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1 **Exposure to pollutants altered glucocorticoid signaling and clock gene expression in**
2 **female mice. Evidence of tissue- and sex-specificity**

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27 **Abstract**

28 Environmental pollutants suspected of disrupting the endocrine system are considered
29 etiologic factors in the epidemic of metabolic disorders. As regulation of energy metabolism
30 relies on the integrated action of a large number of hormones, we hypothesized that certain
31 chemicals could trigger changes in glucocorticoid signaling. To this end, we exposed
32 C57Bl6/J female and male mice between 5 and 20 weeks of age to a mixture of 2,3,7,8-
33 tetrachlorodibenzo-p-dioxin (20 pg/kg body weight/day [bw/d]), polychlorobiphenyl 153 (200
34 ng/kg bw/d), di-[2-ethylhexyl]-phthalate (500µg/kg bw/d) and bisphenol A (40µg/kg bw/d).
35 In female mice fed a standard diet (ST), we observed a decrease in plasma levels of leptin as
36 well as a reduced expression of corticoid receptors *Nr3c1* and *Nr3c2*, of leptin and of various
37 canonical genes related to the circadian clock machinery in visceral (VAT) but not
38 subcutaneous (SAT) adipose tissue. However, *Nr3c1* and *Nr3c2* mRNA levels did not change
39 in high-fat-fed females exposed to pollutants. In ST-fed males, pollutants caused the same
40 decrease of *Nr3c1* mRNA levels in VAT observed in ST-fed females but levels of *Nr3c2* and
41 other clock-related genes found to be down-regulated in female VAT were enhanced in male
42 SAT and not affected in male VAT. The expression of corticoid receptors was not affected in
43 the livers of both sexes in response to pollutants. In summary, exposure to a mixture of
44 pollutants at doses lower than the no-observed adverse effect levels (NoAELs) resulted in sex-
45 dependent glucocorticoid signaling disturbances and clock-related gene expression
46 modifications in the adipose tissue of ST-fed mice.

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50 **Key words:** Pollutant mixture; mouse; Adipose tissue; Glucocorticoid signaling; Clock-
51 related genes

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53 **Highlights**

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- 55 - A mixture of low-dosed pollutants trigger metabolic changes in exposed mice
- 56 - In females, pollutants affect glucocorticoid signaling in VAT and leptin synthesis
- 57 - Mode of action may involve alteration of clock-related genes in female VAT
- 58 - Glucocorticoid signaling was not affected in the female or male liver
- 59 - In males, pollutants mainly affect the signaling of glucocorticoids in SAT

60 **1. Introduction**

61 Metabolic-related diseases, such as diabetes and obesity, are prominent public health
62 challenges of the 21st century. In the last decades, prevalence of these chronic diseases
63 reached epidemic proportions worldwide. Metabolic diseases have multifactorial causes.
64 Genetic predisposition, high calorie intake, poor physical activity and also environmental
65 pollutants are among the etiological factors identified. The capacity of some pollutants to
66 interfere with hormonal action, also known as endocrine disruptors has been well documented
67 (Carpenter, 2008, Diamanti-Kandarakis et al., 2009, Thayer et al., 2012, Ibrahim et al., 2011,
68 Ruzzin et al., 2010, Alonso-Magdalena et al., 2011, Heindel and Blumberg, 2019, Le
69 Magueresse-Battistoni et al., 2015). Endocrine disruptors have been shown to target the
70 estrogen, androgen, thyroid, and steroidogenesis pathways and others (e.g., through binding to
71 estrogen-related receptor, peroxisome proliferator-activated receptor, xenobiotic receptors)
72 (Diamanti-Kandarakis et al., 2009, Reif et al., 2010, Delfosse et al., 2015), depending on the
73 repertoire of co-expressed nuclear receptors and hormonal environment (Casals-Casas and
74 Desvergne, 2011, Le Magueresse-Battistoni et al., 2017). Therefore, chemicals targeting
75 energy homeostasis could have extensive metabolic implications. Indeed, energy homeostasis
76 is a complex process orchestrated by multiple hormones adjusting energy intake to energy
77 expenditure including sex hormones (Mauvais-Jarvis et al., 2013) and glucocorticoids
78 (Vegiopoulos and Herzig, 2007).

