

40 **Keywords:** Ethanol, NAFLD, Liver, Mouse, Lipids, Transcriptomics

41

42 **Introduction**

43

44 Alcohol abuse is associated with a large array of ailments including liver diseases, psychiatric
45 disorders, cardiomyopathy and pancreatitis [1, 2]. Regarding the liver, sustained high alcohol
46 consumption (>20 and 30 grams/day of alcohol for women and men, respectively) almost always
47 leads to steatosis, which can then progress in some patients to steatohepatitis, cirrhosis and
48 hepatocellular carcinoma [2, 3]. Importantly, a recent systematic analysis of data providing from
49 195 different countries and territories indicated that alcohol drinking, regardless of amount, actually
50 increases the overall risk to health across population [4]. However, the latter study and others found
51 some protective effects of light-to-moderate alcohol consumption (i.e. <20 and 30 grams/day of
52 alcohol for women and men, respectively) on ischemic heart disease and diabetes [4–6]. Moreover,
53 several clinical studies suggested that light-to-moderate alcohol intake could have beneficial effects
54 on nonalcoholic fatty liver disease (NAFLD) [7–12], which is commonly associated with obesity.
55 For instance, light-to-moderate alcohol consumption has been reported to reduce the degree of fatty
56 liver [10], the levels of serum transaminases [11, 12] and the risk of developing nonalcoholic
57 steatohepatitis (NASH) [7, 8, 11, 12], which is characterized by the presence of necroinflammation
58 and fibrosis, in addition to fatty liver. However, as pointed out by some authors, clinical data
59 reporting these beneficial effects on NAFLD should be interpreted with caution because of the
60 existence of several potential sources of bias, confounding factors and errors [7, 11]. Moreover,
61 other studies reported no beneficial effects of moderate alcohol drinking on NAFLD [12–14]. For
62 example, a 47-month longitudinal cohort study reported that modest alcohol use was associated
63 with less improvement in steatosis and blood level of aspartate aminotransferase (AST), compared
64 with no alcohol consumption [13], while a Mendelian randomization study showed that moderate

65 alcohol consumption had no beneficial effect on hepatic steatosis and necroinflammatory activity
66 [14].

67 Only 4 experimental studies carried out in rodents previously reported the beneficial effects of
68 light-to-moderate intake of alcohol on NAFLD [15–18]. In 2009, we first reported that moderate
69 ethanol consumption for 6 months reduced the gain of body weight, liver triglycerides and diabetes
70 in leptin-deficient obese and diabetic ob/ob mice [15]. However, these beneficial effects were not
71 associated with reduced levels of plasma transaminases. A recent study also carried out in ob/ob
72 mice reported that moderate alcohol intake for 6 weeks decreased plasma levels of alanine
73 aminotransferase (ALT) and AST, liver triglycerides and hepatic inflammation [16]. Investigations
74 performed in rats fed a high-fat diet (HFD) reported that low ethanol consumption for 12 weeks
75 lessened serum levels of ALT and lactate dehydrogenase (LDH), whereas liver triglycerides were
76 unchanged [17]. Finally, investigations in rats fed a high-fat high-cholesterol diet showed that a
77 moderate ethanol consumption for 8 weeks alleviated liver fibrosis and reduced the mRNA
78 expression of several profibrotic genes [18]. Notably, the beneficial effects of moderate ethanol
79 exposure in rodents are not restricted to the liver and can also be observed in other organs and
80 tissues such heart, colon, white adipose tissue and blood vessels [19–21]. Lower oxidative stress
81 and inflammation as well as improvement of lipid metabolism could be involved in these favorable
82 effects [19–21].

83 The mechanism(s) whereby light-to-moderate alcohol consumption could be beneficial on
84 NAFLD is poorly understood. Hence, the aim of our study was to improve our knowledge regarding
85 these mechanism(s) by carrying out investigations in HFD obese mice submitted or not to moderate
86 ethanol intake (ca. 10 g/kg/day) for 3 months via the drinking water. In particular, we performed a
87 thorough histological examination of livers associated for the first time with hepatic fatty acid
88 analysis and unsupervised genome-wide expression profiling.

89 **Materials and Methods**

90

91 **Animals and ethanol administration**

92 Thirty 7-week-old male C57BL/6J-+/+ mice, weighing 20 to 23 g, were purchased from Janvier
93 (Le-Genest-St-Isle, France). After arrival, the mice were acclimatized in the animal care facility of
94 the University of Rennes 1 (ARCHE), accredited by the French veterinary authorities (agreement
95 n°A35 23840) and fed *ad libitum* on a standard diet (SD) bringing 3 kcal/g of food (Teklad global
96 16% protein rodent diet, ENVIGO, Huntingdon, United Kingdom). After 1 week of acclimatization,
97 mice were split into three groups of 10 animals. While one group was kept on the SD, the two
98 others were fed a 60% high-fat diet (HFD) bringing 5.5 kcal/g (260HF V19, Safe Diet, Augy,
99 France). In this diet, 60% of the energy came from lipids, mainly anhydrous milk fat with 233 g/kg
100 of saturated fatty acids (SFAs). The main nutrient composition of these diets is given in the
101 Supplementary table 1. Details regarding the protocol of ethanol exposure (ca. 10 g/kg/day
102 corresponding to 15.9% of calories from alcohol) and blood withdrawal are provided in the
103 Supplementary methods 1.

104

105 **Serum analyses**

106 Immediately after collection, blood was centrifuged for 10 min at 1000g and serum was
107 immediately used for most biochemical analyses, or stored at -80°C until use for insulin and
108 adiponectin measurement. Activity of alanine aminotransferase (ALT) and aspartate
109 aminotransferase (AST), total cholesterol, triglycerides and glucose levels was measured on an
110 automatic analyzer (Cobas 8000, Roche Diagnostics, Mannheim, Germany) using the appropriate
111 reagents purchased from Roche Diagnostics. Total antioxidant capacity was assessed using the
112 Biovision TAC Colorimetric Assay Kit (K274-100, Milpitas, CA). Ethanol levels were measured
113 using the Ethanol assay kit purchased from Abcam (Paris, France). Insulin and adiponectin were

114 respectively determined using the Ultra-Sensitive Insulin Mouse ELISA Kit and the Adiponectin
115 Mouse ELISA kit purchased from Crystal Chem (Downers Grove, IL).

116

117 **Liver histology and fibrosis quantification**

118 To evaluate steatosis, necroinflammation, apoptosis and fibrosis, liver fragments were fixed in 4%
119 neutral formalin and embedded in paraffin. Then, 4 μm thick sections were cut and stained with
120 hematoxylin-eosin-safran (HES) or Sirius red. The sections were then digitally scanned with the
121 NanoZoomer 2.0 RS (Hamamatsu Corp. Japan) and thoroughly examined by an experienced
122 pathologist (V.T.-S.). The degree of hepatic steatosis was evaluated in 10 different randomly chosen
123 fields (x 200) as the percentage of hepatocytes containing one or several lipid vacuoles. The
124 classification of steatosis into 3 different categories (i.e. microvesicular, mediovesicular and
125 macrovacuolar) was determined as previously described [22, 23]. Necroinflammation, portal
126 fibrosis and perisinusoidal fibrosis were evaluated on the entire slide and scored 0 (absent), 1 (mild)
127 or 2 (moderate), as previously reported [23]. Finally, apoptosis was scored 0 (absent) or 1 (present)
128 depending on the presence of apoptotic bodies in the hepatic parenchyma.

129 In order to have more information regarding fibrosis, deposition of fibrillary collagen was
130 assessed by using second-harmonic generation (SHG) microscopy available at the light microscopy-
131 imaging center (MRic facility of Biosit, University of Rennes 1), as previously described [24, 25].
132 More details on the SHG methodology used in our study are provided in the Supplementary
133 methods 2.

134

135 **Activity of respiratory chain complexes I and II in liver**

136 For protein extraction, liver samples were rinsed in phosphate-buffered saline (PBS) and then lysed
137 using a Dounce homogenizer with the extraction buffer supplied by Abcam, as recommended by the

138 manufacturer. Mitochondrial respiratory chain (MRC) complex I activity was measured with 5 µg
139 of liver proteins by using the Complex I Enzyme Activity Dipstick Assay kit from Abcam (Paris,
140 France), as recently described [26, 27]. Activity of the MRC complex II, also referred to as
141 succinate dehydrogenase (SDH), was measured with 100 µg of liver proteins using the Complex II
142 Enzyme Activity Microplate Assay kit from Abcam, as recently reported [26, 27].

143

144 **Hepatic glutathione, carbonylated proteins, lipid peroxidation and CYP2E1 protein** 145 **expression**

146 Reduced (GSH) and oxidized (GSSG) glutathione were measured in liver tissue extracts using the
147 Glutathione Assay Kit purchased from Cayman Chemicals (Montigny-le-Bretonneux, France).
148 Hepatic carbonylated proteins were also assessed as valuable markers of oxidative stress [28].
149 Protein carbonyl quantification was carried out using the Protein Carbonyl Elisa Kit purchased from
150 Enzo Life Sciences (Villeurbanne, France). Lipid peroxidation was assessed using the Lipid
151 Peroxidation (MDA) Colorimetric/Fluorometric Assay Kit (K739) from BioVision. Protein
152 expression of hepatic cytochrome P450 2E1 (CYP2E1) and heat shock cognate 70 (HSC70) (used
153 as loading control) was assessed by western blot analysis as previously described [15], using
154 respectively the antibodies from Oxford Biomedical Research (Oxford, MI) and Santa Cruz
155 Biotechnology (Dallas, TX). The dilution used for the antibodies was 1:1000 for CYP2E1 and
156 1:500 for HSC70.

157

158 **RNA extraction and transcriptomic analysis**

159 For our transcriptomic analysis, 5 HFD mice and 5 ethanol-treated HFD mice were randomly
160 chosen in each group of mice. Total RNA was extracted from 10-15 mg of mouse liver with the
161 RNeasy Mini Kit (Qiagen, Courtaboeuf, France). Genome-wide expression profiling was then

162 performed using the low-input QuickAmp labeling kit and SurePrint G3 Mouse GE v2 8x60K
163 microarrays purchased from Agilent Technologies (Santa Clara, CA), as previously described [29].
164 Further information on transcriptomic analysis and gene set enrichment analysis (GSEA) is
165 provided in the Supplementary methods 3.

166

167 **Hepatic lipid extraction and fatty acid analysis**

168 For hepatic fatty acid analysis, all HFD and ethanol-treated HFD mice (n=10 per group) were
169 investigated. Hepatic lipid extraction and fatty acid analysis were performed as previously
170 described [30-32]. Further information regarding the experimental procedures is provided in the
171 Supplementary methods 4.

