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1 **Estrogen withdrawal and replacement differentially target liver and adipose tissues in**
2 **female mice fed a high-fat high-sucrose diet. Impact of a chronic exposure to a low-dose**
3 **pollutant mixture.**

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17

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23 **Key words:** high-fat high-sucrose diet; mixture of pollutants; metabolic disorders;
24 ovariectomy; estradiol replacement;

25 **Abbreviations:**

26 ALAT, alanine aminotransferase ; ASAT, aspartate aminotransferase, AUC, area-under-
27 curve; BPA, bisphenol A; bw, body weight ; DEHP, diethylhexyl phthalate; DMSO,
28 dimethylsulfoxide; E2, 17 β estradiol ; FFA, free fatty acid; GTT, glucose tolerance test;
29 HFHS, high- fat high sucrose; PCB, polychlorinated biphenyl; TCDD, 2,3,7,8-
30 tetrachlorodibenzo-p-dioxin; TDI, Tolerable Daily Intake; TG, triglyceride.

31

32 **Abstract**

33 Post-menopausal women may be at particular risk when exposed to chemicals especially
34 endocrine disruptors because of hormonal deficit. To get more insight, ovariectomized
35 C57Bl6/J mice fed a high-fat high-sucrose diet were chronically exposed from 5 to 20 weeks
36 of age to a low-dose mixture of chemicals with one dioxin, one polychlorobiphenyl, one
37 phthalate and bisphenol A. Part of the mice received as well E2 implants to explore the
38 potential estrogenic dependency of the metabolic alterations. With this model, estrogen loss
39 resulted in glucose but not lipid metabolism impairment and E2 replacement normalized the
40 enhanced body and fat pad weight, and the glucose intolerance and insulin resistance linked to
41 ovariectomy. It also altered cholesterol metabolism in the liver concurrently with enhanced
42 estrogen receptor *Esr1* mRNA level. In addition, fat depots responded differently to estrogen
43 withdrawal (e.g., selective mRNA enhancement of adipogenesis markers in subcutaneous and
44 of inflammation in visceral fat pads) and replacement challenges. Importantly, the pollutant
45 mixture impacted lipid deposition and mRNA expression of several genes related to lipid
46 metabolism but not *Esr1* in the liver. Adiponectin levels were altered as well. In addition, the
47 mRNA abundance of the various estrogen receptors was regionally impacted in fat tissues.
48 Besides, xenobiotic processing genes did not change in response to the pollutant mixture, in
49 the liver. The present findings bring new light on estrogen-dependent metabolic alterations
50 with regards to situations of loss of estrogens as observed after menopause.

51

52

53 **Bullet points:**

54 Estrogens differentially impacted hepatic glucose and lipid metabolism in female mice

55 Estrogens differentially impacted visceral and subcutaneous fat depots

56 Exposure to pollutants altered lipid deposition in liver and adiponectin levels

57 Pollutants impacted E2-regulated gene mRNA levels in a tissue-dependent manner

58 Mixture contained pollutants at the tolerable daily intake dose

59

60

61 **1. Introduction**

62 Obesity-related metabolic dysfunctions constitute a major problem in Public Health. Over
63 1.9 billion adults were overweight, of those 650 million were obese in 2016 placing them at
64 high risk for metabolic diseases including type 2 diabetes [1]. Risk of obesity is not equal
65 between males and females. Sex differences appeared as the population aged. In 2005, 16-
66 17% of women *vs* 10-11% of men over 50 were obese [2]. Risk of obesity in females is
67 associated with metabolic changes occurring during the menopausal transition characterized
68 by progressive reduction of ovarian estrogen production. According to different studies, the
69 prevalence of the metabolic syndrome which is a cluster of conditions of dyslipidemia, central
70 adiposity, hypertension, abnormal glucose metabolism and insulin resistance is enhanced
71 among postmenopausal women varying between 30 to 70% compared with 14-45% in
72 younger women [3] delineating the post-menopausal women as a vulnerable population.

73 Metabolic disorders are multifactorial diseases. Besides genetic predispositions and
74 imbalance between energy intake and expenditure, environmental pollutants have been
75 suggested to be etiologic factors as well, based on robust epidemiologic data and experimental
76 studies demonstrating changes in body weight and/or insulin resistance [4-7]. Environmental
77 pollutants may include persistent chemicals which bioaccumulate in the food chain. They are
78 especially present in fatty foods of animal origin (meat, milk, fish) and characterized by half-
79 lives of several years in humans, e.g. dioxins and polychlorobiphenyls [8]. Other pollutants
80 may have short half-lives e.g. phthalates and bisphenols. However, as these chemicals are
81 massively used in plastic goods, the leaching from packaging causes contamination resulting
82 in a chronic exposure [9, 10]. Pollutants are under regulation by international risk assessment
83 evaluation bodies (e.g., the European Food Safety Agency, EFSA). However, the cocktail
84 effect resulting from the chronic exposure to a myriad of chemicals originating from different
85 sources (foods and beverages, air breathing, skin absorption of cosmetics) and to which the
86 human population is involuntary exposed is underappreciated [11-13]. Of striking importance
87 are chemicals identified for their ability to interfere with hormonal action, the so-called
88 endocrine disruptors [14]. Populations vulnerable because of hormonal deficits such as post-
89 menopausal women may thus be at particular risk when exposed to these chemicals. It has
90 consistently been found that among a population of obese and post-menopausal women,
91 plasma levels of persistent organic pollutants (POPs) were associated with insulin resistance
92 [15].

93 The estrogeno-mimetic properties of endocrine disruptors are of particular concern.
94 Characterizing their mechanisms of actions could help to explain their deleterious impact on
95 the reproductive functions but also on metabolic disorders [10]. Indeed, it has been well
96 demonstrated that estrogens exert protective roles against metabolic diseases in addition to
97 regulating reproductive functions. They are for instance known to positively regulate glucose
98 homeostasis and lipid metabolism favoring insulin sensitivity [5, 16-18]. All these beneficial
99 effects progressively disappear with menopause. Estrogens act through specific receptors
100 distributed centrally and peripherally including ER α , ER β and the G-protein-coupled receptor
101 GPR30 which differ in abundance and tissue distribution in a sex-dependent manner [16, 19,
102 20]. Non-genomic actions of estrogens as well as cross-talk with various nuclear receptors
103 and/or transcription factors have also been evidenced [21, 22]. All these signaling pathways
104 result in pleiotropic actions of estrogens in the peripherally and metabolically active tissues
105 including the liver and the adipose tissues which express the estrogen receptors. Thus, these
106 signaling pathways are potential targets for endocrine disruptors in a manner dependent on the
107 age, the sex and the tissue considered [4, 23, 24].

108 We previously demonstrated that a mixture of low-dose pollutants made of dioxin, PCB,
109 phthalate and bisphenol A (BPA) could interfere with estrogen signaling and trigger hepatic
110 insulin resistance and aggravation of glucose intolerance in female mice fed a high-fat high-
111 sucrose diet [25]. In addition, mice fed a standard diet but exposed to the same mixture of
112 pollutants exhibited enhancement of hepatic triglycerides content and insulin resistance [26].
113 The impact of this pollutant mixture depended on the age of the mice, with differences
114 occurring between mature and immature females [25, 27]. However, the metabolic impact of
115 the mixture of pollutants in conditions of hormonal deficit after ovariectomy to mimic
116 menopause and hormonal replacement using 17 β estradiol (E2) pellets implanted
117 subcutaneously, has not yet been explored. The purpose of the current study was to fill this
118 gap. After characterizing the metabolic phenotypes of liver, subcutaneous and visceral
119 adipose tissues in ovariectomized mice, we delineated the estrogenic component using
120 hormonal replacement.

121

122 **2. Materials and methods**

123

124 *2.1. Animals, diets and experimental design*

125 A schematic view of the 3 experimental protocols is described in Supplemental Figure 1.
126 C57Bl/6 female mice (4-week-old) were purchased from Envigo laboratories (Gannat,
127 France), housed by 2 in polypropylene cages (to avoid BPA leaching from polycarbonate
128 cages) at 21°C with a normal light-dark cycle and free access to water (polypropylene
129 bottles) and standard chow (LASvendi-16R; Soest, Germany). After one week of
130 acclimatization, mice of the experimental protocol 1 were fed a high fat-high sucrose (HF)
131 diet (from Envigo) containing (HFp) or not (HF0) a mixture of pollutants (see below for
132 the details). At 7 weeks of age, mice were sham-operated (HF0 sham) or underwent a
133 bilateral ovariectomy after general anesthesia, as previously described [28] followed by
134 the implantation, under the neck skin, of Silastic implants filled either with vehicle (HF0
135 Ovx and HFp Ovx) or with 50µg of 17β-estradiol-3-benzoate, E2 (Sigma-Aldrich, Saint-
136 Quentin Fallavier, France) in 30µl of sesame oil (HF0 Ovx+E2 and HFp Ovx+E2) as
137 described [29]. The implants were renewed at 13 weeks of age (8 weeks of diet) as the
138 release of E2 was effective only 6 weeks based on body weight follow-up performed in a pilot
139 experiment (data not shown). In the course of the study, two other protocols were
140 conducted to assess the individual effects of the HF diet and of the ovariectomy
141 (Supplemental Figure 1). All procedures were performed with the approval of the
142 Regional Committee of Ethics for Animal Experiments and the French Ministry for
143 Higher Education and Research. Body weight (for all protocols) and food intake (for
144 protocol 1) were recorded weekly throughout the protocol until mice were 20 weeks of
145 age. Validation of the surgery was assessed at the time of mouse euthanasia with the
146 observation of uterine atrophy in the Ovx females (measurement of uterus weight;
147 Supplemental Figure 2). Food intake measured in Protocol 1 did not change between
148 groups, regardless of treatments (data not shown).

149 The pollutant mixture was made of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, CAS
150 n° 1746-01-6 from LGC-Promochem, Molsheim, France), polychlorinated biphenyl (PCB)
151 153 (CAS n°35065-27-1), bisphenol A (BPA, CAS n°80-05-7) and di-[2-ethylhexyl]-
152 phthalate (DEHP, CAS n°117-81-7) (all three from Sigma-Aldrich). Each pollutant was
153 used at a dose close to its tolerable daily intake (TDI) reference dose of either the
154 pollutant itself (DEHP, BPA) or representative congeners (TCDD, PCB153) [25].
155 Pollutants were dissolved in dimethylsulfoxide (DMSO) and diluted in corn oil to
156 facilitate uniform homogenization in the food and the making of pellets. Pollutant-free
157 diet contained identical volumes of DMSO and corn oil as the pollutant-containing food.

158 HF0 and HFp diets contained 63% fat, 23.6% carbohydrate and 13.4% protein in kcal, as
159 detailed previously [26]. To globally ensure the daily exposure to 2pg/kg bw/d of TCDD,
160 80 ng/kg bw/d of PCB153, 50µg/ kg bw/d of DEHP and 5 µg/ kg bw/d of BPA 1 g of
161 contaminated food per 17 g of body weight was given per day to mice otherwise fed ad
162 libitum as previously described [25, 27].

163 2.2. *Metabolic tests*

164 After 13 wks of diet, 16 h-fasted mice were injected intraperitoneally with glucose (1
165 mg/g of body weight) for the glucose tolerance test (GTT). Blood glucose was measured
166 using the Accu-Chek Performa glucometer (Roche Diabetes Care France, Meylan).