79 Over the course of a lifetime, humans are exposed to a plethora of chemicals that can interact
80 in vivo by modifying each other's effect. In addition, some chemicals such as bisphenol A
81 (BPA) were shown to cause adverse effects in experimental studies (Vandenberg et al., 2012,
82 Angle et al., 2013) even at doses lower than those considered to be safe in humans (Koch and
83 Calafat, 2009, Schafer and Kegley, 2002). Therefore, the cocktail effect resulting from
84 exposure to complex mixtures makes it difficult to predict the health consequences (Le

85 Magueresse-Battistoni et al., 2018b, Kortenkamp, 2008, Heindel et al., 2017b). Interestingly,
86 we have recently implemented a model in which mice were exposed through diet to a mixture
87 of pollutants made of BPA, di-[2-ethylhexyl]-phthalate (DEHP), polychlorinated biphenyl
88 (PCB) 153 and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). These chemicals are part of a
89 large group of archetypal endocrine disruptors present in food (Casals-Casas and Desvergne,
90 2011, Heindel et al., 2017a, Vandenberg et al., 2012) with different characteristics regarding
91 half-lives, endocrine disrupting activities and activation of nuclear receptors/transcription
92 factors (Le Magueresse-Battistoni et al., 2018a).

93 BPA is probably the most emblematic chemical since humans are almost continuously
94 exposed to it, with estimated daily doses of 0.4 to 5 μ /kg body weight (Vandenberg et al.,
95 2012). BPA has large industrialized applications, particularly in plastics and resins, and its
96 global production exceeds millions of tons per year (Vandenberg et al., 2007). Initially
97 described as pro-estrogenic because of its binding to estrogen receptors (ERs), the modes of
98 action of BPA may involve non-estrogenic receptors including the peroxisome proliferator
99 activator receptor (PPAR) γ , the glucocorticoid receptor (GR) or the thyroid receptor, all
100 intricately linked to metabolic health and body weight (Le Magueresse-Battistoni et al.,
101 2018a, Zoeller et al., 2005, Casals-Casas and Desvergne, 2011). Disruptive effects on glucose
102 and lipid metabolism or insulin resistance may also result from mechanisms linked to
103 hormonal metabolism, oxidative stress, inflammation, mitochondrial dysfunction and
104 epigenetic changes (Vandenberg et al., 2012, Vom Saal et al., 2012, Ma et al., 2019, Nadal et
105 al., 2017). Phthalates are other short-lived chemicals that are used primarily to soften plastics.
106 They are of great concerns for human health with an average exposure of 0.5 to 25 μ /kg body
107 weight per day for DEHP (Vandenberg et al., 2012). As with BPA, the diet is the main source
108 of contamination and results from the chemical leaching of food and drink containers and
109 packaging (Koch and Calafat, 2009, Vandenberg et al., 2007). Experimental studies have

110 shown that DEHP exposure can cause adverse effects on energy balance and glucose
111 homeostasis through binding to PPARs (Feige et al., 2007). Phthalates can also bind to
112 xenobiotic receptors but not to androgen receptors although they exert anti-androgenic effects
113 (Schug et al., 2011, Casals-Casas and Desvergne, 2011, Le Magueresse-Battistoni et al.,
114 2017).

115 Dioxins and polychlorinated biphenyls (PCBs) are lipophilic and belong to the group of
116 persistent organic pollutants (POP). Epidemiological and experimental studies demonstrated
117 that exposure to POPs could lead to detrimental metabolic effects including obesity and type 2
118 diabetes (Wahlang et al., 2013, La Merrill et al., 2013, Gauthier et al., 2014, Ibrahim et al.,
119 2011, van Esterik et al., 2015). Dioxins result from incomplete combustion, which occurs
120 during industrial processes, forest fires or volcanic eruptions. PCBs had a large number of
121 industrial applications because of their low-flammability and high-thermal conductivity
122 properties. Today, the production of PCBs is banned and industrial emissions of dioxins are
123 limited. However, they still contaminate food by bio-amplification in the food chain and are
124 concentrated in fatty foods due to their low degradability (Guo et al., 2019). It was reported
125 that average daily exposure to TCDD (the most toxic dioxin congener that includes the
126 coplanar PCBs) was below the tolerable daily intake (TDI) dose but it was up to 80 ng/kg
127 body weight per day for non-coplanar PCBs thus exceeding the toxicological reference values
128 for certain groups of consumers (Baars et al., 2004, EFSA, 2005). Interestingly, PCB153 a
129 non-coplanar PCB is considered as a marker for the total PCB exposure (Hagmar et al., 2006).
130 TCDD and co-planar PCBs can bind to the aryl hydrocarbon receptor (AHR) (Barouki et al.,
131 2012, Bock, 2019, Van den Berg et al., 1998) while non-coplanar PCBs cannot bind to AHR
132 although they can interact with its signaling mechanisms. They may as well activate the
133 pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) (Chen and Liu,
134 2019). Dioxins and PCBs can also interact with the estrogen and thyroid signaling pathways,