172

173 **Statistical analysis**

174 All results are expressed as mean \pm SEM (standard error of mean). Comparisons between groups
175 were performed using one-way analysis of variance (ANOVA) with a post-hoc Newman-Keuls test,
176 two-way ANOVA with a post-hoc Bonferroni test, Student *t*-test or nonparametric Mann-Whitney
177 U test, as appropriate. All statistical analyses and graphics were performed using GraphPad Prism5
178 software (GraphPad Software, San Diego, CA, USA).

179 **Results**

180

181 **Body weight, food consumption and energy intake**

182 As expected, mice fed the HFD (henceforth referred to as HFD mice) gained more body weight
183 compared with mice fed the SD (SD mice) and the body weight gain was significant from the
184 second week of HFD (Figure 1a). However, HFD mice consuming ethanol gained significantly less
185 body weight during the last month compared with naive HFD mice (Figure 1a). This difference was
186 apparently not related to lower food consumption in ethanol-treated obese mice in this period of
187 time (Figure 1b). HFD induced a transient increase in food consumption (Figure 1b), as already
188 observed in different rodent models of diet-induced obesity (DIO) [33, 34]. Caloric intake was
189 lower in SD mice compared with HFD mice and within the latter group, ethanol-treated HFD mice
190 consumed overall more energy than HFD mice (Figure 1c), due to the calories provided by ethanol
191 (7 kcal/g).

192

193 **Serum parameters and liver weight**

194 Different serum parameters were serially measured during the 4-month experiment (Figure 2a).
195 Serum ethanol concentrations in ethanol-treated HFD mice were comprised between 0.5 and 0.7 g/l
196 (ca. 11 and 15 mM) throughout the experiment. However, it should be underlined that ethanol
197 concentrations were measured in the morning after an overnight fast. Hence, it cannot be excluded
198 that these concentrations might have been higher if measured in the fed state and during the dark
199 cycle when the highest ethanol consumption usually occurs in mice [35, 36]. Serum glucose levels
200 were increased in naive and ethanol-treated HFD mice during the course of the experiment although
201 hyperglycemia was reduced in ethanol-treated HFD mice after 3 months but not afterwards. Serum
202 total cholesterol was increased in naive and ethanol-treated HFD mice throughout the experiment

203 but hypercholesterolemia was lower in ethanol-treated HFD mice after 4 months. Serum
204 triglyceride levels were enhanced in ethanol-treated HFD mice during the first 3 months but
205 returned to normal levels after 4 months. Although serum levels of triglycerides were enhanced in
206 ethanol-treated HFD mice compared with HFD mice at months 2 and 3, it seemed unlikely that this
207 unfavorable metabolic profile might be due to the higher energy intake brought only by ethanol
208 during the overnight fast. Indeed, this higher caloric intake would also have induced higher serum
209 triglycerides at month 4 but also unfavorable profiles on serum glucose and total cholesterol
210 throughout the experiment. Serum ALT activity was higher in naive and ethanol-treated HFD mice
211 during the experiment but a significant reduction of this activity was observed in ethanol-treated
212 HFD mice after 3 and 4 months. At these periods of time, AST activity was significantly increased
213 in naive HFD mice, but not in ethanol-treated HFD mice, compared with SD mice. There was no
214 modification of the serum total antioxidant capacity whatever the groups of mice and the timing of
215 measurement (Supplementary figure 2). At the end of the 4-month experiment, serum insulin levels
216 and liver weight were enhanced in HFD mice compared with the SD mice (Figure 2b and 2c).
217 However, there was no difference between naive and ethanol-treated HFD mice (Figure 2b and 2c).
218 Interestingly, a trend ($p=0.055$) toward higher serum adiponectin levels was found in HFD mice
219 treated with ethanol compared to the other groups of mice (Figure 2b).

220

221 **Liver steatosis, necroinflammation and apoptosis**

222 A thorough evaluation of steatosis, necroinflammation, apoptosis and fibrosis was performed at the
223 end of the experiment in 9 SD mice, 10 naive HFD mice and 10 ethanol-treated HFD mice. As
224 expected, steatosis was present in a vast majority of hepatocytes in DIO mice (Figure 3a). However,
225 the percentage of hepatocytes with steatosis was slightly but significantly reduced in ethanol-treated
226 HFD mice (87%) compared with naive HFD mice (94%) (Figure 3a). More strikingly, the pattern of
227 steatosis was modified in ethanol-treated HFD mice, which presented significantly less

228 microvesicular steatosis compared to naive HFD mice (Figure 3b and 3c). Indeed, the percentage of
229 steatotic hepatocytes presenting microvesicular steatosis was respectively 49 and 23% in naive and
230 ethanol-treated HFD mice (Figure 3b). On the other hand, ethanol-treated HFD mice presented
231 significantly more mediovesicular and macrovacuolar steatosis compared to naive HFD mice
232 (Figure 3b and 3c). Regarding necroinflammation, there was however no difference between naive
233 and ethanol-treated HFD mice. Indeed, in each group, there were 9 mice scored 1 and 1 mouse
234 scored 2 for this liver lesion. The inflammatory infiltrates, which were observed in the portal tracts
235 and the lobules, consisted mainly of lymphocytes and Kupffer cells, with few neutrophils. In
236 contrast, ethanol drinking reduced the apoptosis score. Indeed, there were 4 naive HFD mice
237 presenting apoptotic bodies in the hepatic parenchyma (score 1), whereas this pathological feature
238 was not observed in ethanol-treated HFD mice. In keeping with these results, our microarray data
239 showed a significant ($p < 0.05$) downregulation of different genes involved in apoptosis in ethanol-
240 treated HFD mice compared with naive HFD mice. This was for instance the case for *Card11* (-
241 43%), *Bcl2l14* (-36%), *Aen* (-26%), *Tnfsf12* (-23%), *Acin1* (-22%) and *Bid* (-11%). In contrast, there
242 was a significant upregulation of some anti-apoptotic genes such as *Faim2* (+35%), *Aven* (+19%),
243 *Tmbim4* (+13%) and *Dad1* (+11%). However, GSEA did not reveal an overall change in the
244 expression of genes involved in cell death.

245

246 **Assessment of liver fibrosis by histology and SHG microscopy**

247 Portal and perisinusoidal fibrosis was either absent (score 0) or mild (score 1) in all DIO mice (data
248 not shown). Whereas the mean score of perisinusoidal fibrosis was similar between naive and
249 ethanol-treated HFD mice, that of portal fibrosis tended to be lower in the last group (Figure 4a). In
250 order to have a quantitative assessment of fibrosis, liver fibrillar collagen deposition was assessed
251 by using SHG microscopy and the percentage of liver area with fibrosis was calculated for each
252 mouse, as previously reported [24, 25]. A trend towards reduced area of fibrosis was observed in

253 ethanol-treated HFD mice compared to naive HFD mice (Figure 4b and 4c) but the difference did
254 not reach statistical difference (p=0.10).

255

256 **Investigations on liver lipids and fatty acids**

257 Liver fatty acid analysis was subsequently carried out in order to better understand the altered
258 pattern of fatty liver in ethanol-treated HFD mice. Whereas the mass of fatty acids from total lipids
259 was similar between ethanol-treated HFD mice and naive HFD mice, more fatty acids were found in
260 triglycerides and phospholipids (Figure 5a). The 5 most abundant hepatic fatty acids in HFD mice
261 were, by decreasing order of magnitude, oleic acid (C18:1 n-9; 44.1%), palmitic acid (C16:0;
262 27.1%), cis-vaccenic acid (C18:1 n-7; 6.7%), palmitoleic acid (C16:1 n-7; 6.0%) and linoleic acid
263 (C18:2 n-6; 4.8%) (Figure 5b and data not shown). The proportion of the remaining fatty acids was
264 near or below 2% (data not shown). There was a significant decrease in the proportion of palmitic
265 acid and an increase in that of oleic acid in ethanol-treated HFD mice compared with naive HFD
266 mice (Figure 5b). Overall, the proportion of SFAs was significantly reduced in ethanol-treated HFD
267 mice whereas that of monounsaturated fatty acids (MUFAs) was enhanced (Figure 5b). Moreover,
268 the $\Delta 9$ -desaturation index, calculated as the ratio $(C16:1\ n-7 + C18:1\ n-9)/(C16:0 + C18:0)$, was
269 significantly enhanced in ethanol-treated HFD mice compared with naive HFD mice (Figure 5c),
270 suggesting higher hepatic stearoyl-CoA desaturase (SCD1) activity by ethanol intake. However,
271 *Scd1* mRNA expression was not increased in ethanol-treated HFD mice (data not shown).
272 Interestingly, our genome-wide expression profiling showed in ethanol-treated HFD mice an
273 upregulation of genes encoding 4 enzymes involved in glycolysis and conversion of pyruvate to
274 acetyl-coenzyme A (*Gpi1*, *Aldob*, *Pklr*, *Pdhb*), 4 enzymes implicated in fatty acid synthesis and
275 elongation (*Acy*, *Acaca*, *Me1*, *Elovl6*) and one enzyme participating in triglyceride formation
276 (*Lpin1*) (Figure 5d). In addition, we found an increased mRNA expression of thyroid hormone

277 responsive protein (*Thrsp*, also referred to as *Spot14*), cell death-inducing DFFA-like effector a
278 (*Cidea*) and adipogenin (*Adig*), three proteins involved in lipid synthesis and storage (Figure 5d).

279

280 **Microarray analysis, GSEA and MRC complex activity**

281 Our microarray analysis complemented by GSEA revealed in naive HFD mice a significant
282 enrichment of the GO gene sets corresponding to “collagen fibril organization” and “extracellular
283 structure organization”, in comparison with ethanol-treated HFD mice (Figure 6a). Accordingly, a
284 marked reduction of *Coll1a1*, *Coll1a2*, *Col2a1* and *Col3a1* mRNA levels was for instance observed
285 in these mice (Figure 6a). GSEA also disclosed in naive HFD mice a significant enrichment of the
286 GO gene sets related to “cell chemotaxis” and “leukocyte chemotaxis”, in comparison with ethanol-
287 treated HFD mice with reduced *Cx3cl1*, *Cxcl1*, *Ccr2* and *Cx3cr1* mRNA expression in the latter
288 group of mice (Figure 6b). In contrast, GSEA showed in ethanol-treated HFD a significant
289 enrichment of the GO gene sets corresponding to “oxidative phosphorylation” (OXPHOS) and
290 “MRC complex assembly”, compared with naive HFD mice (Figure 7a). For example, increased
291 mRNA levels of *Nd1*, *Nd3*, *Nd5*, *Cox5a*, *Cox7a2*, *Atp5d*, *Atp8* and *Ndufs4* were observed in
292 ethanol-treated HFD mice (Figure 7a). Regarding MRC complexes activity, no difference was
293 found between the groups for complex I activity, whereas HFD-induced reduction of complex II
294 activity was significantly restored in ethanol-treated HFD mice (Figure 7b).