167 2.3. *Body composition, blood and tissue collection*

168 One day before euthanasia, weight and whole body composition were analyzed using
169 nuclear magnetic resonance (Minispec TD-NMR, Bruker BioSpin). Following a 6 hour
170 fasting, blood was collected by retro-orbital sampling. Mice were euthanized by cervical
171 dislocation and liver, inguinal subcutaneous (SAT) and visceral (periovarian+parametrial,
172 VAT) adipose tissue were quickly dissected, snap-frozen in nitrogen liquid and conserved
173 at -80°C until experiments. Small pieces of SAT and VAT were fixed in 4% buffered
174 formaldehyde and embedded in paraffin for histology studies.

175 Blood glucose concentrations (Accu-Chek Performa glucometer) as well as plasma
176 levels of insulin (Mouse Ultrasensitive ELISA, Eurobio), triglycerides (Biolabo, Maizy,
177 France), cholesterol, free fatty acids (FFA), alanine aminotransferase (ALAT) and aspartate
178 aminotransferase (ASAT) activities (Sigma-Aldrich), leptin and adiponectin (Crystal
179 Chem, Zaandam, Netherlands) were measured. Total cholesterol and triglycerides were
180 also measured in the liver after extraction of lipids from frozen liver samples using a
181 lipid Extraction kit chloroform-free (Clinisciences, Nanterre, France).

182 *Adipose tissue histology*

183 Paraffin-embedded adipose tissues were cut and adipocyte area was assessed by
184 immunostaining of the basal membrane realized on 4µm sections using the rabbit anti-
185 laminin antibody (1:100, Merck, L9393). Image acquisition was obtained with the
186 AXIOVERT 200 M inversed fluorescent microscopes (Carl Zeiss S.A.S., Marly le Roi,

187 France) equipped with the AxioVision 4.8.2 software. Analyses were performed using the
188 ImageJ software.

189 2.4. *Real time PCR analysis*

190 Total RNA extracted from frozen tissue samples was reverse-transcribed and
191 analyzed by real-time PCR as described [30] using a set of specific primers
192 (Supplemental Table 1). In each tissue data were normalized relatively to TBP (encoding
193 TATA-Box Binding Protein).

194 2.5. *Statistical analysis*

195 All results are expressed as means \pm SEM and differences between means were
196 considered significant at p -value <0.05 . To test for statistical significance, one-way
197 ANOVA followed by Tukey post hoc test was used when comparing HF0 sham, HF0
198 Ovx and HF0 Ovx+E2 groups. To test for pollutant effects, two-way ANOVA for non-
199 repeated measures with E2 and pollutants as factors was used, followed by a Bonferroni
200 post hoc test. When significant effects without interaction were obtained, differences
201 were tested using Student's t-test between the pollutant-exposed group and the non-
202 exposed group. GraphPad Prism 5.0 software was used for all statistical analysis.

203

204 3. Results

205

206 3.1. *Metabolic characterization of sham and ovariectomized females fed a HF diet and* 207 *effect of 17 β estradiol replacement*

208 Sham-operated (sham) and ovariectomized (Ovx) females having received E2 or vehicle
209 supplementation, all fed a HF diet were thoroughly characterized (Protocol 1). Non-operated
210 females fed either a standard diet or a HF diet were monitored in parallel (Protocol 3). As
211 expected, females fed the HF diet were significantly heavier than ST fed mice with high
212 levels of blood glucose and plasma insulin levels resulting in a 3-fold increase of the HOMA-
213 IR (Fig. 1A-B; Supplemental Figure 3) which is an index of insulin resistance. Ovariectomy
214 induced an additional increase of body weight and fat mass, a hyperglycaemia after a 16h
215 fasting (time 0 of the GTT) with a trend after a 6h fasting (blood sample at euthanasia), and
216 elevated insulin plasma levels, all resulting in glucose intolerance (GTT) and insulin

217 resistance (enhanced HOMA-IR) (Fig. 1). Conversely, triglycerides (TG) and cholesterol
218 levels (both plasma and liver) were not significantly impacted by ovariectomy (Table 1).
219 These metabolic features were also observed in Protocol 2 in which ovariectomy was
220 performed in mice fed a standard diet (Supplemental Figure 4). Neither ovariectomy nor E2
221 treatment induced liver toxicity as there was no increase in plasma AST or ALT in HF0 Ovx
222 and HF0 Ovx+E2 mice (data not shown).

223 Estradiol supplementation to HF0 Ovx mice led to a restoration of insulin and FFA plasma
224 levels as well as glucose tolerance to values measured in HF0-sham mice (Fig. 1; Table 1).
225 Other metabolic parameters including glycaemia, body weight, VAT and SAT weight, plasma
226 cholesterol (total and cholesteryl esters) and leptin levels were significantly lower than those
227 measured in HF0 sham mice (Fig. 1 and Table 1). For some parameters, the range of values
228 was identical to those in standard-fed mice (Fig. 1). Besides, impedance analyses revealed
229 enhanced lean mass and reduced fat mass in E2-replaced Ovx mice compared to HF-fed sham
230 mice (Fig. 1D) which is probably associated to the reduced adipocyte diameter observed in
231 both AT depots (Fig. 1D and Supplemental Figure 5) with E2 replacement. Moreover,
232 adiponectin levels, not affected by ovariectomy, were reduced in E2-replaced females (Table
233 1) as reported elsewhere [31].

234

235 ***3.2. Fat depots were differently affected in response to ovariectomy and 17 β estradiol*** 236 ***supplementation***

237 The metabolic explorations showed that the adipose tissue was highly responsive to
238 ovariectomy and E2 replacement consistent with estrogens playing major roles in fat tissue
239 deposition and expansion in females [16, 32]. To go further in-depth, we analyzed by RT-
240 qPCR a panel of genes encoding the estrogen receptors and markers of adipogenesis or
241 inflammation. We observed that the expression level of *Esr1* and *Esr2* genes encoding the
242 nuclear estrogen receptor (ER) α and β , respectively and *Gper1* encoding the G protein
243 coupled estrogen receptor 1 (GPR30) responded differently to estrogen withdrawal and
244 replacement. *Esr1* mRNA levels were unchanged in Ovx females but significantly increased
245 (2-fold) upon E2 replacement in both SAT and VAT. *Gper1* and *Esr2* mRNA levels were
246 significantly higher and lower, respectively, in Ovx than in sham females. E2 replacement
247 corrected these effects in SAT but not in VAT (Fig. 2). The pattern of expression of *Nr3c4*
248 (encoding the androgen receptor) and *Esr1* mRNA was quite similar in SAT and VAT (i.e.,

249 almost no change following ovariectomy and augmentation following E2 replacement relative
250 to sham mice) (Fig 2). Additionally, ten-fold more *Esr1* and 1.5-fold more *Nr3c4* mRNA
251 levels were found in VAT than in SAT, and 2-fold more *Gper1* mRNA levels were found in
252 SAT than in VAT. As for *Esr2*, mRNA levels were low in both fat pads with large
253 fluctuations in the VAT of sham mice (Fig. 2).

254 Plasma leptin levels of Ovx females (Table 1) which correlated with obesity were consistent
255 with the increase of *lep* mRNA levels. They returned to sham levels in SAT and VAT of E2-
256 treated mice (Fig. 3). A similar pattern of mRNA expression was observed for *Ccl2* which
257 encodes the monocyte chemoattractant protein 1 (MCP1) along with estrogen loss and
258 recovery challenges in both fat pads (Fig. 4). Conversely, fluctuations in level of
259 adipogenesis, lipogenesis, lipolysis and inflammation markers were found to vary in fat
260 depots according to body localization i.e., subcutaneous or visceral (Fig. 3, 4). Specifically,
261 we surveyed *Nr1c3* encoding peroxisome proliferator-activated receptor PPAR γ , *Fabp4*
262 encoding fatty-acid binding protein4, *Lipe* encoding hormone-sensitive lipase (HSL), *Lpl*
263 encoding lipoprotein lipase (LPL), *Pnpla2* encoding Patatin Like Phospholipase Domain
264 Containing 2 also known as Adipose triglyceride lipase (ATGL) and inflammatory cytokines,
265 *Tnfa*, *Il1 β* and *Il12a* encoding tumor necrosis factor (TNF) α and the interleukins 1 β and 12a,
266 respectively. On the one hand, no variations were observed in the level of *Nr1c3*, *Pnpla2*,
267 *Tnfa* and *Il12a* mRNA in SAT across the groups, while the same genes were up-regulated in
268 VAT in the Ovx group. Specifically, VAT samples from E2-replaced females had higher
269 mRNA levels of *Nr1c3* and *Pnpla2* than samples from Ovx females which was consistent
270 with PPAR γ being a direct transcriptional regulator of ATGL [33] while inflammatory marker
271 mRNA levels increased in Ovx females and returned to sham levels in E2-replaced females
272 (Fig. 3, 4). On the other hand, *Fabp4*, *Lipe* and *Lpl* had a profile of gene expression consistent
273 with estrogens negatively regulating these genes in SAT but not in VAT samples (Fig. 3, 4).
274 Regarding *Il1 β* , the profile was identical to the other inflammatory markers in VAT while the
275 mRNA levels were enhanced following E2 replacement in SAT.

276

277 3.3. Estrogens differentially impact glucose and lipid metabolism in the liver

278 Estrogens act in liver to positively regulate insulin sensitivity and glucose tolerance [16]. ER α
279 being the primary estrogen receptor expressed in mouse liver [34], we explored the hepatic
280 expression of the *Esr1* gene in ovariectomized females. We observed a significant decrease

281 of *Esr1* mRNA levels (Fig. 5A). Interestingly, in E2-replaced Ovx mice a significant doubling
282 in *Esr1* mRNA levels was measured compared to those detected in sham mice. We next
283 performed RT-qPCR analyses to discriminate, among genes regulating glucose and lipid
284 metabolism, those under negative and positive estrogen regulation. Consistent with E2
285 positively regulating glucose tolerance, the expression levels of *G6pc* encoding the catalytic
286 subunit of the gluconeogenic enzyme G6Pase (glucose-6 phosphatase) doubled in Ovx mice
287 and was restored in E2-replaced females, relative to the level of sham mice (Fig. 5B).
288 Expression levels of *Gck* encoding Glucokinase, mirrored those of *G6pc* (Fig. 5B) consistent
289 with an adaptive role of *Gck* in counteracting hyperinsulinemia and insulin resistance and
290 with the reduction of glycaemia [35].

291 To complete the study and although we did not observe significant changes in hepatic TG and
292 cholesterol levels in ovariectomized mice (Table 1), we undertook a comprehensive analysis
293 of genes encoding proteins involved in hepatic lipid metabolism. Specifically, genes surveyed
294 included *Nr1h3* encoding Liver X receptor (LXR) α and its downstream target gene *Srebf1*
295 encoding sterol regulatory element binding protein 1c (SREBP1c), a master regulator of *de*
296 *novo* lipogenesis; *Scd1* encoding the stearoyl-CoA desaturase-1 (SCD1), a rate-limiting
297 enzyme of lipogenesis; *Dgat2* encoding diacylglycerol O-acyltransferase 2 which catalyzes
298 the final reaction in the synthesis of triglycerides; *Cd36* encoding Cluster of differentiation 36
299 involved in fatty acid uptake; *Nr1c1* encoding peroxisome proliferator-activated receptor
300 (PPAR) α which regulates fatty acid oxidation and its target gene *Cpt1a* encoding carnitine
301 palmitoyltransferase 1 α ; *Srebf2* which encodes the transcription factor Sterol Regulatory
302 Element Binding Transcription Factor 2 and its downstream target genes *Hmgcr* and *Pcsk9*
303 encoding 3-Hydroxy-3-Methylglutaryl-CoA Reductase, the rate limiting enzyme of
304 cholesterol metabolism and Proprotein convertase subtilisin/kexin type 9 which regulates
305 lipoprotein metabolism, respectively. We surveyed *Ldlr* encoding the low density lipoprotein
306 receptor which mediates the endocytosis of cholesterol-rich LDL; *Nr1h4* encoding the bile
307 acid receptor Farnesoid X receptor (FXR), and *Cyp7a1* encoding the cholesterol 7 α
308 hydroxylase which is the rate-limiting enzyme in the synthesis of bile acids [36].