135 respectively (Diamanti-Kandarakis et al., 2009, Casals-Casas and Desvergne, 2011, Pavek,
136 2016).
137 Using the exposure model to a mixture of BPA, DEHP, PCB153 and TCDD, we previously
138 demonstrated that the metabolic adverse impact in mice was strongly sex-dependent altering
139 estrogen signaling in the liver of exposed females and hepatic cholesterol metabolism in
140 males (Naville et al., 2013). In addition, the mixture of pollutants affected other metabolic
141 tissues including the adipose tissues and involved other nuclear receptors than the estrogen
142 and xenobiotic receptors (Julien et al., 2018, Julien et al., 2019, Naville et al., 2015, Naville et
143 al., 2019). The purpose of the current study was to address the implication of glucocorticoid
144 signaling in mediating the effects of the pollutants in female mice. We also looked for sexual
145 dimorphism benefiting from male samples taken in a previous study (Naville et al., 2019).
146 Glucocorticoids (GC) are steroid hormones with strong anti-inflammatory properties. They
147 are produced by the adrenal glands under the negative feed-back involving the hypothalamic-
148 pituitary-adrenal (HPA) axis. They exert vital roles on behavior, immunity and metabolism
149 through binding to corticoid receptors in target cells including the liver and adipocytes
150 (Vegiopoulos and Herzig, 2007). Recent reports highlighted that xenobiotics could bind to
151 GR (Gulliver, 2017), and that BPA and phthalates had a weak GR agonist activity in
152 adipocytes (Gulliver, 2017, Le Magueresse-Battistoni et al., 2017, Casals-Casas and
153 Desvergne, 2011, Sargis et al., 2010).

154

155 **2. Materials and Methods**

156 2.1. Animals, diet and experimental design

157 All procedures were performed with the approval of the Regional Committee of Ethics for
158 Animal Experiments. Four-week (wk) old C57Bl/6J female and male mice (Envigo; Gannat,

159 France) were randomly housed two per polypropylene cage at 21°C with normal light/dark
160 cycle and free access to water and standard chow diet (from Genestil, Royaucourt, France) for
161 a one- week acclimatization period. Mice were next randomly assigned to four different
162 groups (n=7-8 / group). Two groups were fed a standard diet (ST; 10% Fat and 35.6%
163 maltodextrin) and two groups were fed a high-fat high-sucrose diet (HF; 39.4% Fat and
164 16.6% maltodextrin +16.6% sucrose, Envigo). The STp and HFp groups correspond to the ST
165 or HF diet, containing respectively a mixture of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD,
166 CAS N° 1746-01-6 from LGC Promochem, Molschein, France), polychlorinated biphenyl
167 (PCB) 153 (CAS N° 35065-27-1), bisphenol A (BPA, CAS N° 80-05-7) and di-[2-
168 ethylhexyl]-phthalate (DEHP, CAS N° 117-81-7) (all three from Sigma-Aldrich). The ST0 and
169 HF0 groups correspond to the ST or HF diet containing the vehicle, only. To prepare the
170 contaminated food, stock solutions of pollutants dissolved in DMSO were diluted in corn oil
171 and they were mixed into diet (316 µl DMSO in 60 ml corn oil/kg diet) using a food-
172 processor to ensure good distribution in the food. Specifically, mice were exposed to 20 pg
173 TCDD /kg bw/day, 200 ng PCB153/kg bw/day, 500µg DEHP/kg bw/day and 40µg BPA/kg
174 bw/day (Supplementary Table 1). These doses were at least 10 times lower than the NoAEL
175 (No-observed Adverse Effect Level) dose for each pollutant when taken individually (DEHP,
176 BPA) or representative congener (TCDD, PCB153) (Beausoleil et al., 2018, van Leeuwen et
177 al., 2000, WHO, 2003). To ensure that animals ingested the correct amount of polluted
178 food/kg bw/day and were fed *ad libitum*, the calculations of intake of pollutants in the diet
179 were based on 1 g of contaminated food /17 g of body weight / day, and pollutant-free food
180 was provided to the animals at libitum. Food was given on Mondays, Wednesdays and
181 Fridays. Body weight was recorded weekly and the amount of polluted food distributed was
182 adjusted, accordingly over a 15 weeks period. It should be noted that the male samples used in
183 the present study have previously been analyzed for lipid metabolism in the liver and jejunum,

184 and adipogenesis and inflammation of the fat pads (Naville et al., 2019), but not
185 glucocorticoid signaling.