295

296 **Investigations on oxidative stress and proteasome gene expression**

297 Investigations were also carried out on oxidative stress and cellular defense systems in liver.
298 Whereas there was no difference between the groups for total liver GSH, the GSSG/GSH ratio was
299 significantly decreased in ethanol-treated HFD mice compared with SD mice (Figure 8a). Liver
300 carbonylated proteins were enhanced in DIO mice compared with SD mice but there was no

301 difference between naive and ethanol-treated HFD mice (Figure 8a). Hepatic levels of
302 malondialdehyde (MDA), reflecting lipid peroxidation, tended to be higher in HFD mice compared
303 to SD mice but there was no statistical difference between the different groups of animals. The
304 protein expression of CYP2E1 was slightly (18%) but significantly reduced in ethanol-treated HFD
305 mice compared with naive HFD mice (Figure 8b), while *cyp2e1* mRNA expression was not
306 different between these groups (1.00 ± 0.06 and 0.98 ± 0.07 in naive HFD mice and ethanol-treated
307 HFD mice, respectively). Our microarray analysis complemented by GSEA also revealed in naive
308 HFD mice a moderate but significant enrichment ($p=0.016$) of the GO gene set corresponding to
309 “response to oxidative stress” in comparison with ethanol-treated HFD mice (Supplementary figure
310 3a). A reduction of *Gpx3*, *Gpx7*, *Gpx8*, *Hmox1*, *Ogg1* and *Sod3* mRNA expression was for example
311 observed in the latter group of mice (Supplementary figure 3b). Finally, GSEA showed in ethanol-
312 treated HFD a significant enrichment of the GO gene set corresponding to the “proteasome
313 complex”, compared with naive HFD mice (Figure 8c). For instance, increased mRNA levels of
314 *Psmb4*, *Psmb7*, *Psmc1*, *Psmc4*, *Psmc9* and *Psmc13* were observed in ethanol-treated HFD mice
315 (Figure 8c).

316 **Discussion**

317

318 Light-to-moderate chronic ethanol consumption could have beneficial effects on NAFLD in patients
319 [7, 8, 10–12], although some studies did not confirm these effects [12–14]. In this study, we used a
320 mouse model of DIO in an attempt to better understand how moderate alcohol consumption could
321 be beneficial on NAFLD. Thanks to different biochemical, analytical and transcriptomic analyses
322 performed in mouse liver, our investigations suggested that moderate chronic ethanol consumption
323 may alleviate NAFLD by several mechanisms including the generation of non-toxic lipid species
324 such as triglycerides enriched in MUFAs, reduced expression of profibrotic and proinflammatory
325 genes and restoration of mitochondrial function.

326

327 **Murine models of NAFLD and moderate ethanol consumption**

328 Our DIO model fulfilled the different recommended items required for a relevant mouse model of
329 NASH, in particular regarding overweight, hyperinsulinemia, hyperglycemia and raised serum
330 transaminase activity [37]. However, it is noteworthy that our naive HFD mice presented borderline
331 NASH rather than definite NASH, as discussed afterwards.

332 Regarding ethanol exposure (ca. 10 g/kg/day corresponding to 15.9% of calories from alcohol),
333 it might be considered as high when compared to humans. However, previous investigations
334 indicated that our protocol of alcohol administration can be considered as a *bona fide* model of
335 moderate chronic ethanol consumption. First, although data are scarce, it has been shown that mice
336 metabolize ethanol at a greater rate than do rats and humans [38]. Second, serum ethanol
337 concentrations in ethanol-treated HFD mice were between 0.5 and 0.7 g/l, which are below the toxic
338 range in humans (starting from 0.8-1 g/l) [39, 40]. Third, exposure of the livers to high ethanol
339 concentrations would have led to increased CYP2E1 expression at the mRNA and protein levels

340 [41–43], which was not observed in ethanol-treated HFD mice. Finally, ethanol exposure in our
341 murine model was similar (or even lower) to previous studies in rodents investigating the effects of
342 moderate alcohol consumption on different tissues and parameters [19, 44–46].

343 A caveat of our investigations was that ethanol-treated HFD mice ingested ethanol before blood
344 and liver collection since these animals were allowed to drink during the different episodes of
345 overnight fast scheduled in the study. Thus, some results observed in ethanol-treated HFD mice
346 might have been directly induced by the presence of ethanol (and possibly the corresponding
347 calories) during the few hours preceding the biological sample collection, in addition to the long-
348 term effects of the moderate consumption of ethanol.

349

350 **Moderate chronic ethanol consumption alleviates NAFLD in obese mice**

351 Moderate chronic ethanol consumption in HFD mice significantly decreased serum ALT and AST
352 activity, thus indicating NAFLD alleviation, although the mean necroinflammation score was
353 similar between ethanol-treated and naive HFD mice. However, no apoptotic bodies was observed
354 in ethanol-treated HFD mice, in contrast to naive HFD mice. Because hepatic apoptosis can be
355 associated with increased serum transaminase activity [47, 48], our data suggested that lower serum
356 ALT and AST activity in ethanol-treated HFD mice could be linked to reduced hepatic apoptosis. In
357 keeping with this hypothesis, these mice presented a downregulation of several genes involved in
358 apoptosis and an upregulation of some anti-apoptotic genes. Finally, ethanol consumption tended to
359 reduce hepatic fibrosis, in particular in portal areas. Interestingly, a recent study in a rat model of
360 NAFLD reported that a moderate chronic ethanol consumption improved liver fibrosis and
361 decreased the mRNA expression of several profibrotic genes such as TGF- β , α -SMA and collagen
362 [18].

363

364 **NAFLD alleviation is associated with increased serum adiponectin and higher hepatic levels of**
365 **triglycerides and MUFAs**

366 The mechanism(s) whereby light-to-moderate alcohol consumption could be beneficial on NAFLD
367 is poorly understood, possibly because only a few experimental investigations have tackled this
368 issue [15–17]. Some clinical and experimental investigations suggested a role of insulin resistance
369 improvement, possibly via enhanced circulating adiponectin levels [15, 49]. Interestingly,
370 adiponectin is able not only to alleviate fatty liver but also hepatic necroinflammation and fibrosis
371 [50, 51]. In this study, serum adiponectin levels tended to be higher in ethanol-treated HFD mice
372 compared to naive HFD mice but this was not associated with lower serum insulin concentrations.

373 Our fatty acid analysis revealed that moderate chronic ethanol consumption significantly
374 increased the amount of both hepatic triglycerides and phospholipids (as measured by their fatty
375 acid amounts), consistent with larger lipid droplets. This was accompanied with a significant
376 decrease in the proportion of palmitic acid and an increase in that of oleic acid in ethanol-treated
377 HFD mice compared with naive HFD mice as well as a greater $\Delta 9$ -desaturation index, suggesting
378 higher SCD1 activity. Thus, ethanol-induced NAFLD improvement might be related to the lower
379 hepatic levels of palmitic acid and higher levels of triglycerides since these lipid species are
380 respectively harmful and beneficial for the hepatocytes, as discussed afterwards. Interestingly,
381 higher SCD1 activity in ethanol-treated HFD mice might also have explained the significant
382 increase in serum triglyceride levels in these mice. Indeed, high SCD1 activity favors triglyceride
383 secretion from hepatocytes [52, 53]. However, further investigations will be required in order to
384 explain why hypertriglyceridemia was transient in ethanol-treated HFD mice and no longer
385 observed after 4 months. Nonetheless, reduced body weight and possibly fat mass might have
386 played a role.

387

388 **NAFLD improvement may occur through lower levels of SFAs including palmitic acid**

389 Numerous investigations previously reported that excess of palmitic acid is deleterious for the liver,
390 in particular by inducing hepatocyte oxidative stress, endoplasmic reticulum (ER) stress and
391 eventually apoptosis [54–56]. Moreover, lipotoxicity-induced mitochondrial dysfunction could play
392 a major role in the pathogenesis of NASH [55–58]. By inducing cell death and favoring the release
393 of extracellular vesicles, palmitic acid could also play a key role in the pathogenesis of NASH by
394 promoting inflammation and fibrosis [55, 56, 59]. It is noteworthy that a lipidomic analysis in
395 NAFLD patients showed a trend toward higher hepatic levels of palmitic acid and lower levels of
396 oleic acid in NASH compared with simple fatty liver [60]. On the other hand, increased conversion
397 of palmitate to MUFAs via higher SCD1 activity favors accretion of neutral lipids (triglycerides or
398 cholesterol ester) and protects against palmitate-induced cell death [61–63]. Interestingly, SCD1
399 knockout mice fed a methionine-choline deficient (MCD) diet had less steatosis but increased
400 hepatocellular apoptosis, liver injury, and fibrosis compared with wild-type mice [64]. Similarly,
401 diacylglycerol acyltransferase 2 (DGAT2) antisense oligonucleotide treatment in MCD diet-fed
402 mice decreased steatosis but enhanced hepatic free fatty acids, oxidative stress, lobular
403 necroinflammation and fibrosis [65]. Hence, these investigations and others [54–56] support a
404 protective role of triglyceride accumulation against SFA-induced lipotoxicity.

405

406 **NAFLD alleviation is associated with larger lipid droplets and less microvesicular steatosis**
407 **suggesting restoration of mitochondrial function**

408 NAFLD alleviation in ethanol-treated HFD mice was associated with larger lipid droplets. Lipid
409 droplet growth is deemed to be due to the relocalization of several triglyceride synthesis enzymes
410 (e.g. GPAT4, AGPAT3, DGAT2) from the ER to lipid droplets [66, 67]. Further investigations will
411 be needed in order to determine whether this mechanism occurred in ethanol-treated HFD mice.
412 Nevertheless, these mice presented higher expression of several enzymes involved in lipogenesis
413 such as ATP citrate lyase (*Acly*), acetyl-CoA carboxylase 1 (*Acaca*), malic enzyme (*Me1*) and Lipin

414 1 (*Lpin1*). Thus, besides enzyme relocalization, enhanced expression of lipogenic enzymes might
415 have favored lipid droplet expansion in ethanol-treated HFD mice. In keeping with this assumption,
416 our previous investigations in ob/ob mice showed that moderate chronic ethanol consumption led to
417 an increase in the hepatic mRNA levels of *Acaca*, fatty acid synthase (*Fas*) and *Scd1*, which was
418 associated with an enlargement of the lipid droplet size [15]. Lipid droplet growth is also dependent
419 on the expression of the CIDE proteins, which promote the formation of large lipid droplets in
420 different tissues including liver [68, 69]. Interestingly, *Cidea* mRNA expression was increased by
421 2.5-fold in ethanol-treated HFD mice compared with naive HFD mice.