309 On this panel of genes, *Nr1h4* was unique in that the mRNA level was enhanced by estrogen
310 withdrawal compared to sham mice. There was reduction in mRNA abundance of *Srebf1*,
311 *Dgat2* and *Ldlr* in the Ovx mice (Fig. 5). All returned to sham levels after E2 replacement.
312 Lower *Nr1c1* and *Cd36* mRNA levels were found in E2 replaced mice than in sham mice
313 (Fig. 5); mRNA levels of *Nr0b2* encoding small heterodimeric partner (SHP) and those of

314 *Cpt1a*, *Nr1h3*, *Scd1* remained the same between the different groups (data not shown). In
315 addition, several genes belonging to the cholesterol metabolism pathway had their mRNA
316 levels strongly increased following E2 supplementation. This included *Srebf2* and the target
317 genes *Hmgcr*, *Pcsk9* and *Cyp7a1* (Fig. 5E).

318

319 ***3.4 A low-dose mixture of pollutants differentially impacted the expression levels of E2-*** 320 ***regulated genes depending on the metabolic tissue explored***

321 The expression levels of estrogen receptors and estrogen-regulated genes were analyzed in the
322 liver and adipose tissues of mice exposed to a mixture of low-dose pollutants previously
323 shown to exhibit estrogeno-mimetic activities [25, 27, 28].

324 Metabolic exploration revealed that exposure to pollutants did not change food intake (not
325 shown), body weight and fat pad weight of the exposed animals (Supplemental Figure 6). No
326 effect of pollutants was observed on blood glucose and plasma insulin levels, HOMA-IR,
327 glucose tolerance, as well as on TG (plasma and hepatic) and FFA levels (Table 1).

328 Notwithstanding, cholesterol (plasma and hepatic) and leptin levels were significantly
329 increased in HFp-Ovx females compared to HF0 sham mice while these parameters were not
330 significantly changed by ovariectomy itself arguing for a pollutant effect. Moreover, liver TG
331 accumulation in HFp Ovx mice was 1.5-fold higher than in HF0 Ovx mice although it did not
332 reach significance. In addition, its level significantly decreased following E2 replacement
333 (HFp Ovx+E2 *versus* HFp Ovx) which was not observed in pollutant-free conditions (Table
334 1). Adiponectin levels were significantly different in HFp Ovx+E2 *versus* levels measured in
335 HF0-Ovx+E2 mice which also indicated a pollutant effect (Table 1).

336 Regarding gene expression, exposure to pollutants resulted in the increase of *Esr1* mRNA
337 levels in SAT and in the ratio of *Esr1* to *Nr3c4* expression in SAT and VAT of Ovx mice
338 (Fig. 6). In conditions of E2 supplementation, effects of pollutant exposure included the
339 augmentation of *Esr2* mRNA levels in both fat pads as well as *Esr1* /*Nr3c4* ratio in SAT (HFp
340 Ovx+E2 *versus* HF0 Ovx+E2) (Fig. 6). Among genes involved in cholesterol metabolism, we
341 observed a significant doubling of *Nr1h3*, *Nr0b2* and *Cd36* in pollutant-exposed Ovx mice
342 compared to non-exposed Ovx mice. Interestingly, in E2-replaced females, pollutant exposure
343 triggered significant changes in *Cyp7a1* (half-decrease) and *Nr1h3* (a doubling) mRNA levels

344 (Fig. 6C). These pollutant-induced effects were different than those exerted by E2
345 replacement (Fig.4).

346 To complete the study, we measured the expression levels of genes encoding xenobiotic
347 receptors including the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor
348 CAR (Nr1i3) and the pregnane xenobiotic receptor PXR (Nr1i2) as well as CAR and PXR
349 immediate target genes which encode the cytochrome P450 enzymes CYP2B10 and
350 CYP3A11 [37]. These genes were not found impacted by the pollutant mixture as compared
351 to non-exposed mice except *Cyp2b10* which mRNA abundance slightly increased
352 (Supplemental figure 7).

353

354 **Discussion**

355 In the present study, we developed a model allowing the exploration of the metabolic
356 consequences of ovarian withdrawal and E2 replacement on liver and adipose tissues along
357 with investigating the impact of a low-dose mixture of pollutants previously reported to
358 exhibit some estrogeno-mimetic properties in female mice [25, 27]. Because of differences in
359 the literature regarding the extent of the ovariectomy-induced deleterious metabolic effects,
360 the experimental model developed herein was first characterized. Accordingly [17, 19, 38-40],
361 ovariectomy induced body weight increase, glucose intolerance and insulin resistance and
362 tended toward an increase of leptin and insulin plasma levels. No changes in TG plasma
363 levels and cholesterol or in hepatic lipid deposition could be measured in the ovariectomized
364 mouse model.

365 Importantly, treatment of ovariectomized mice with E2 either reversed the ovariectomy-
366 induced adverse effects (e.g., glucose tolerance) to sham levels or improved metabolic
367 functions over the physiological levels (e.g., adipose tissue weight and leptin levels). It
368 suggested that diet-induced obesity could impair estrogenic signaling in the adipose tissues in
369 female mice and that part of the HF effects were counter-balanced by E2 supplementation,
370 extending previous data [31]. Importantly, while ovariectomy and E2 replacement similarly
371 regulated the ERs and AR encoding mRNA expression in SAT and VAT depots, adipogenesis
372 and inflammatory markers were differently regulated in response to estrogen withdrawal and
373 E2 replacement. Specifically, our findings were consistent with estrogens negatively
374 regulating adipogenesis in SAT but not in VAT, and inflammation in VAT but not in SAT. In

375 addition, enhanced *Nr1c3* and *Atgl* mRNA expression in VAT, two markers of adipocyte
376 differentiation, suggested a positive effect of E2-treatment on this tissue in Ovx mice.
377 Extending the literature [41, 42], the herein findings underlined distinct functions of SAT and
378 VAT in the development of obesity-induced metabolic disorders in females, with estrogens
379 regulating fat distribution and expansion preferably in subcutaneous depots and opposing
380 inflammation particularly in VAT thus protecting from insulin resistance. Noticeably, the
381 mRNA abundance changes of the estrogen receptors induced by ovariectomy and E2
382 replacement emphasized that, in physiological conditions, circulating estrogens could regulate
383 *Gper1* and *Esr2* at a transcriptional level. In addition, if considering the reported phenotypes
384 of enhanced adiposity in *Esr2*-deficient female mice [43] and the protection from diet-induced
385 obesity in *Gper1*-deficient female mice [44], the alterations (herein observed) of *Gper1* and
386 *Esr2* expression in the adipose tissues could well be linked to the obesity phenotype of the
387 Ovx mice. The E2 replacement data suggested that the up-regulation of *Esr1* and *Nr3c4*
388 expression in both fat pads and the restored *Esr2* and *Gper1* mRNA abundance (at least in
389 SAT) could be linked to the observed decreased in adiposity in line with previous data [19,
390 43, 45]. Moreover, the data presented herein indicated that the described estrogen anti-
391 inflammatory properties [19, 43] could be mediated through ER β in VAT extending previous
392 reports in female mice [46]. Interestingly, SAT and VAT share identically regulated genes
393 including *Ccl2* (in line with previous findings [28]) and *Lep* which could be positively
394 regulated by estradiol in adipose tissues via *Gper1* or negatively via ER β . Such regulations
395 remain to be demonstrated.

396 Within the present study, ovariectomy clearly impacted glucose metabolism but not
397 hepatic steatosis while E2 replacement resulted in altered cholesterol metabolism concurrently
398 with marked changes in liver *Esr1*, *Nr1c1* and *Cd36* mRNA abundance linking estrogen
399 signaling to these effects. It could indicate that impairment of glucose metabolism might be an
400 early metabolic consequence of estrogen loss while alteration of lipid metabolism could be
401 indirectly affected. This hypothesis is consistent with the phenotype of hepatosteatosis
402 observed after a long-term ovariectomy [47] but not in the present study. The finding that TG
403 accumulation did not change with ovariectomy is in agreement with the observation that the
404 mRNA levels of FXR, a protein known to oppose hepatic steatosis and hyperlipidemia [48],
405 was increased while the abundance of genes related to lipogenesis (*Srebp1*, *Dgat2*) was
406 reduced. Noticeably, the impairment of cholesterol metabolism observed following E2
407 supplementation extended previous data demonstrating that *Esr1* overexpression could trigger

408 a loss in the fine tuning of negative feedback regulation of cholesterol synthesis [49].
409 Deregulation of lipid metabolism may as well result from the lack of cycles in E2-replaced
410 females since it has been demonstrated that reproduction and lipid metabolism are tightly
411 coupled in cycling females [47]. Whether changes in the mRNA levels of *Esr1*, *Nr1c1* and of
412 *Cd36* in the E2-replaced Ovx mice also resulted from a loss in feed-back regulatory
413 mechanism will await further studies. Collectively, it could be concluded that regulation of
414 glucose metabolism is tightly controlled by estradiol but that lipid metabolism escaped
415 physiological regulatory feedback in E2-treated females. In addition, because ovaries produce
416 progestogens as well as androgens, E2 replacement is not expected to normalize all disorders
417 induced by ovariectomy.

418 The use of a model of estrogen withdrawal and replacement has allowed the
419 identification of a set of estrogen-regulated genes. We expected these genes to exhibit
420 changes in mRNA levels in response to chronic exposure to a mixture of archetypal endocrine
421 disruptors previously identified as bearing some estrogeno-mimetic activities as well as
422 metabolic disrupting effects [25, 27, 50]. Specifically, the protocol used herein was adapted
423 from the published previous model [25, 26] except that female mice were not exposed to the
424 mixture of pollutants from fetal life onwards but continuously from week 5 to week 20
425 through diet. As reviewed previously [7, 51, 52], the mixture of pollutants was designed to
426 question the metabolic health consequences of a multi-exposure scenario to various pollutants
427 at low dosage to approach a real life situation. Specifically, the mixture was made of shortly
428 metabolized (BPA, DEHP) and persistent pollutants (TCDD, PCB153) contaminating food
429 either through bioaccumulation along the food chain (TCDD, PCB153) or leaching from
430 packaging (BPA, DEHP), activating a wide range of signaling molecules, not to mention the
431 many cross-talks occurring between them and triggering estrogeno-mimetic and anti-androgen
432 activities among others [4, 6, 10]. Pollutants constituent parts of the mixture were also chosen
433 because of high concern for links with metabolic diseases in epidemiological and
434 experimental studies [4, 7, 53-55]. Interestingly, we herein observed modest but significant
435 effects in the set-up model of Ovx and E2 replacement in response to the mixture of
436 pollutants. Phenotypically, and in agreement with our previous studies [25, 27, 50], the
437 mixture did not impact body weight. No changes were observed in glucose tolerance or
438 insulin resistance in the mice exposed compared to those not exposed to the pollutant mixture.
439 Nonetheless, adiponectin levels were found to be lower in E2-replaced and pollutant-exposed
440 mice than in E2-replaced and non-exposed females. This finding is indicative of an adverse

441 hormonal and metabolic effect triggered by pollutant exposure since adiponectin is an
442 adipokine with positive metabolic properties [56]. In addition, TG accumulation in liver of
443 HFp Ovx mice was trending upward compared to non-exposed Ovx mice. Some genes
444 involved in lipid metabolism in the liver and which mRNA levels were not modified by
445 ovariectomy, were found impacted in response to the mixture of pollutants in Ovx mice (i.e.,
446 HFp Ovx *versus* HF0 Ovx mice). All these findings indicated that the mixture of pollutants
447 had some metabolic impact but did not exert estrogeno-mimetic activities at least on the set of
448 hepatic genes studied. These data do not fully reproduce previous findings in which the same
449 mixture of pollutants was found to enhance *Esr1* gene expression in the liver of
450 ovariectomized females and alleviate glucose intolerance [28]. One possible explanation
451 resides in the timing of exposure to the mixture encompassing gestation and lactation in the
452 previous protocol [28] but not in the present study. Indeed, the maternal period has been
453 found to be a highly vulnerable period [57, 58]. For example, it was demonstrated that hepatic
454 metabolic gene expression in adult mice including *Esr1* was strongly impacted in mice
455 exposed *in utero* to BPA as compared to control mice [59]. In addition, the vulnerability of
456 the fetal period towards exposure to the presently used mixture of pollutants has been
457 evidenced in male mice [50].