422 Whatever its etiology, microvesicular steatosis is thought to be deleterious for the liver, in
423 particular as the consequence of mitochondrial dysfunction and energy shortage [70–72].
424 Interestingly, a previous study in NAFLD patients showed that the presence of microvesicular
425 steatosis was significantly associated with megamitochondria, ballooning cell injury, more
426 advanced fibrosis and diagnosis of NASH [73]. In the present study, the occurrence of less
427 microvesicular steatosis in ethanol-treated HFD mice might be linked to an improvement of
428 mitochondrial function via transcriptional mechanisms. Indeed, GSEA revealed in these mice a
429 significant enrichment of the expression of genes involved in OXPHOS and MRC complex
430 assembly. Moreover, this transcriptional effect might have explained why MRC complex II activity
431 was no longer decreased in ethanol-treated HFD mice compared with naive HFD mice. However, it
432 cannot be excluded that restoration of mitochondrial function in ethanol-treated HFD mice might
433 also be the mere consequence of reduced levels of SFAs including palmitic acid.

434

435 **Other potential mechanisms involved in NAFLD alleviation**

436 GSEA also disclosed that ethanol drinking lessened the hepatic expression of a wide range of genes
437 involved in fibrosis and leucocyte chemotaxis. Although moderate chronic ethanol consumption
438 might have directly lessened the expression of these genes, a reduction of apoptosis might have

439 been indirectly involved. Indeed, hepatocyte apoptosis can favor both inflammation and fibrosis
440 [74, 75]. Nevertheless, the downregulation of proinflammatory and profibrotic genes in ethanol-
441 treated HFD mice was not associated with a significant improvement of the necroinflammation and
442 fibrosis scores, although there was a trend toward less fibrosis in these mice. However, it is
443 noteworthy that both necroinflammation and fibrosis was mild in our DIO mouse model, indicating
444 borderline NASH. Hence, any improvement of these lesions might be difficult to appraise.
445 Accordingly, more prolonged exposure to HFD and ethanol might have been needed in order to
446 significantly alleviate these lesions.

447 Liver GSSG/GSH was reduced in ethanol-treated HFD mice compared to naive HFD mice, thus
448 suggesting an alleviation of some components of oxidative stress such as reduced CYP2E1 protein
449 expression. This assumption was also supported by a global reduction in ethanol-treated HFD mice
450 of the mRNA expression of different genes involved in the response to oxidative stress.
451 Nevertheless, ethanol consumption significantly enhanced the mRNA levels of several genes
452 encoding proteasome subunits, which may reflect an adaptive response in order to get rid of some
453 damaged proteins [76, 77]. Since the proteasome plays a major role in cell protection, our results
454 suggest that NAFLD alleviation by moderate chronic ethanol consumption might be, at least in part,
455 linked to proteasome stimulation. Previous investigations reported that low ethanol exposure or
456 moderate oxidative stress could activate the proteasome [78, 79]. In our study, however, ethanol-
457 treated HFD mice presented evidence of lower oxidative stress and increased proteasome activity at
458 the gene-expression level. Because the transcription factor nuclear factor erythroid 2-like 2
459 (NFE2L2, also referred to as Nrf2) is able to activate the expression of genes involved in both
460 biological processes [76, 77], our data suggest a lack of robust Nrf2 activation in ethanol-treated
461 HFD mice. Accordingly, a supervised GSEA with a dataset of 481 Nrf2 target genes (data set
462 NFE2L2.V2) [80] did not reveal in these mice a significant enrichment of genes related to the Nrf2
463 pathway. Further investigations will be needed in order to determine why oxidative stress and
464 proteasome activity seemed to be disconnected in ethanol-treated HFD mice. Nevertheless, our

465 previous investigations in ob/ob mice showed that fatty liver improvement induced by moderate
466 ethanol consumption over 6 months was associated with some signs of mild oxidative stress such as
467 reduced aconitase activity and higher MnSOD protein expression [15]. Hence, additional
468 investigations will be required to determine whether further prolongation of the moderate alcohol
469 drinking protocol might lead to stronger hepatic oxidative stress and loss of the beneficial effects on
470 liver afforded by ethanol. Moreover, it should be underlined that hepatic fat accumulation can favor
471 in the long term the occurrence of unfavorable extra-hepatic effects such as dyslipidemia (including
472 hypertriglyceridemia and hypercholesterolemia), systemic inflammation and cardiovascular
473 diseases [81, 82].

474

475 **Conclusion**

476 Our study performed in DIO mice suggests that moderate chronic ethanol consumption may
477 alleviate NAFLD progression, in particular as regards hepatic cytolysis and fibrosis. Several
478 mechanisms could be involved including a reduction in the levels of deleterious SFAs (including
479 palmitic acid) and a concomitant generation of non-toxic lipid species such as MUFAs. NAFLD
480 alleviation in ethanol-treated HFD mice was associated with larger lipid droplets and less
481 microvesicular steatosis, in keeping with a possible improvement of mitochondria function.
482 Genome-wide expression profiling and GSEA revealed an overall upregulation of genes involved in
483 the proteasome complex, thus suggesting possible stimulation of proteasome activity. Because there
484 was also evidence of lower oxidative stress in ethanol-treated HFD mice, further investigations will
485 be required in order to determine why moderate chronic ethanol intake could induce an opposite
486 effect on proteasome activity and oxidative stress.

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497

498 **Conflict of interest statement**

499 On behalf of all authors, the corresponding author states that there is no conflict of interest.

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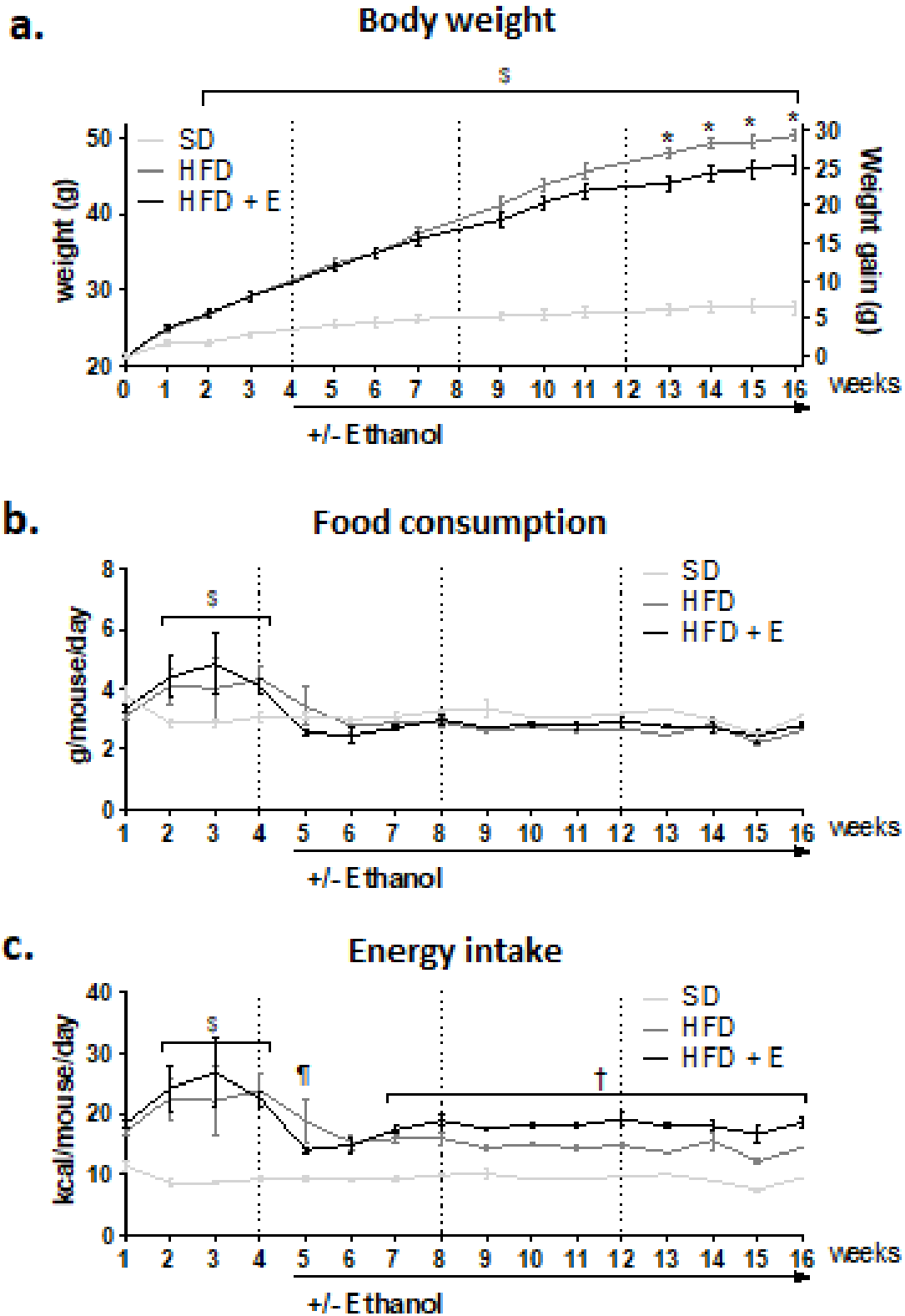


Figure 1

721 Fig. 1. Body weight, body weight gain, food consumption and energy intake in mice fed the
722 standard diet (SD), mice fed the high-fat diet (HFD) and ethanol-treated HFD mice (HFD+E). (a)
723 Body weight (mean \pm SEM for 9-10 mice per group) was measured every week or two weeks (left
724 y-axis) and the body weight gain (right y-axis) was subsequently calculated. [§]Significantly different
725 between HFD and SD mice, *significantly different between HFD+E and naive HFD mice, $p < 0.01$
726 with a two-way ANOVA test. (b) Food consumption (mean \pm SEM for 9-10 mice per group) was
727 measured every week. [§]Significantly different between HFD and SD mice, $p < 0.05$ with a two-way
728 ANOVA test. (c) Energy intake (kcal/mouse/day) corresponding to food and ethanol (if any)
729 consumption in the different groups of animals (mean \pm SEM for 9-10 mice per group).
730 [§]Significantly different between HFD and SD mice, [¶]Significantly different between HFD and SD
731 mice, [†]Significantly different between HFD+E and SD mice, $p < 0.05$ with a two-way ANOVA test.