458 Importantly, we observed that the response to the mixture of pollutants was highly
459 tissue-dependent extending our previous data [26, 50]. While we did not identify estrogeno-
460 mimetic activities of the pollutant mixture in the liver, the situation was clearly distinct in
461 SAT. Indeed, the estrogen receptors *Esr1* and *Esr2* were found regulated the same way by
462 either the pollutant mixture or E2 supplementation. Conversely, the mixture of pollutants
463 tended to oppose E2 effects in VAT with an up-regulation of *Esr2* and *Il12a* mRNA
464 abundance in E2-replaced mice. The meaning of these distinct effects depending on fat
465 depots, remains to be investigated but may be linked to the distinct functions of the fat depots
466 regarding adipogenesis and inflammation [41, 42]. In addition, it should be reminded that the
467 dosage of the mixture was based on the tolerable daily intake (TDI) doses defined empirically
468 by risk assessment agencies, e.g., the European Food Safety Authority (EFSA) or the U.S.
469 Food and Drug administration (FDA) [60], thus not expected individually to generate a
470 metabolic effect. Therefore, the herein demonstration that several genes were impacted (at
471 least at the mRNA level) by a mixture of low-dose pollutants together with previous data on
472 the adverse metabolic effects of low-dosed mixture [52], emphasizes that current legislation
473 on chemical risk assessment may underestimate the risk and should be updated.

474 In summary, analysis of female mice in conditions of withdrawal of estrogen, E2 replacement
475 or exposure to a mixture of pollutants with reported estrogeno-mimetic activities, extended
476 knowledge on the estrogenic regulation of glucose and lipid metabolism in the liver and of
477 adipogenesis and inflammatory markers in the fat depots. Importantly, this model highlighted
478 that the disrupting effect of the mixture of low-dosed pollutants which did not affect body
479 weight, glycaemia, plasma insulin and lipidemia was different in the liver and adipose tissues.
480 Noticeably, alterations concerned adiponectin plasma levels, estrogen receptors in the adipose
481 tissue and lipid metabolism in the liver. The present findings may have important implications
482 for the understanding of the estrogen-dependent metabolic alterations with regards to
483 situations of loss of estrogens as observed with menopause, especially if considering the
484 worldwide context of exposure to chemicals.

485

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637

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643 **Conflict of interest**

644 The authors have declared that no competing interests exist.

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647 **Legends**

648 **Figure 1:** (A) Body weight and weight of visceral and subcutaneous adipose tissues (VAT
649 and SAT, respectively) measured at the end of the protocols (after 6 h of fasting). (B)
650 Biochemical parameters: Blood glucose, plasma insulin and HOMA-IR. (C) Glucose
651 Tolerance test performed after 13 wks of diet and corresponding AUC. (D) Measurement of
652 whole body composition by NMR and of the adipocyte area.

653 Values are means \pm SEM with n=7-8. ***P<0.001; ** P<0.01; *P<0.05 (one-way ANOVA
654 followed by Tukey post hoc test). For the adipocyte area, values are means \pm SEM with n=3.
655 A significant overall effect was obtained using a Two-way ANOVA followed by the
656 Bonferroni test. Standard: standard diet; HF0: pollutant-free high-fat high-sucrose diet; Sham:
657 sham-operated mice; Ovx: ovariectomized mice; Ovx+E2: ovariectomized mice with E2
658 replacement.

659 **Figure 2:** Effect of ovariectomy alone (Ovx) or with 17 β -estradiol (E2) supplementation
660 (Ovx+E2) on the expression of genes encoding the estrogen receptors ER α , ER β , GPR30 and
661 the androgen receptor AR in subcutaneous (A) and visceral (B) adipose tissues (SAT and
662 VAT, respectively). Values are means \pm SEM with n=6-8. ***P<0.001; ** P<0.01; *P<0.05.
663 Sham: sham -operated mice.

664 **Figure 3:** Effect of ovariectomy alone (Ovx) or with 17 β -estradiol (E2) supplementation
665 (Ovx+E2) on the expression of genes related to adipogenesis and lipogenesis (A, A'),
666 lipolysis (B, B') in subcutaneous (SAT, A, B) and visceral (VAT A', B') adipose tissues.
667 Values are means \pm SEM with n=6-8. ***P<0.001; ** P<0.01; *P<0.0; Sham: sham-operated
668 mice.

669 **Figure 4:** Effect of ovariectomy alone (Ovx) or with 17 β -estradiol (E2) supplementation
670 (Ovx+E2) on the expression of genes encoding inflammatory markers in subcutaneous (SAT)
671 and visceral (VAT) adipose tissues. Values are means \pm SEM with n=6-8. ***P<0.001; **
672 P<0.01; *P<0.0. Sham: sham-operated mice.

673 **Figure 5:** Effect of ovariectomy alone (Ovx) or with 17 β -estradiol (E2) supplementation
674 (Ovx+E2) on the expression of genes encoding the nuclear estrogen receptor ER α (A), genes
675 related to glucose metabolism (B), fatty acid metabolism (C), triglyceride metabolism (D),
676 cholesterol metabolism (E) in liver. Values are means \pm SEM with n=6-8. ***P<0.001; **
677 P<0.01; *P<0.05. Sham: sham-operated mice.

678 **Figure 6:** Effect of the the mixture of pollutants on the expression of genes expressed in
679 subcutaneous (SAT, A), visceral (VAT, B) adipose tissues, or liver (C). Values (expressed as
680 fold-change relative to the HF0 group) are means \pm SEM with n=6-8. ***P<0.001; **
681 P<0.01; *P<0.05. Ovx: ovariectomized mice; Ovx+E2: ovariectomized mice with E2
682 replacement. HF0: pollutant-free high-fat high-sucrose diet; HFp: high-fat high-sucrose diet
683 containing the mixture of pollutants.

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689 **Table 1:** Biochemical parameters

	HF0 sham	HF0 Ovx	HF0 Ovx + E2	HFp Ovx	HFp Ovx + E2
Plasma					
Total cholesterol (mg/mL)	1.54 ± 0.06	1.72 ± 0.08	1.32 ± 0.09 ^β	1.84 ± 0.08	1.16 ± 0.17 ^β
Cholesteryl esters (mg/mL)	1.17 ± 0.04	1.31 ± 0.06	1.02 ± 0.07 ^β	1.38 ± 0.07	0.87 ± 0.12 ^β
Triglycerides (mM)	0.70 ± 0.04	0.63 ± 0.03	0.62 ± 0.10	0.73 ± 0.06	0.46 ± 0.05 ^β
Free Fatty Acids (μM)	325 ± 36	500 ± 43 ^α	352 ± 44 ^β	468 ± 50	281 ± 39 ^β
Leptin (ng/mL)	23.2 ± 3.8	32.3 ± 4.5	11.4 ± 1.2 ^{α,β}	41.3 ± 4.0	12.1 ± 1.9 ^β
Adiponectin (μg/mL)	14.7 ± 0.4	15.1 ± 0.4	12.6 ± 0.6 ^{α,β}	15.0 ± 0.8	9.82 ± 0.63 ^{β,γ}
Liver					
Cholesterol (μg/mg)	3.19 ± 0.13	3.59 ± 0.40	3.70 ± 0.21	3.67 ± 0.15	3.27 ± 0.16
Triglycerides (nmol/mg)	18.6 ± 3.6	25.8 ± 4.8	15.6 ± 1.4	38.6 ± 8.1	14.8 ± 1.7 ^β

690

691 Values are means ± SEM with n=6-8. (**α**): significant effect vs HF0 sham; (**β**) significant E2 effect;
 692 (**γ**) significant pollutant effect (using a one-way ANOVA followed by Tukey post hoc test).

693 HF0: pollutant-free high-fat high-sucrose diet; HFp: high-fat high-sucrose diet containing the
 694 mixture of pollutants; Sham: sham-operated mice; Ovx: ovariectomized mice; Ovx+E2:
 695 ovariectomized mice with E2 replacement.

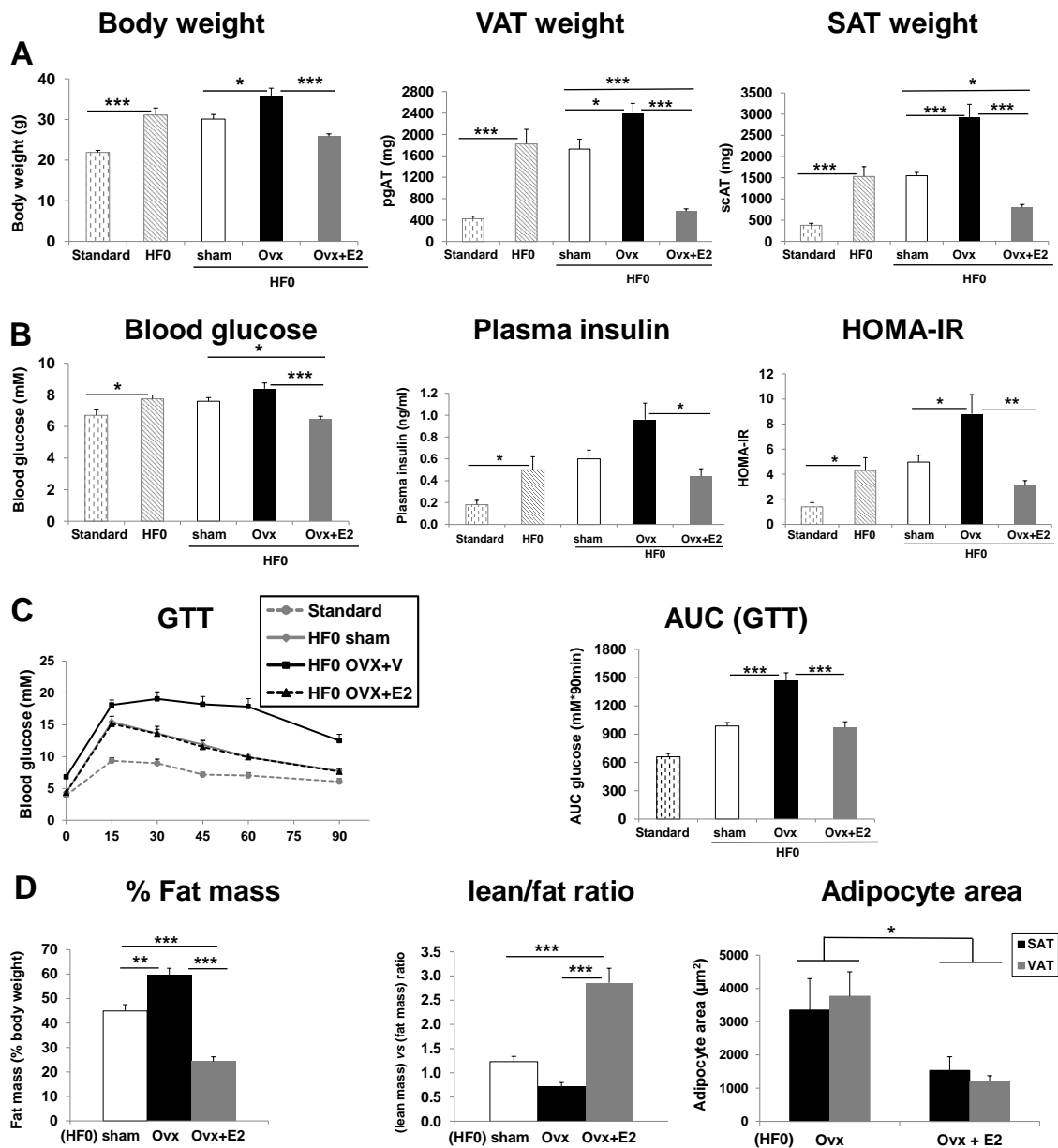


Figure 1

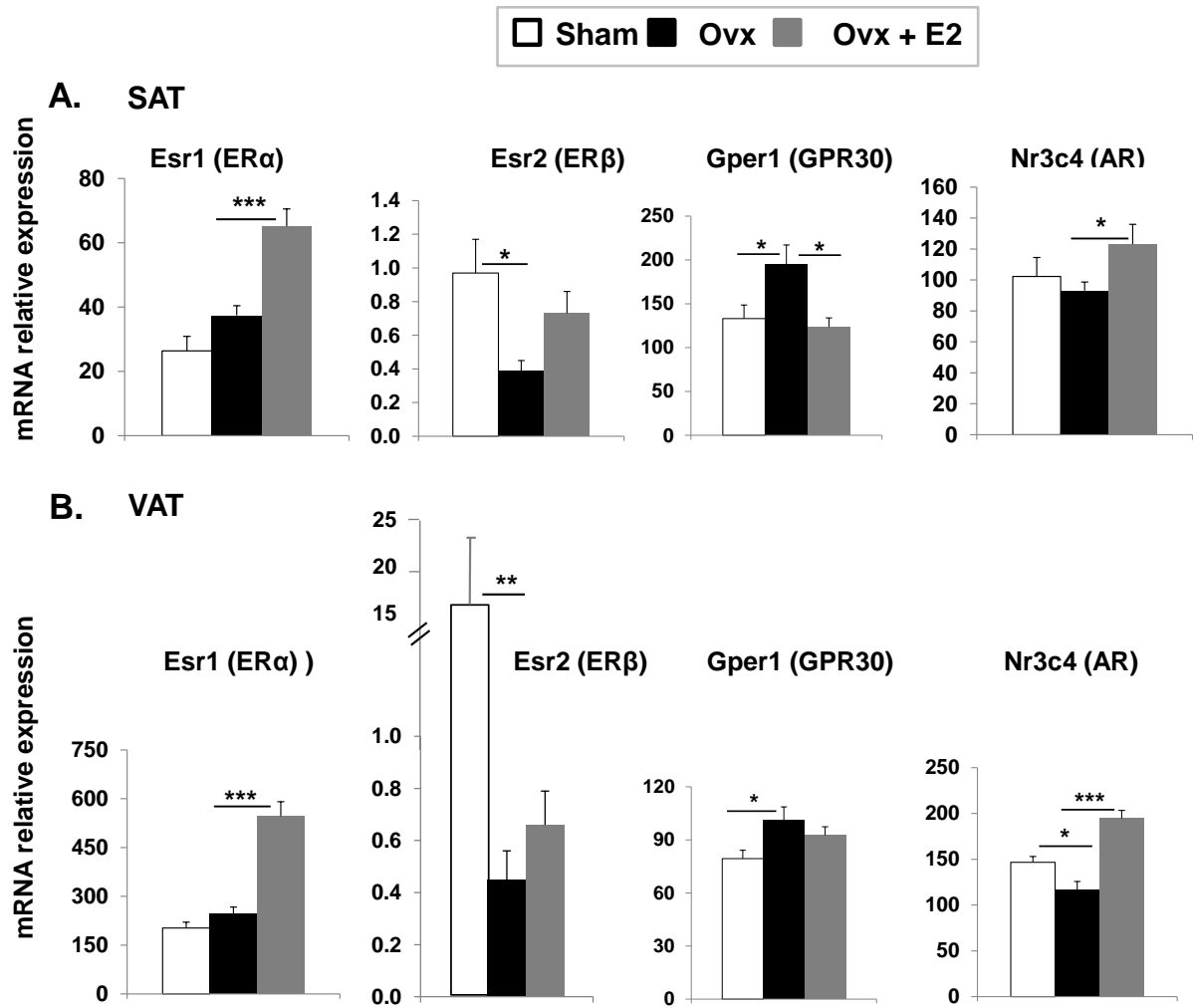


Figure 2

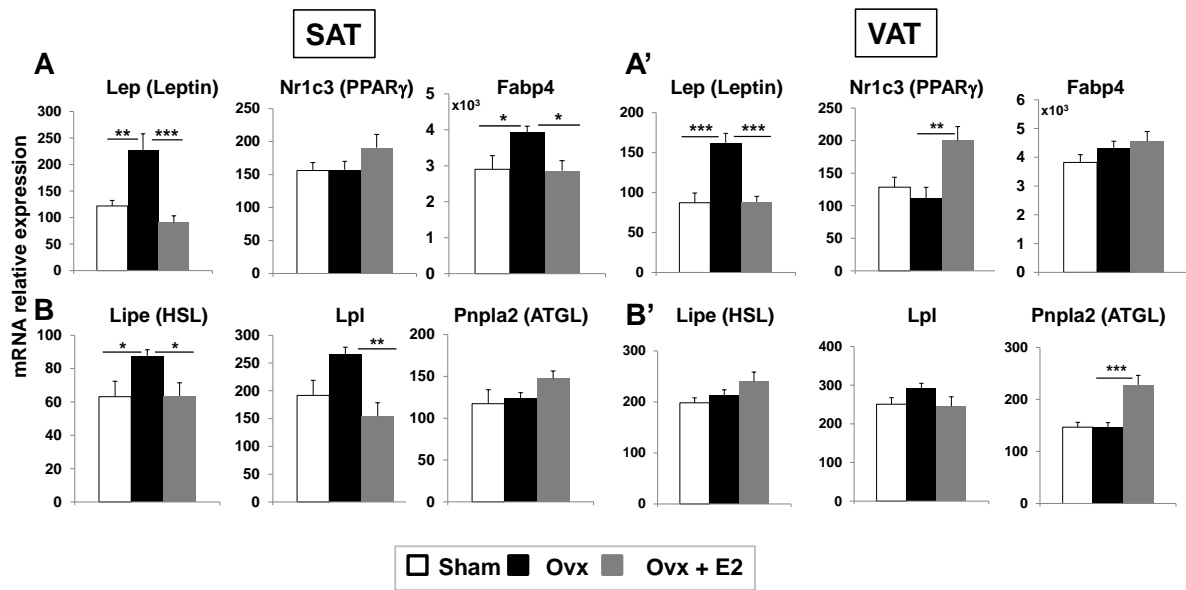


Figure 3

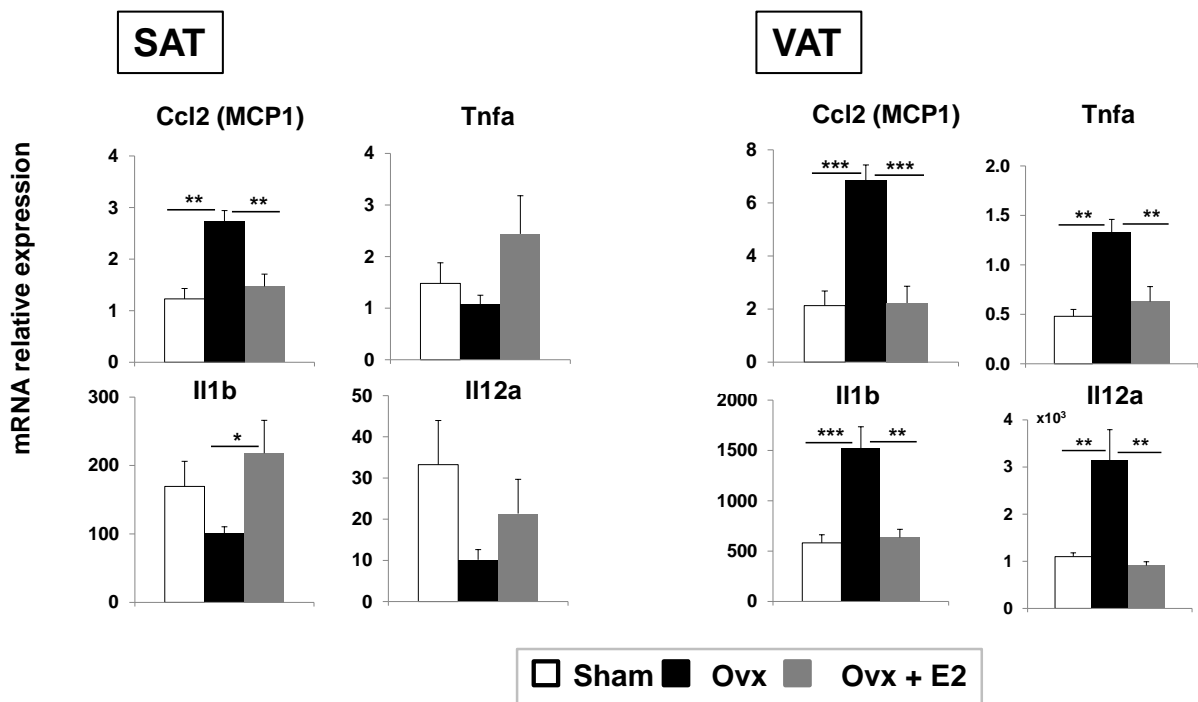


Figure 4

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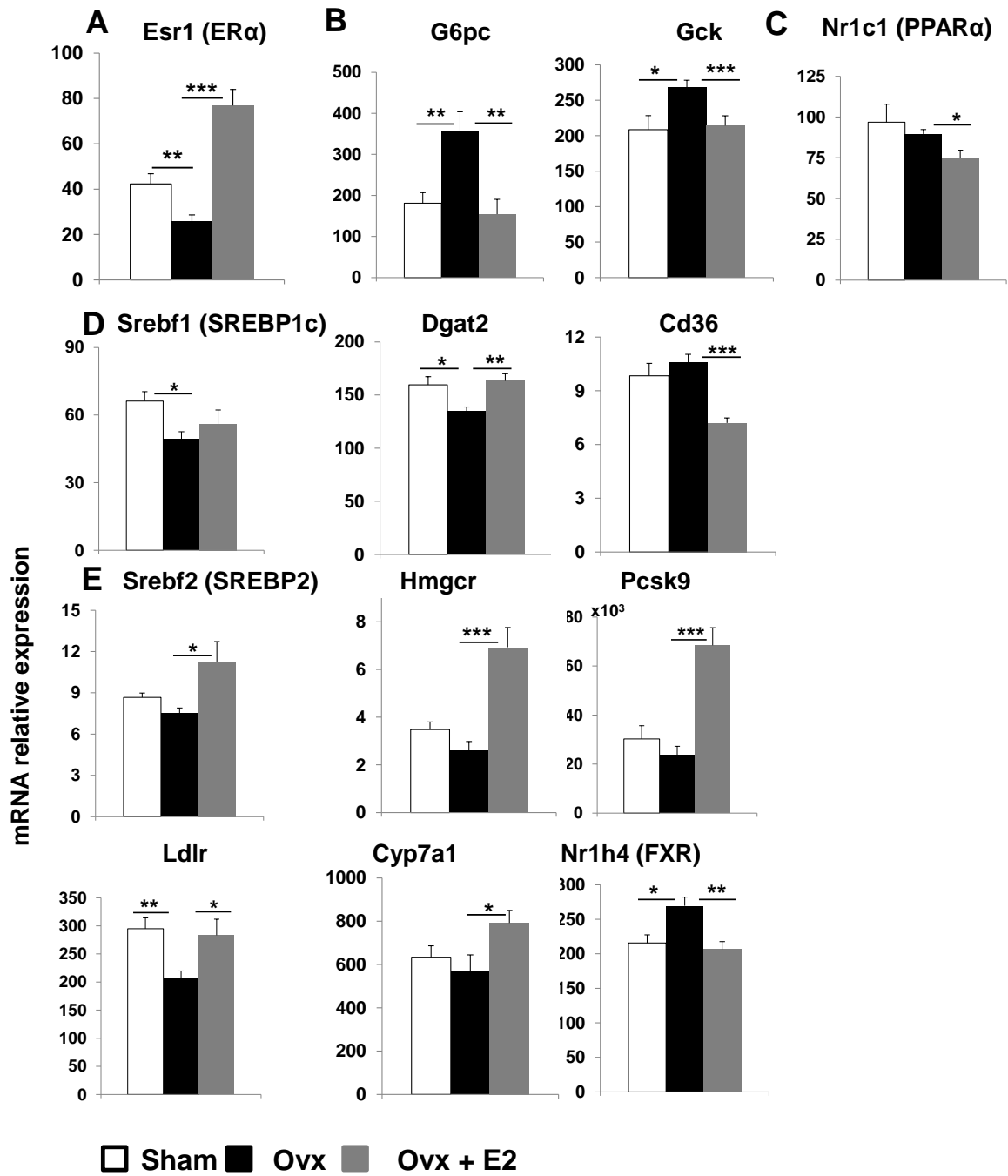


Figure 5

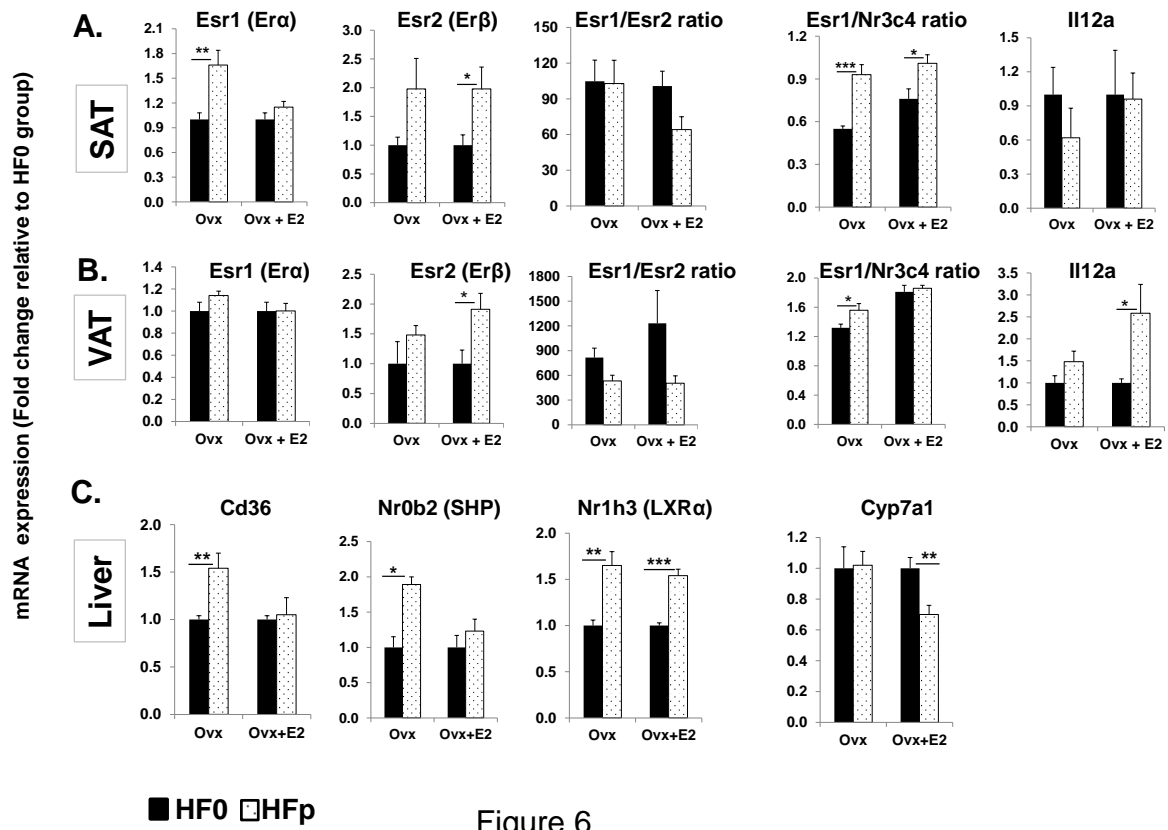
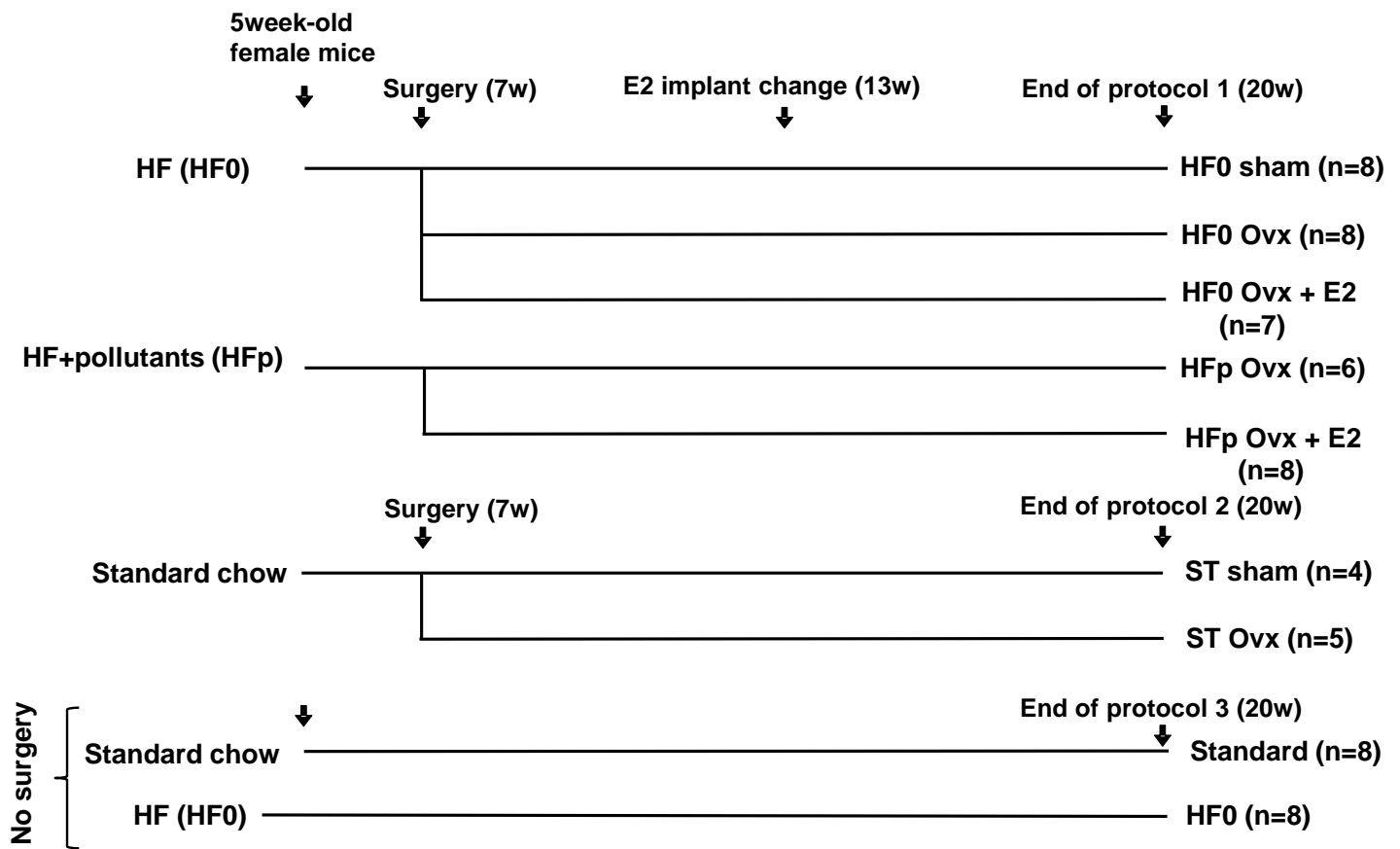


Figure 6

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703 **Supplemental Figures**



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706 **Supplemental Figure 1:** Experimental protocols. ST: standard chow diet; HF: high-fat high-
 707 sucrose diet; HF0: pollutant-free high-fat high-sucrose diet; HFp: high-fat high-sucrose diet
 708 containing the mixture of pollutants. Sham: sham-operated mice; Ovx: ovariectomized mice;
 709 Ovx+E2: ovariectomized mice with E2 replacement.