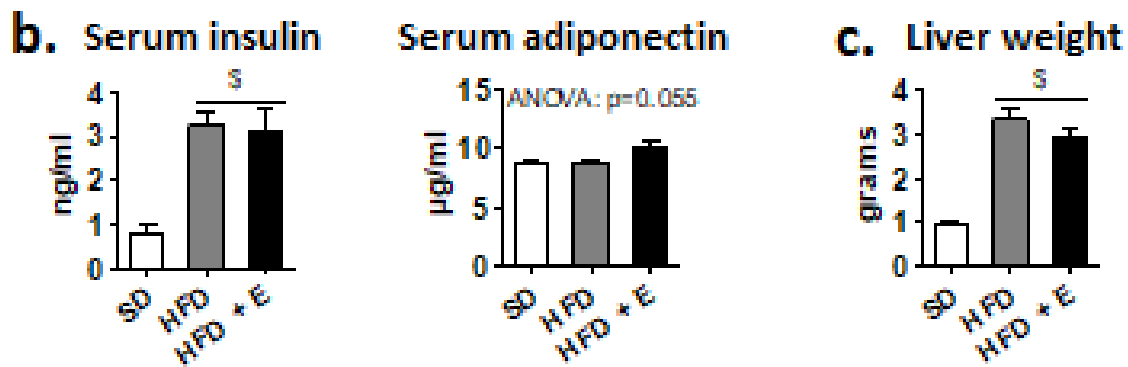
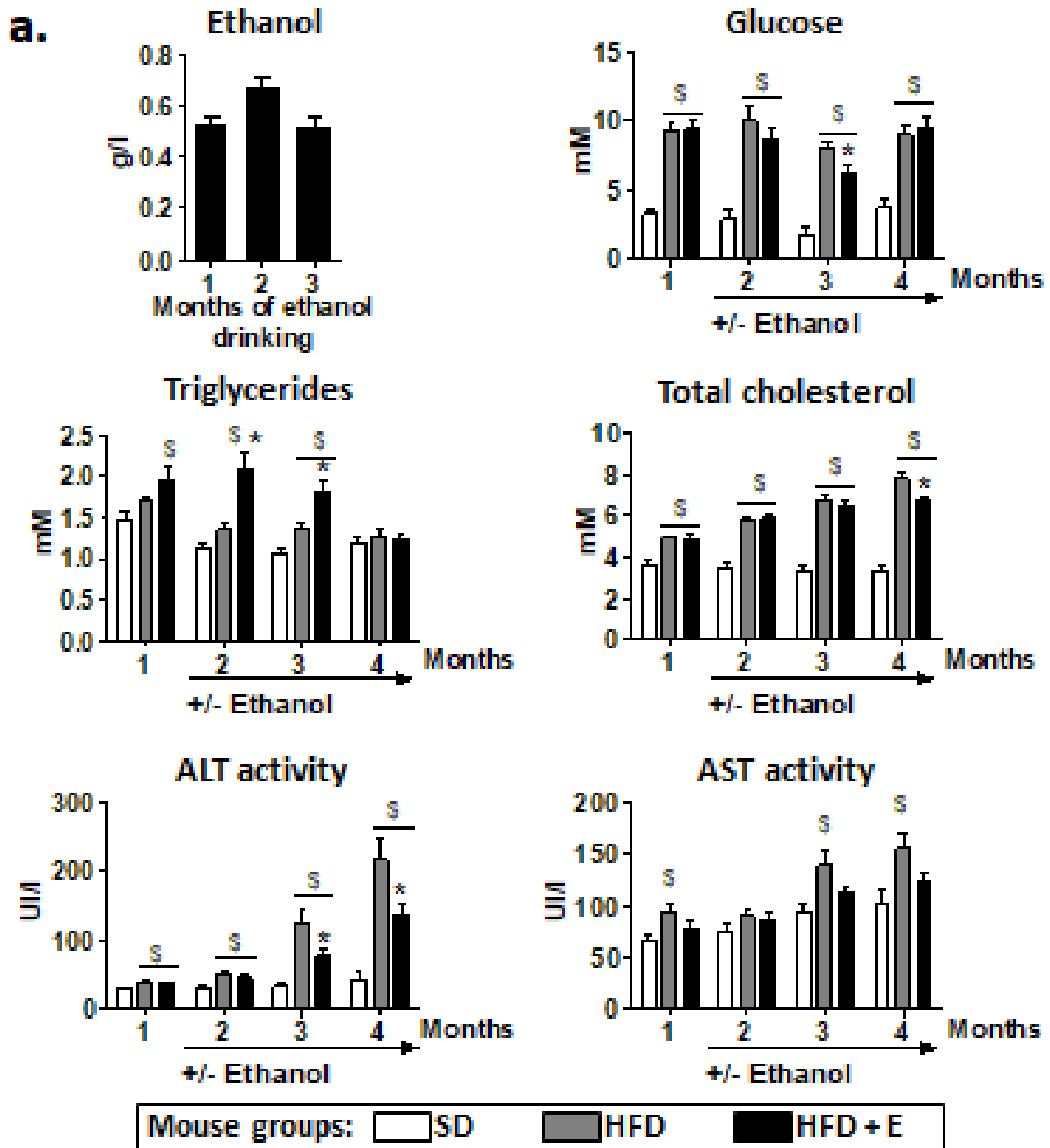
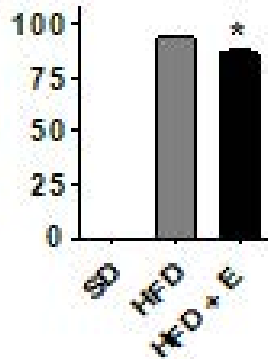


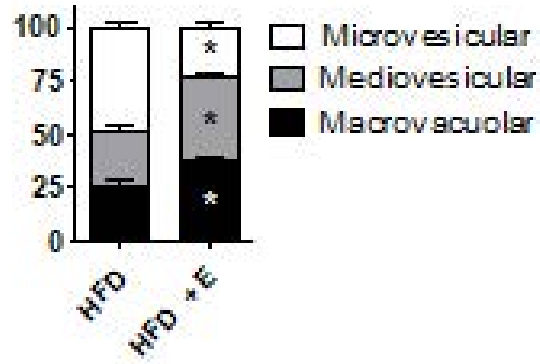
Figure 2

733 Fig. 2. (a) Serum parameters serially measured during the 4-month experiment in mice fed the
734 standard diet (SD), mice fed the high-fat diet (HFD) and ethanol-treated HFD mice (HFD+E).
735 Serum ethanol levels were measured only in the 10 ethanol-treated HFD mice. Values are means \pm
736 SEM for 8-10 mice per group for glucose, triglycerides and cholesterol and 7-10 mice per group for
737 alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. [§]Significantly
738 different from SD mice, *significantly different from naive HFD mice, $p < 0.05$ with a one-way
739 ANOVA test. (b) Serum insulin and adiponectin (mean \pm SEM for 7-10 mice per group) measured
740 at the end of the 4-month experiment. [§]Significantly different from SD mice, $p < 0.001$ with a one-
741 way ANOVA test. (c) Liver weight (mean \pm SEM for 9-10 mice per group) measured at the end of
742 the 4-month experiment. [§]Significantly different from SD mice, $p < 0.001$ with a one-way ANOVA
743 test.

a. Hepatocytes with steatosis (%)



b. Type of steatosis (%)



c.

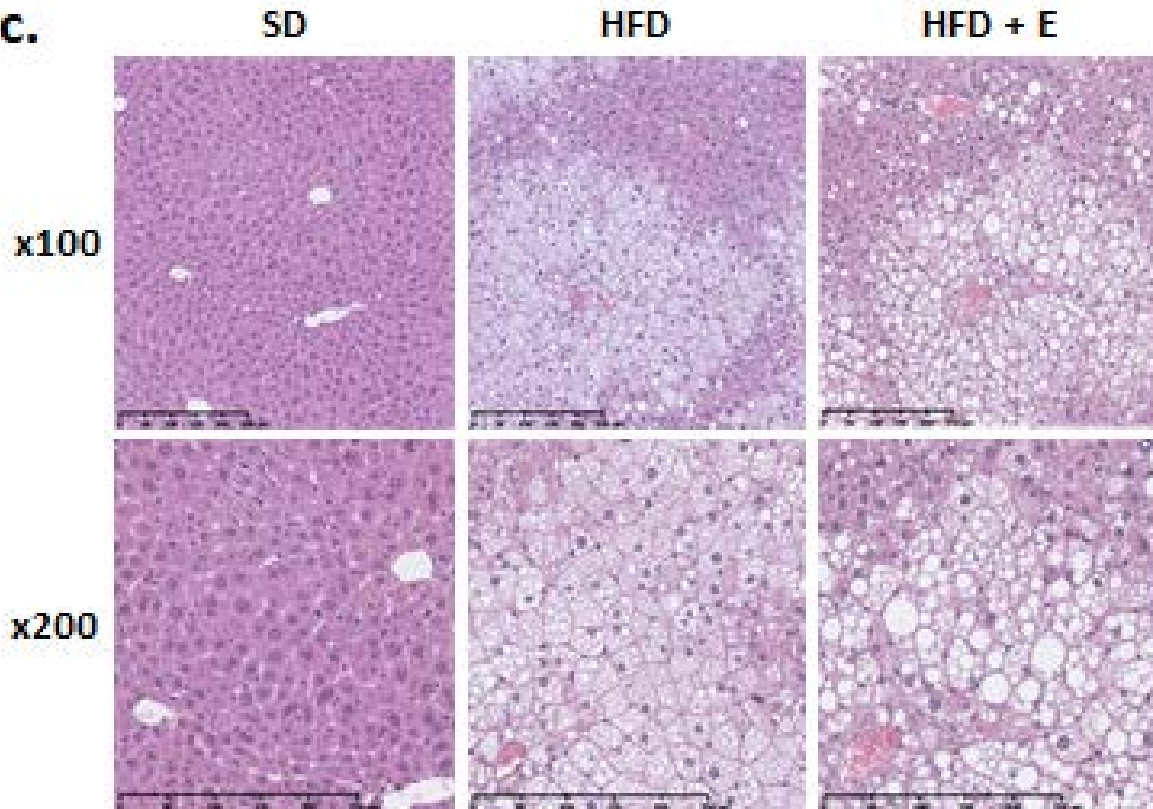
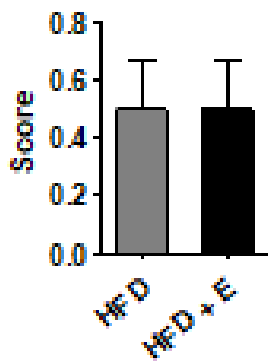


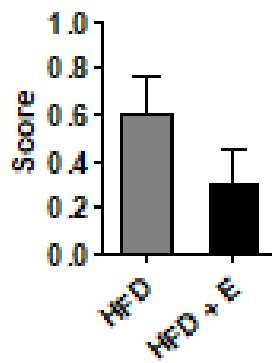
Figure 3

745 Fig. 3. Features of hepatic steatosis in mice fed the standard diet (SD), mice fed the high-fat diet
746 (HFD) and ethanol-treated HFD mice (HFD+E). (a) Percentage of hepatocytes presenting steatosis.
747 Values are means \pm SEM for 9-10 mice per group. *Significantly different from naive HFD mice,
748 $p < 0.01$ with a one-way ANOVA test. (b) Pattern of steatosis in HFD and HFD+E mice. Percentage
749 of steatotic hepatocytes presenting microvesicular, mediovesicular and macrovacuolar steatosis,
750 respectively. Values are means \pm SEM for 10 mice per group. *Significantly different from naive
751 HFD mice, $p < 0.01$ with a two-way ANOVA test. (c) Representative pictures of a naive SD mouse
752 liver with no steatosis, a naive HFD mouse liver with predominant microvesicular steatosis and an
753 ethanol-treated HFD mouse liver with predominant mediovesicular and macrovacuolar steatosis.
754 Sections were stained with HES and pictures were taken at 100x and 200x magnification, as
755 indicated. Scale bars (250 μ m) are indicated on each picture.

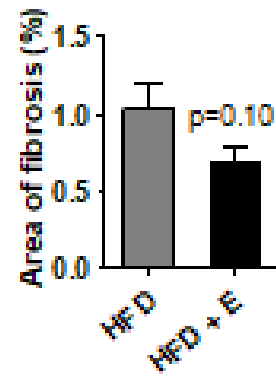
a. Perisinusoidal fibrosis score



Portal fibrosis score



b. Area of fibrosis assessed with SHG (%)



c.

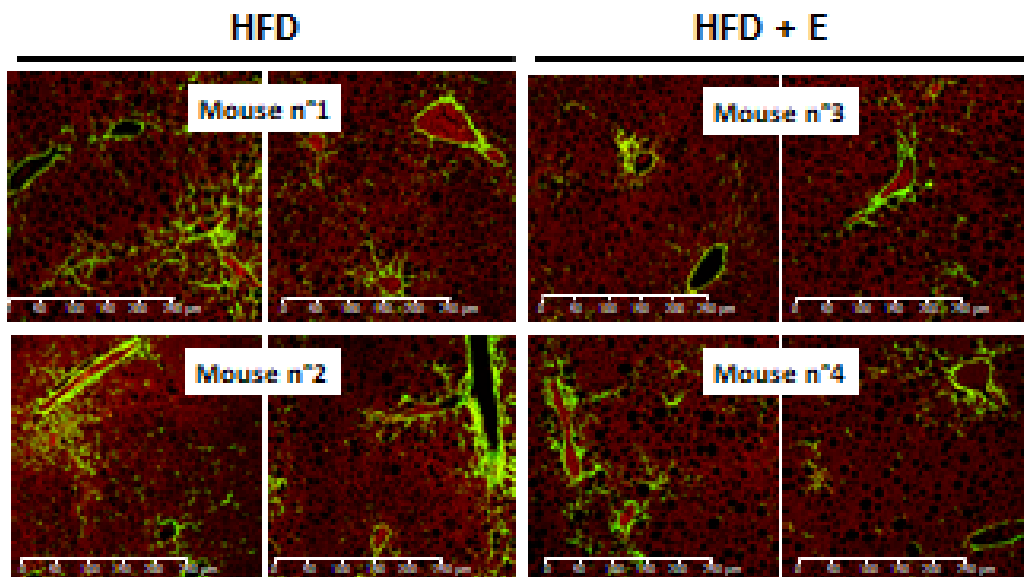
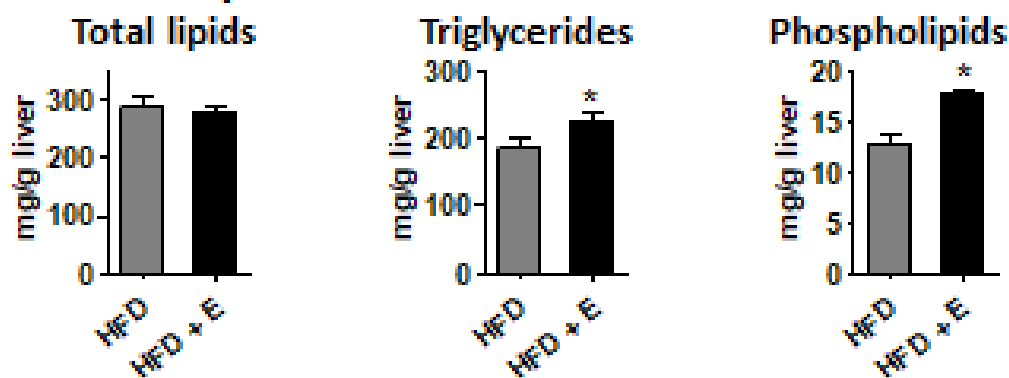


Figure 4

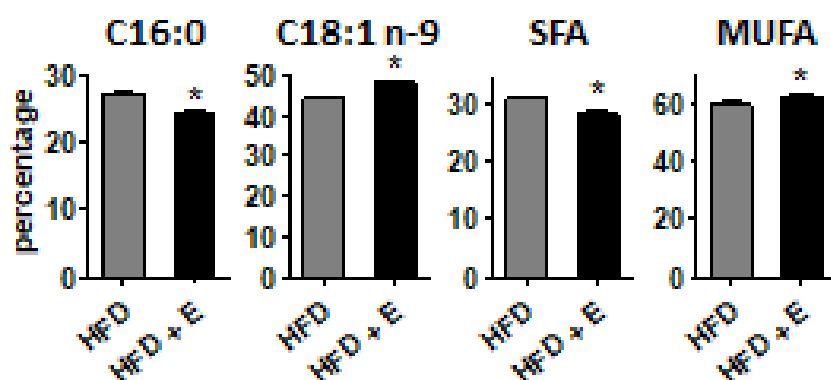
757 Fig. 4. Hepatic fibrosis in mice fed the high-fat diet (HFD) and ethanol-treated HFD mice
758 (HFD+E). (a) Score of perisinusoidal and portal fibrosis. Values are means \pm SEM for 10 mice per
759 group. (b) Percentage of area of fibrosis assessed by second-harmonic generation (SHG)
760 microscopy. Values are means \pm SEM for 10 mice per group, $p=0.10$ with a *t*-test. (c)
761 Representative pictures of two naive HFD mouse livers and two HFD+E mouse livers with less
762 portal fibrosis. Scale bars (250 μ m) are indicated on each picture.

Revised manuscript

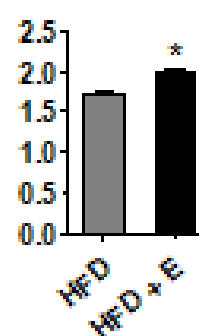
a. Mass of fatty acids



b. Proportion of fatty acids in total lipids



c. $\Delta 9$ -Desaturation index



d. Expression of genes involved in glycolysis and lipogenesis

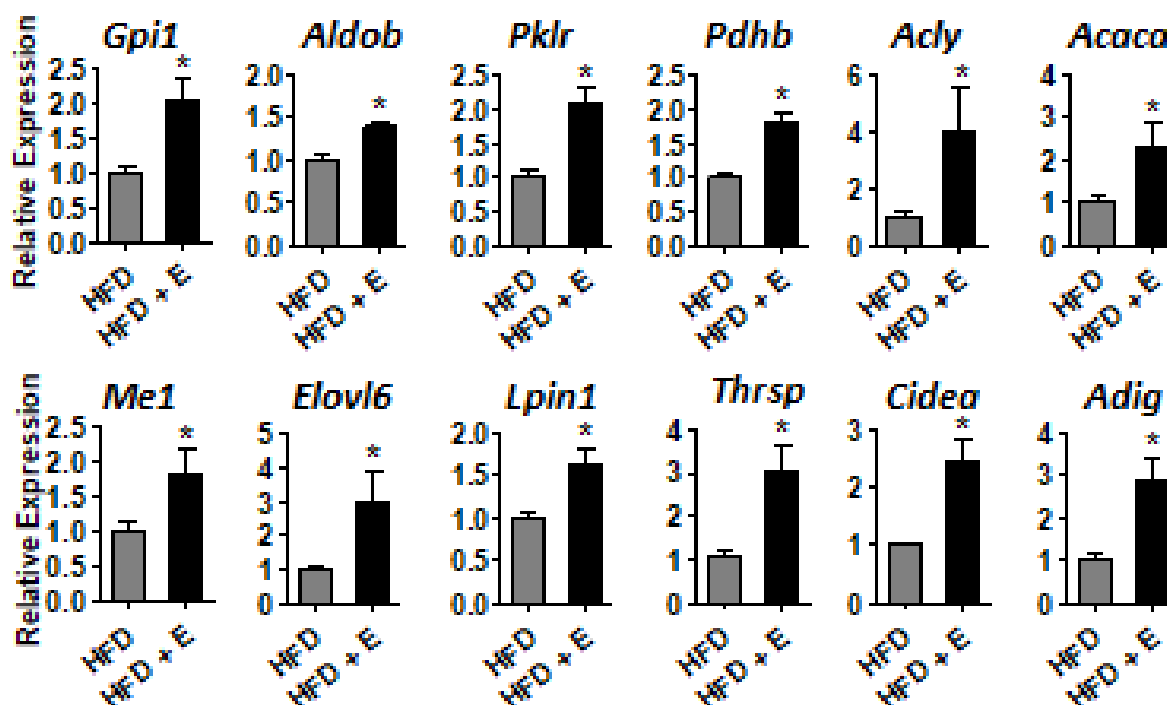


Figure 5

764 Fig. 5. Analysis of hepatic fatty acids in high-fat diet (HFD) and ethanol-treated HFD mice
765 (HFD+E). (a) Mass of fatty acids from total lipids and in the triglyceride and phospholipid
766 fractions. Values are means \pm SEM for 10 mice per group. *Significantly different from naive HFD
767 mice, $p < 0.05$ with a *t*-test. (b) Proportion of palmitic acid (C16:0), oleic acid (C18:1 n-9), saturated
768 fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) in total lipids. Values are means \pm
769 SEM for 10 mice per group. *Significantly different from naive HFD mice, $p < 0.05$ with a *t*-test. (c)
770 $\Delta 9$ -Desaturation index calculated as the ratio $(C16:1\ n-7 + C18:1\ n-9)/(C16:0 + C18:0)$. Values are
771 means \pm SEM for 10 mice per group. *Significantly different from naive HFD mice, $p < 0.05$ with a
772 *t*-test. (d) Hepatic mRNA levels of genes involved in glycolysis and lipogenesis extracted from the
773 transcriptomic analysis (GSE116417). Values are means \pm SEM for 5 mice per group.
774 *Significantly different from naive HFD mice, $p < 0.05$ with a Mann-Whitney test.