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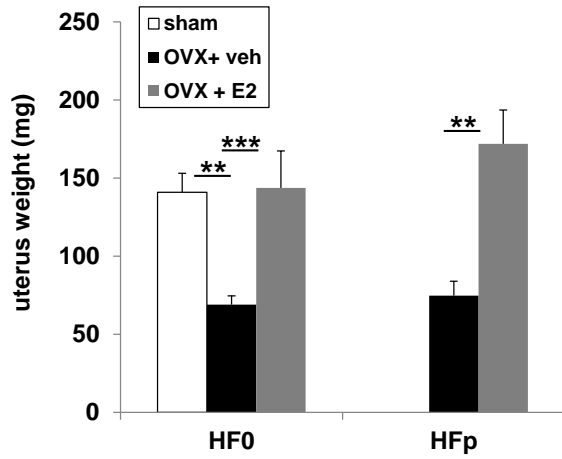
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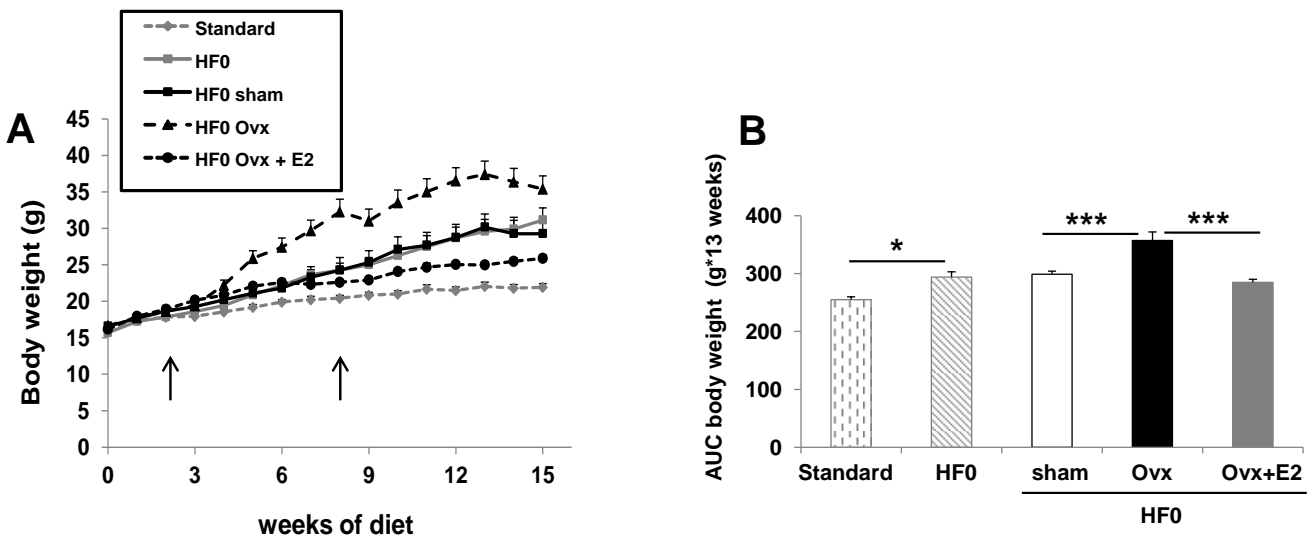
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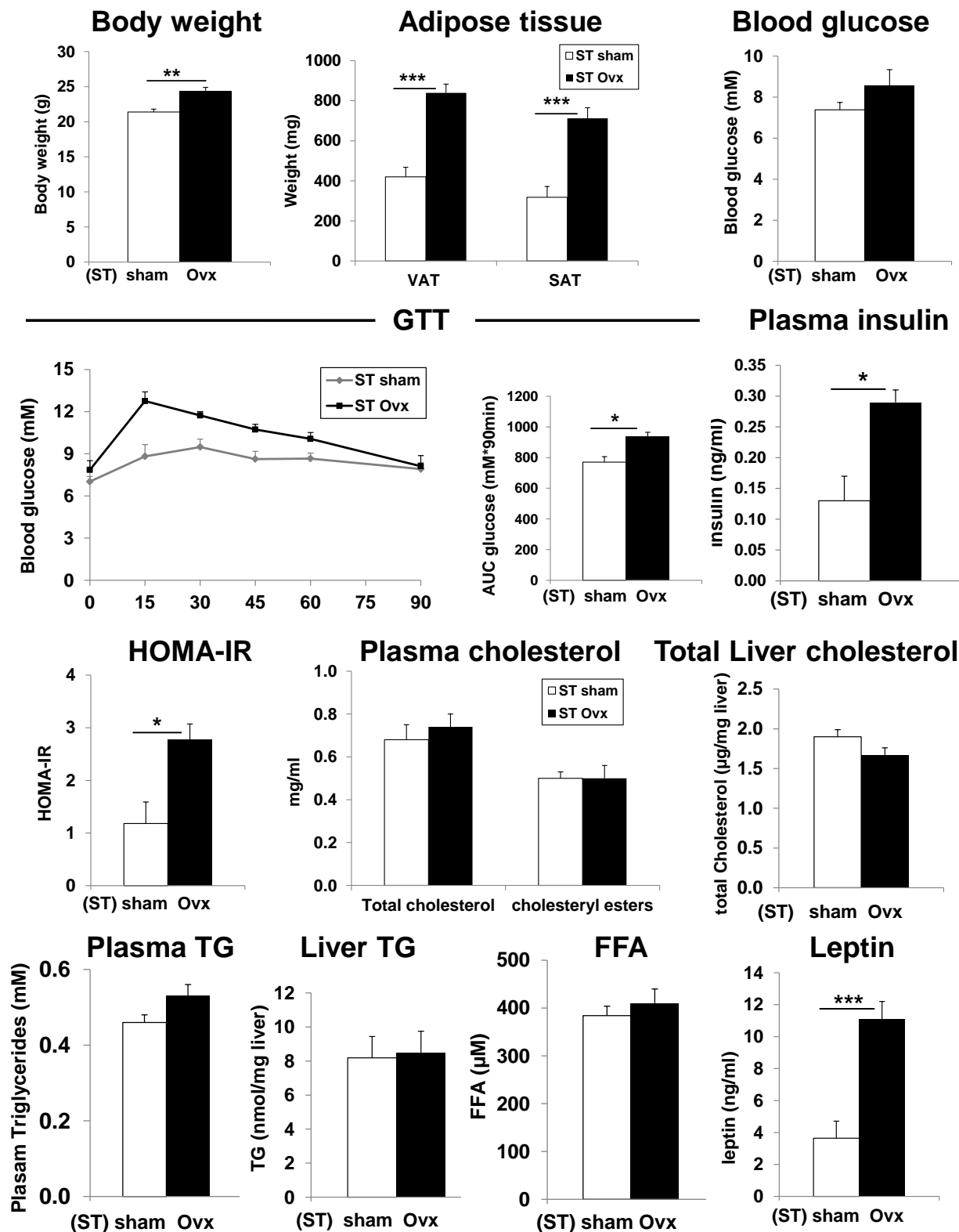
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Supplemental Figure 2: Uterus was weighed at the end of the protocol to validate ovariectomy and delivery of E2 after surgery. HF0: pollutant-free high-fat high-sucrose diet; HFp: high-fat high-sucrose diet containing the mixture of pollutants. Sham: sham-operated mice; Ovx: ovariectomized mice; Ovx+E2: ovariectomized mice with E2 replacement. Values are mean \pm SEM (n=6-8). **p<0.01, ***p<0.001 (using a one-way ANOVA followed by Tukey post hoc test).



Supplemental Figure 3: (A) Body weight curves along the 15 weeks of diet. Arrows indicate weeks when mice (sham, Ovx and Ovx+E2) were submitted to anesthesia (ovariectomy and implant after 2 weeks of diet and implant replacement at 8 weeks). Standard: standard chow diet; HF0: pollutant-free high-fat high-sucrose diet; HFp: high-fat high-sucrose diet containing the mixture of pollutants. Sham: sham-operated mice; Ovx: ovariectomized mice; Ovx+E2: ovariectomized mice with E2 replacement. Values are mean \pm SEM (n=8). *p<0.05; **p<0.01, ***p<0.001.



738 **Supplemental Figure 4:** Phenotype and biochemical characterization of Standard-fed Ovx
 739 versus standard-fed sham-operated mice. Standard: standard chow diet; Sham: sham operated
 740 mice; Ovx: ovariectomized mice; SAT: subcutaneous adipose tissue; VAT: visceral adipose
 741 tissue. Values are mean \pm SEM (n=4-5). *p<0.05; **p<0.01, ***p<0.001.

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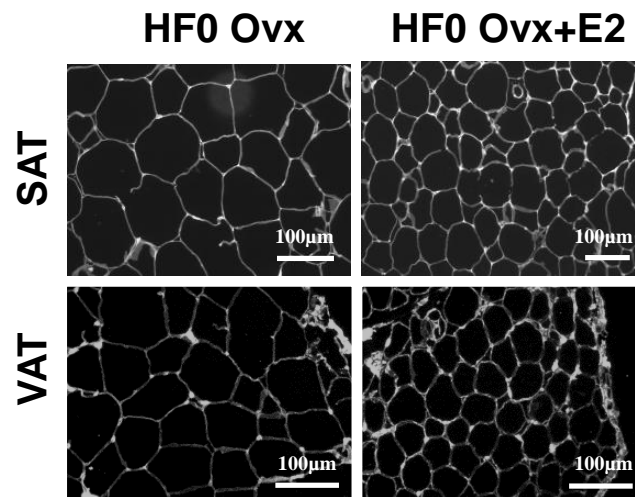
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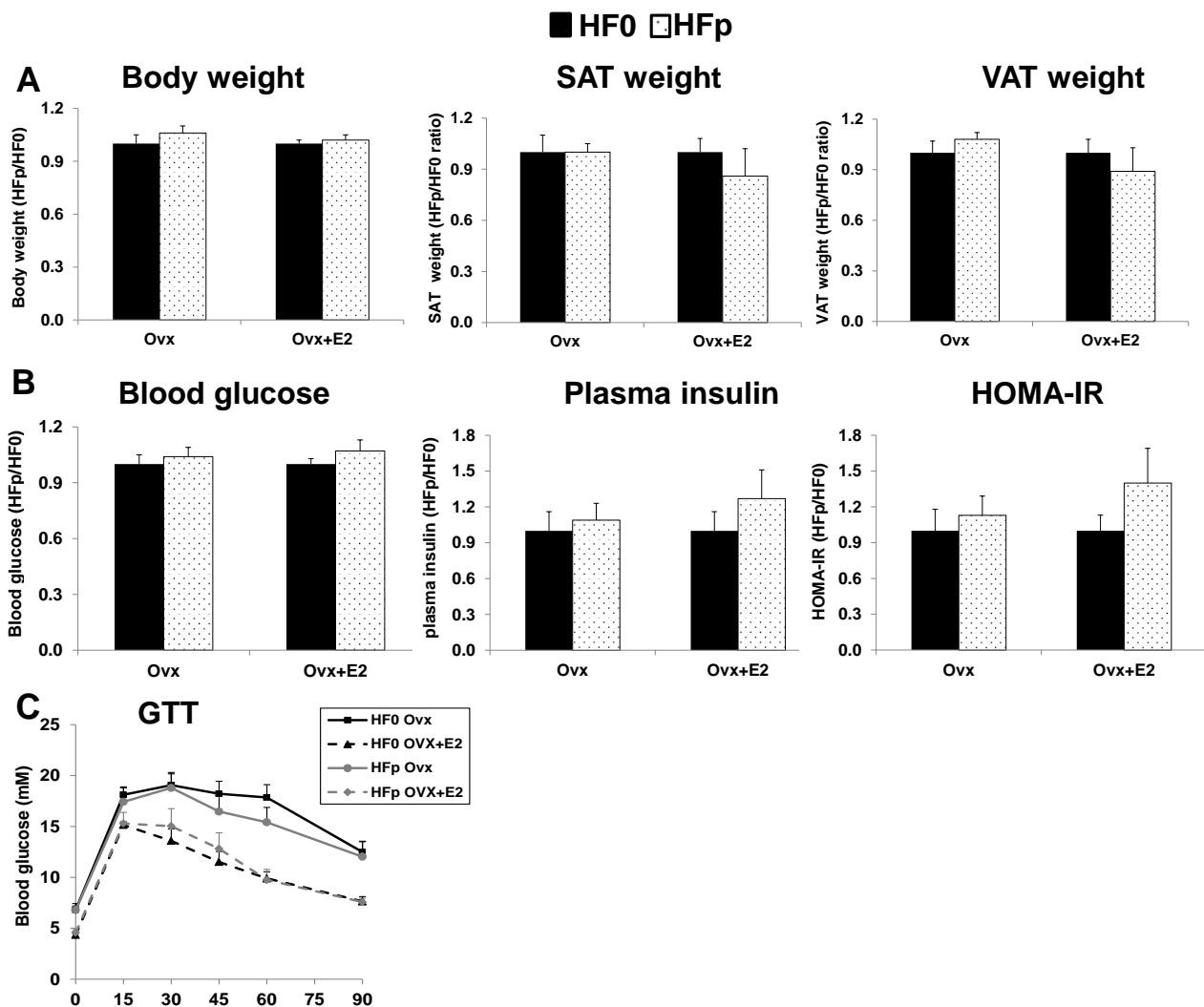
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Supplemental Figure 5: Impact of E2 replacement on adipocyte size. Representative laminin immunostaining of paraffin section of adipose tissues obtained from mice fed HFHS diet and ovariectomized without (Ovx) or with (Ovx+E2) estrogen replacement. Adipose tissue acquisition is represented for subcutaneous (SAT) and visceral (VAT) depots using objective x10. Scale bar=100µm