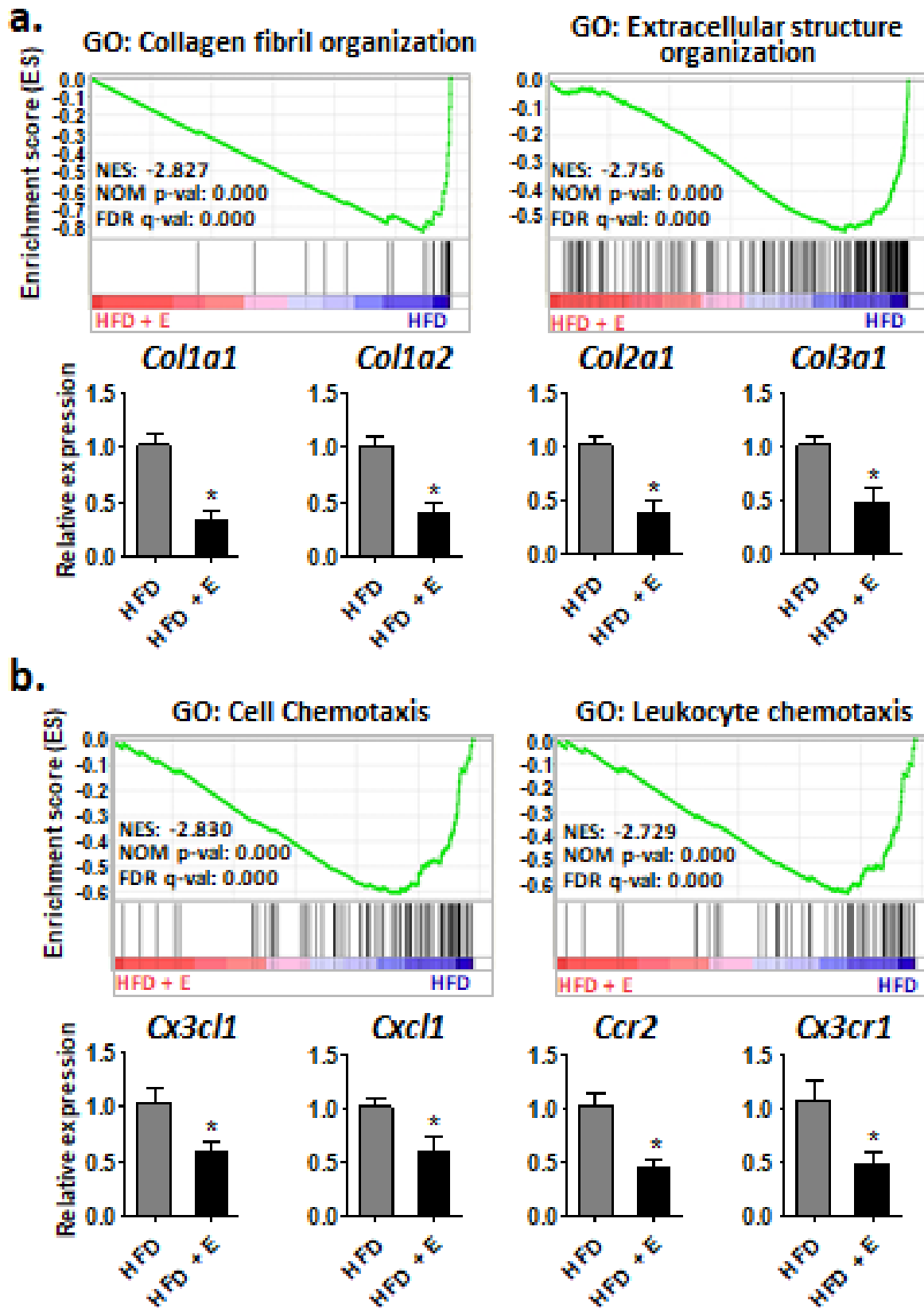


Figure 6

776 Fig. 6. mRNA expression of hepatic genes involved in fibrosis and cell chemotaxis in high-fat diet
777 (HFD) and ethanol-treated HFD mice (HFD+E). Genome-wide expression profiling performed in
778 naive (n=5) and ethanol-treated (n=5) HFD mice (GSE116417) was used for subsequent gene set
779 enrichment analysis (GSEA). (a) GSEA revealing in naive HFD mice a significant enrichment of
780 the gene ontology (GO) gene sets corresponding to “collagen fibril organization” and “extracellular
781 structure organization”, in comparison with ethanol-treated HFD mice. The hepatic mRNA
782 expression of 4 representative genes involved in fibrosis, extracted from the transcriptomic analysis
783 (GSE116417), is shown below the GSEA plots. *Significantly different from naive HFD mice,
784 $p < 0.05$ with a Mann-Whitney test. (b) GSEA revealing in naive HFD mice a significant enrichment
785 of the GO gene sets related to “cell chemotaxis” and “leukocyte chemotaxis”, in comparison with
786 ethanol-treated HFD mice. The hepatic mRNA expression of 4 representative genes involved in cell
787 chemotaxis, extracted from the transcriptomic analysis (GSE116417), is shown below the GSEA
788 plots. *Significantly different from naive HFD mice, $p < 0.05$ with a Mann-Whitney test.

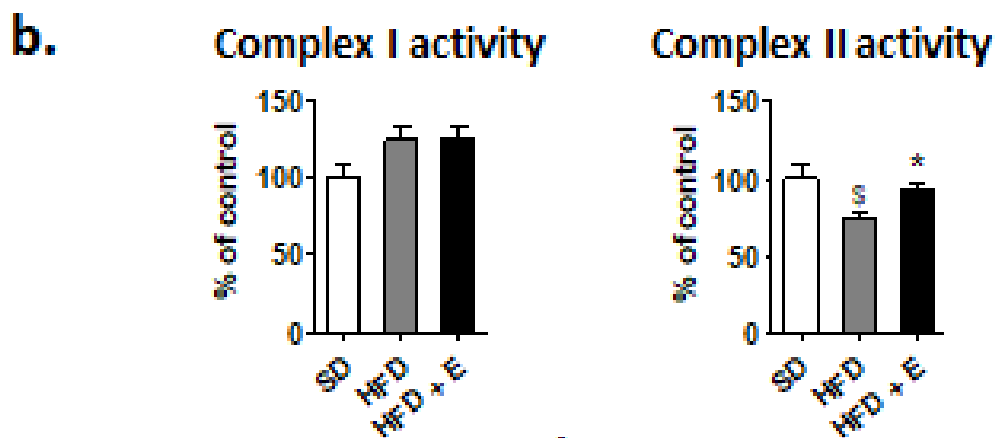
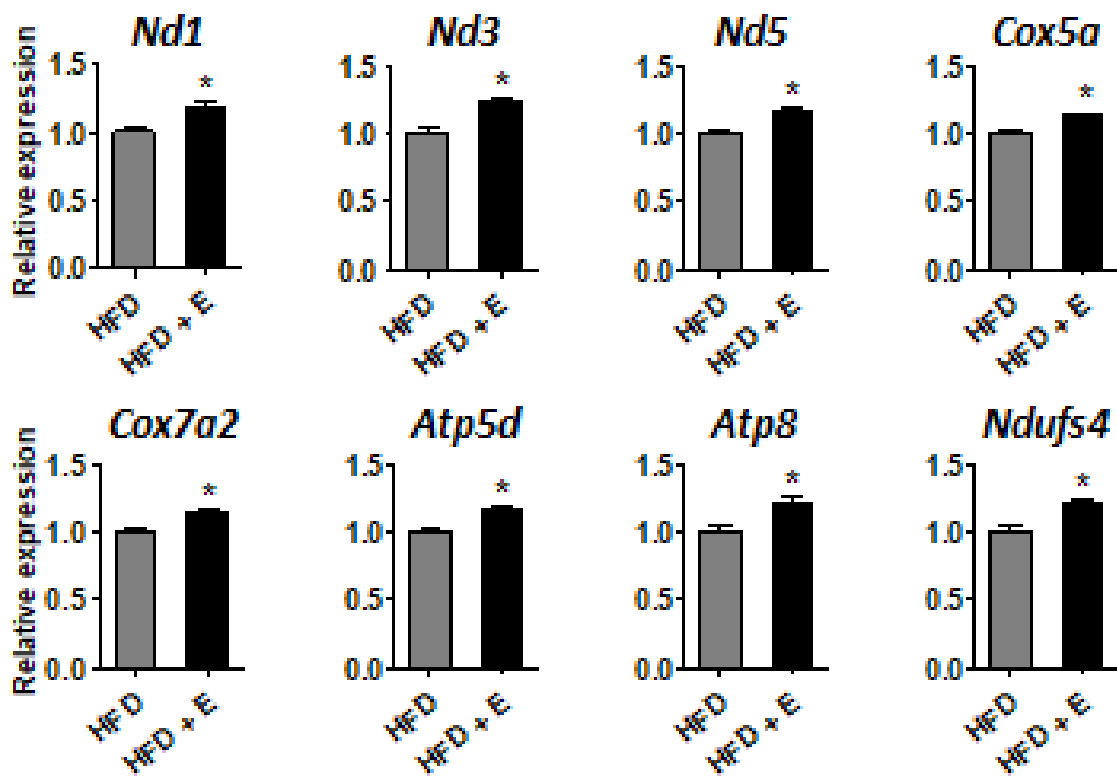
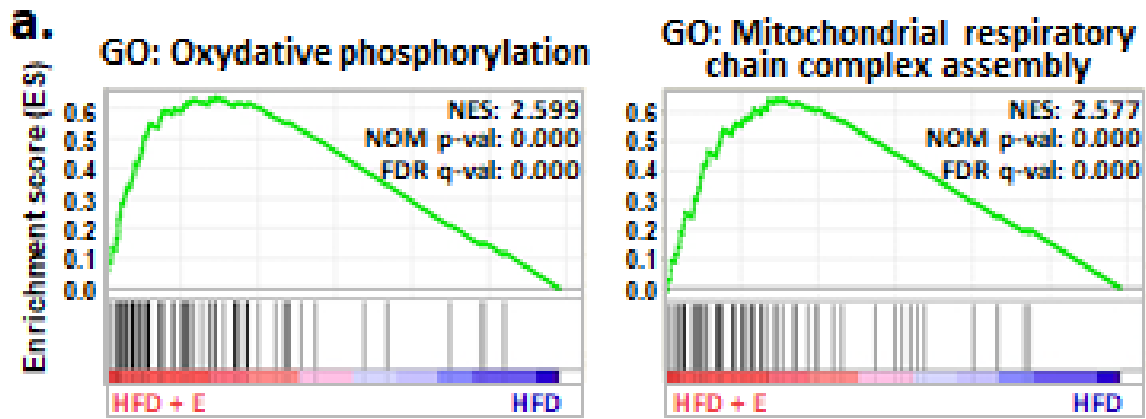