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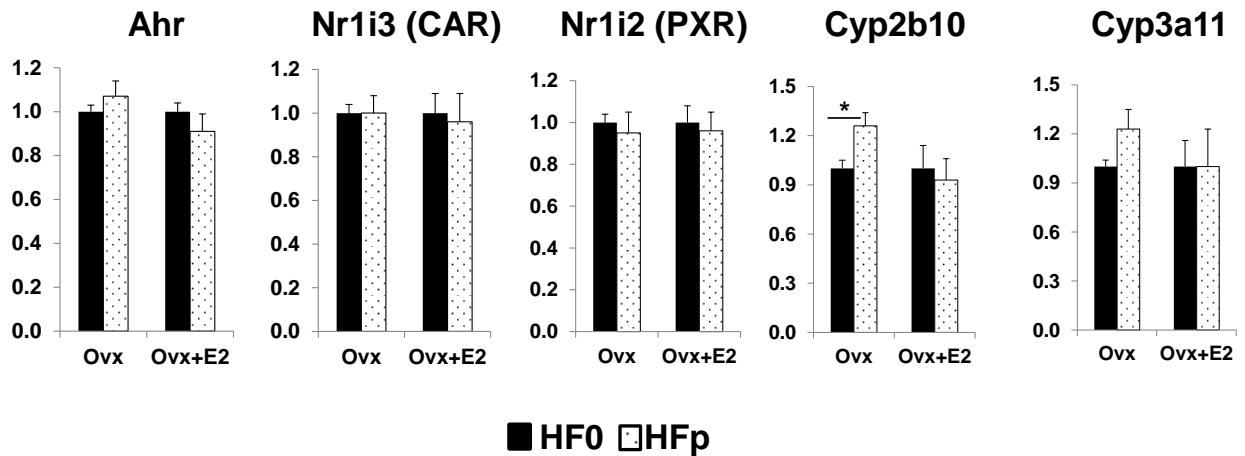


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762 **Supplemental Figure 6:** Comparison between treatment-matched HF0-fed and HFp-fed
763 mice. **A.** Body and adipose tissue weights; **B.** Blood glucose, plasma insulin and HOMA-IR.
764 **C.** Glucose Tolerance Test. HF0: pollutant-free high-fat high-sucrose diet; HFp: high-fat
765 high-sucrose diet containing the mixture of pollutants. Sham: non-operated mice; Ovx:
766 ovariectomized mice; Ovx+E2: ovariectomized mice with E2 replacement.; SAT:
767 subcutaneous adipose tissue; VAT: visceral adipose tissue. Values are mean \pm SEM (n=6-8).

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772 **Supplemental Figure 7:** Effect of the mixture of pollutant on the expression of xenobiotic
 773 processing genes in liver. Results are expressed as HFp/HF0 ratio for each group of mice
 774 with ratio equal to 1.0 for the HF0 groups. HF0: high-fat high-sucrose diet pollutant-free;
 775 HFp: high-fat high-sucrose diet containing the mixture of pollutants. Sham: not operated
 776 mice; Ovx: ovariectomized mice; Ovx+E2: ovariectomized and E2 replaced mice. Values are
 777 mean \pm SEM (n=6-8). *p<0.05.

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781 Supplemental table

782 Supplemental table 1: List of the primers used for qPCR analyses

gene	reference	sequences: 5'->3'	
		sense	antisense
<i>Ahr</i>	NM_013464.4	TCA-TCT-GGT-TTC-CTG-GCA-ATG-AAT	ATA-AGC-TGC-CCT-TTG-GCA-TC
<i>Atoh1</i>	NM_007500	CAG-CGA-TGA-TGG-CAC-AGA-AG	TGG-TTG-TCT-CAG-TTT-TCA-GG
<i>Ccl2</i>	NM_011333	TGG-AGC-ATC-CAC-GTG-TTG-GC	ACT-ACA-GCT-TCT-TTG-GGA-CA
<i>Cd36</i>	NM_001159558.1	AAG-ATC-CAA-AAC-TGT-CTG-TA	GTC-CTG-GCT-GTG-TTT-GGA-GG
<i>Cldn2</i>	NM_016675	CCT-TCG-GGA-CTT-CTA-CTC-GC	TCA-CA-CAT-ACC-CAG-TCA-GGC
<i>Cpt1a</i>	NM_001876;NM_001031847	TAC-AAC-AGG-TGG-TTT-GAC-A	GGC-TGT-CAA-TGG-ACA-TGA-CG
<i>Cyp3a11</i>	NM_007818	ACG-CCT-CTC-CTT-GCT-GTC-ACC	TTG-CCT-TCT-GCC-TCA-AGT-AC
<i>Cyp7a1</i>	NM_007824.2	TAC-AGA-GTG-CTG-GCC-AAG-AG	AGT-GAA-GTC-CTC-CTT-AGC-TG
<i>Cyp2b10</i>	NM_009999	TTC-TGC-CCT-TCT-CAA-CAG-GA	CCT-TAG-GAG-CAA-CAT-GGC-TT
<i>Dgat2</i>	NM_026384	AAG-GGC-TTT-GTG-AAA-CTG-GC	CCT-CCT-CGA-AGA-TCA-CCT-GC
<i>Esr1</i>	NM_000125;NM_001122740;NM_001122741;NM_001122742	TGT-TTG-CTC-CTA-ACT-TGC-TC	CCT-TCT-CTT-CCA-GAG-ACT-TC
<i>Esr2</i>	NM_207707; NM_010157	CTC-TTC-CCA-GCA-GCA-GTC-AGT-C	AGC-ATC-TCC-AGC-AGC-AGG-T
<i>Fabp4</i>	NM_024406	CAG-AAG-TGG-GAT-GGA-AAG-TCG	CGA-CTG-ACT-ATT-GTA-GTG-TTT-GA
<i>Gck</i>	NM_010292	CAA-CTG-GAC-CAA-GGG-CTT-CA	CCA-GCT-CCA-CAT-TCT-GCA-TC
<i>Got2</i>	NM_010325	ATG-GTG-AAG-GAT-GCC-TGG	TTC-ATC-CGC-ATC-TTT-GCA-GAC-C
<i>G6pc</i>	NM_008061	TTA-CCA-AGA-CTC-CCA-GGA-CTG	GAG-CTG-TTG-CTG-TAG-TAG-TC
<i>Gper1</i>	NM_029771	AGC-TGA-TCA-GAT-CTA-GGG-AG	GTC-CTG-GGA-GCC-TGT-TAG-TC
<i>Hmgcr</i>	NM_008255	CCG-GCC-TGT-GTG-TCG-CTG-GT	CCA-GCG-ACT-ATG-AGC-GTG-AA
<i>I11b</i>	NM_008361	ACT-GTT-CCT-GAA-CTC-AAC-TG	CTT-GTT-GAT-GTG-CTG-CTG-CG
<i>I12a</i>	NM_001159424, NM_008351	GAC-ATC-GAT-CAT-GAA-GAC-ATC-AC	TAC-CAA-GGC-ACA-GGG-TCA-TC
<i>Lcn2</i>	NM_008491	TCA-CTC-TGG-GAA-ATA-TGC-AC	ATG-ATG-TTG-TCG-TTG-AG
<i>Ldlr</i>	NM_001252658, NM_001252659, NM_010700	GTT-GAT-TCC-AAA-CTC-CAC-TC	TTC-ACA-TCT-GAA-CCC-GTG-AG
<i>Lep</i>	NM_008493	CAC-CAG-GAT-CAA-TGA-CAT-TTC	TGC-CAG-TGT-CTG-GTC-CAT-CTT-G
<i>Lipe</i>	NM_001039507, NM_010719	GTG-TGT-CAG-TGC-CTA-TTC-AG	GTC-AGC-TTC-TTC-AAG-GTA-TC
<i>Lpl</i>	NM_008509	GGT-CGA-AGC-ATT-GGA-ATC-CAG	TAG-GGC-ATC-TGA-GAA-CGA-GTC
<i>Mmp7</i>	NM_010810; NM_001319986	ACG-ACA-TTG-CAG-GCA-TTC-AG	GTT-CAT-GCC-AGC-TGA-GGG-AT
<i>Muc2</i>	NM_023566	GCT-ATG-ACG-TCT-GTG-TGA-AG	CAA-ACA-CAG-TCC-TTG-CAG-TC
<i>Ngn3</i>	NM_009719	GCT-ATC-CAC-TGC-TGC-TTG-AC	TGG-AAT-TGG-AAC-TGA-GCA-CTT
<i>Npc1l1</i>	NM_207242	GCT-AGC-AGC-CAA-CAT-CAC-AG	CAG-TAG-GAG-GTA-GCA-GAC-CA
<i>Nr0b2</i>	NM_011850	CTG-CCT-GGA-GTC-TTT-CTG-GA	GTG-CCT-GGA-ATG-TTC-TTG-AG
<i>Nr1c1</i>	NM_011144; NM_001113418.1	AAG-GGC-TTC-TTT-CGG-CGA-AC	GTT-CAT-GTT-GAA-GTT-CTT-CAG
<i>Nr1c3</i>	NM_001127330, NM_011146	TCT-CTC-CGT-AAT-GGA-AGA-CC	GCA-TTA-TGA-GAC-ATC-CCC-AC
<i>Nr3c4</i>	NM_013476.3	AGA-AGA-CCT-GCC-TGA-TCT-G	GCT-GGC-ACA-TAG-ATA-CTT-CTG
<i>Nr1h3</i>	NM_013839	CCG-GGA-AGA-CTT-TGC-CAA-AGC	GGA-GCT-GGT-CCT-GCA-CGT-TG
<i>Nr1h4</i>	NM_001163700, NM_001163504, NM_009108	GCA-ACC-TGT-TGG-AAG-AAA-G	GTC-TGT-CTG-GAG-AGA-GGA-TG
<i>Nr1i2</i>	NM_010936.3	AGG-AGG-AGT-ATG-TGC-TGA-TG	CTT-CAG-GAA-CAG-GAA-CCT-GTG
<i>Nr1i3</i>	NM_001243063; NM_001243062; NM_009803	GTC-CCA-TCT-GTC-CGT-TTG-C	AGG-GCT-TCT-GAC-AGT-ATC
<i>Pcsk9</i>	NM_153565	AAC-TGC-AGC-ATC-CAC-AAC-AC	CAG-CAG-GAA-GCA-TAG-ACA-CT
<i>Pnpla2</i>	NM_025802, NM_001163689	TCA-TC-CAG-GCC-AAT-CTC-TGC	TGG-ATG-TTG-GTG-GAG-CTG-TC
<i>Scd1</i>	NM_009127	GAT-ACA-CTC-TGG-TGC-TCA-AC	AAC-GTG-GTG-AAG-TTG-ATG-TG
<i>Slc2a2</i>	NM_031197	TGG-GTA-CTC-TTC-ACC-AAC-TG	AGG-ATG-TGC-CAA-TGA-TCC-TG
<i>Slc5a1</i>	NM_019810	GCT-CCT-TGA-CCT-CCA-TCT-TC	CAG-GCA-ATG-CTG-ATG-CCA-AT
<i>Srebf1</i>	NM_011480	ACG-GAG-CCA-TGG-ATT-GCA-CA	AAG-GGT-GCA-GGT-GTC-ACC-TT
<i>Srebf2</i>	NM_033218	CCT-GTG-ATG-ATG-GGG-CAA-GAG	CCT-CAG-AAC-GCC-AGA-CTT-G
<i>Tbp</i>	NM_013684	TGG-TGT-GCA-CAG-GAG-CCA-AG	TTC-ACA-TCA-CAG-CTC-CCC-AC
<i>Tjp1</i>	NM_009386	ACG-CAT-CAC-AGC-CTG-GTT-G	TGG-CTC-CTT-CCT-GTA-CAC-C
<i>Tnfa</i>	NM_013693	CCA-GAC-CCT-CAC-ACT-CAG-ATC	CAC-TTG-GTG-GTT-TGC-TAC-GAC

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