Figure 7

790 Fig. 7. mRNA expression of hepatic genes involved in mitochondrial function and mitochondrial
791 respiratory chain (MRC) complex I and II activities in high-fat diet (HFD) and ethanol-treated HFD
792 mice (HFD+E). (a) Genome-wide expression profiling performed in naive (n=5) and ethanol-treated
793 (n=5) HFD mice (GSE116417) was used for subsequent gene set enrichment analysis (GSEA).
794 GSEA showing in ethanol-treated HFD a significant enrichment of the gene ontology (GO) gene
795 sets corresponding to “oxidative phosphorylation” (OXPHOS) and “MRC complex assembly”,
796 compared with naive HFD mice. The hepatic mRNA expression of 8 representative genes involved
797 in oxidative phosphorylation and MRC complex assembly, extracted from the transcriptomic
798 analysis (GSE116417), is shown below the GSEA plots. *Significantly different from naive HFD
799 mice, $p < 0.05$ with a Mann-Whitney test. (b) MRC complex I and II activities. Values are means \pm
800 SEM for 9-10 mice per group. [§]Significantly different from SD mice, *significantly different from
801 naive HFD mice, $p < 0.05$ with a one-way ANOVA test.

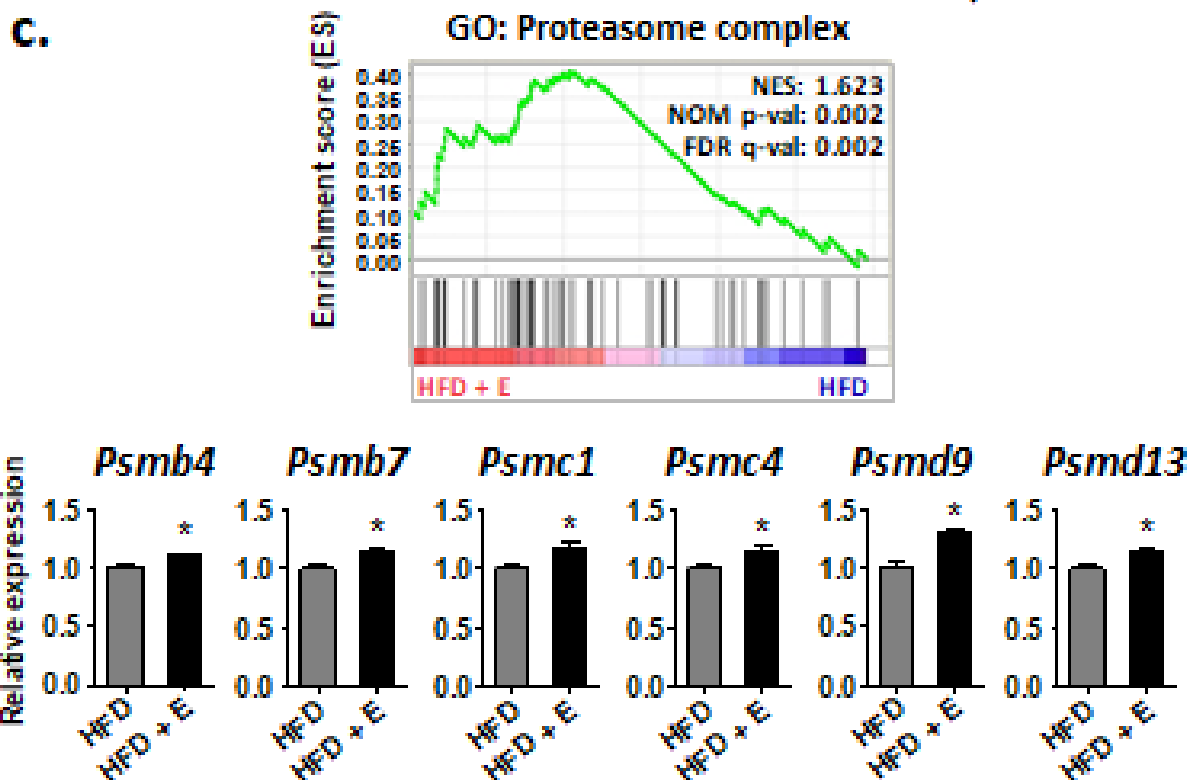
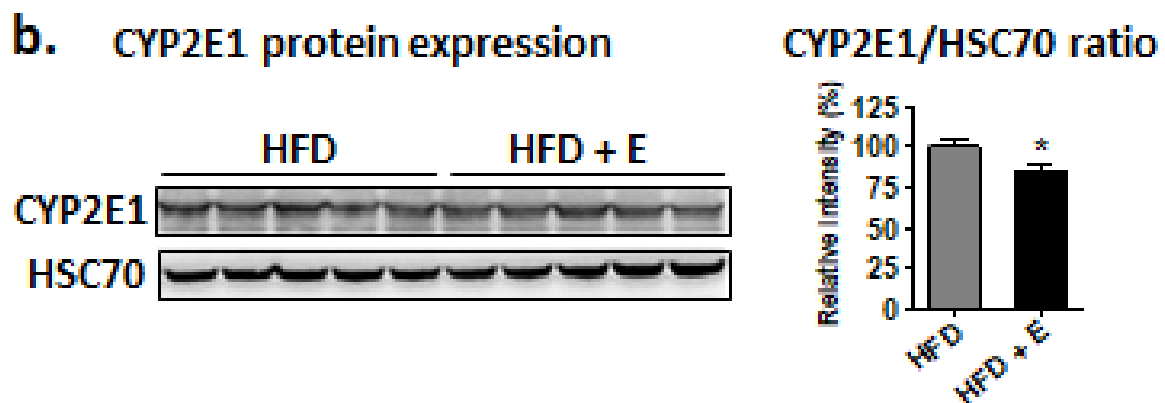
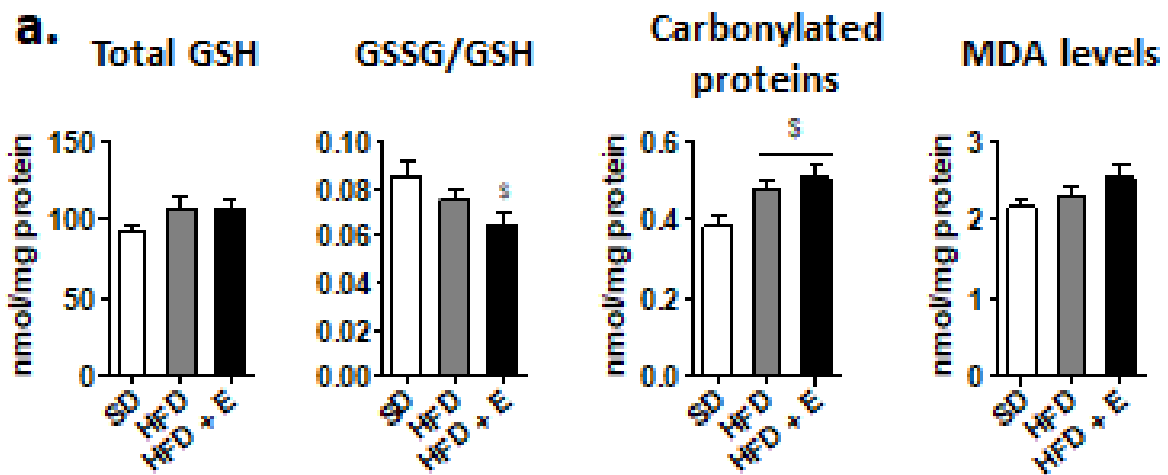


Figure 8

803 Fig. 8. Markers of hepatic oxidative stress and cellular defense systems in mice fed the standard diet
804 (SD), mice fed the high-fat diet (HFD) and ethanol-treated HFD mice (HFD+E). (a) Total
805 glutathione, namely reduced glutathione (GSH) plus oxidized glutathione (GSSG), GSSG/GSH
806 ratio and carbonylated proteins and levels of malondialdehyde (MDA). Values are means \pm SEM
807 for 9-10 mice per group for GSH and carbonylated proteins and 7-10 mice per group for MDA
808 levels. [§]Significantly different from SD mice, $p < 0.05$ with a *t*-test. (b) Protein expression of
809 cytochrome P450 2E1 (CYP2E1) and heat shock cognate 70 (HSC70), used as loading control.
810 Representative western blot for 5 mice per group. CYP2E1/HSC70 ratios showed in the graph are
811 means \pm SEM for 10 mice per group. *Significantly different from naive HFD mice, $p < 0.05$ with a
812 *t*-test. (c) Gene set enrichment analysis (GSEA) showing in ethanol-treated HFD a significant
813 enrichment of the gene ontology (GO) gene set corresponding to the “proteasome complex”,
814 compared with naive HFD mice. The hepatic mRNA expression of 6 representative genes involved
815 in the proteasome complex, extracted from the transcriptomic analysis (GSE116417), is shown
816 below the GSEA plots. Values are means \pm SEM for 5 mice per group. *Significantly different from
817 naive HFD mice, $p < 0.05$ with a Mann-Whitney test.