186 2.2. Tissue collection and blood and plasma analyses

187 Before sampling, mice were fasted six-hour and weighed. Blood was collected by retro-orbital
188 sampling and mice were euthanized by cervical dislocation. Adipose tissues and liver were
189 quickly removed, weighed, frozen in liquid nitrogen and stored at -80°C. Blood glucose
190 concentrations (Accucheck Performa glucometer; Roche Diabetes Care France, Meylan), and
191 plasma levels of insulin and corticosterone (Mouse Ultrasensitive ELISA, Eurobio,
192 Courtaboeuf, France), leptin and adiponectin (ELISA kits from Crystal Chem Europe,
193 Zaandam, Netherlands), triglycerides (Biolabo, Maizy, France) and free fatty acids, total
194 cholesterol and cholesteryl esters (all kits from Sigma-Aldrich) were measured.

195 2.3. RNA extraction and real-time PCR analyses

196 Total RNA was extracted from subcutaneous (SAT) and visceral (VAT) adipose tissue using
197 TRI Reagent (Applied Biosystems, Courtaboeuf, France). RNA integrity was determined with
198 the Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Massy,
199 France). Samples were reverse-transcribed using the Prime Script RT Reagent kit (Takara
200 Bio Europe SAS, Saint-Germain-en-Laye, France). We performed real time PCR using a set
201 of specific primers specifically designed to encompass an intron (Supplemental Table 2). Data
202 were normalized using Tbp encoding TATA-Box Binding Protein as described for adipose
203 tissues and beta-glucuronidase (Gusb) for liver samples (Naville et al., 2011, Naville et al.,
204 2019).

205 2.4. Western blotting analyses

206 Proteins prepared from adipose tissues were separated by SDS-10% polyacrylamide gel
207 electrophoresis and transferred to a polyvinylidene difluoride membrane. Immunoblotting was
208 performed as previously described (Naville et al., 2013, Longin et al., 2001) using a rabbit
209 polyclonal anti-glucocorticoid receptor (dilution 1/5000, Ab3580; Abcam France) or β -Actin
210 (dilution 1/10,000, A5060, Sigma). Blots were revealed using Luminata Classico Western
211 HRP substrate (Millipore, Molsheim, France), detected using the ChemiDoc XRS+ imaging
212 system (Biorad), and analyzed with Image Lab software (Bio-Rad). Data were normalized
213 relative to β -Actin.

214 2.5. Statistical analyses

215 Results are presented as means \pm S.E.M. Graphpad Prism 8.01 software was used for
216 statistical analyses. The overall impact of the pollutant exposure and of the diet was
217 determined using the two-way Anova analysis followed by the Bonferroni's multiple
218 comparisons post-hoc test to determine significant differences between relevant groups. In
219 experiments only assessing the impact of pollutant exposure, differences were tested using the
220 non-parametric Mann-Whitney test. Differences between means were considered significant
221 at p -value < 0.05 .

222 3. Results

223 3.1. Metabolic phenotyping of female mice

224 We explored the metabolic alterations of female mice in response to pollutant exposure
225 including the analysis of the systemic levels of corticosterone. No changes in body weight,
226 blood glucose, plasma insulin and corticosterone levels and no dyslipidemia were observed
227 between control and pollutant-exposed mice. As expected, female mice fed a HF diet showed
228 enhanced body weight, blood glucose, plasma insulin levels, and dyslipidemia as compared to
229 ST-fed mice. Plasma corticosterone levels were in the control range (Fig. 1).

230 3.2. Expression of various nuclear receptor/transcription factors and of 11 β -
231 hydroxysteroid dehydrogenase1 (11 β HSD1) in the visceral adipose tissues of female mice fed
232 a ST or a HF diet and exposed to the mixture of pollutants

233 Adipose tissues express several nuclear receptor/transcription factors that pollutants can bind
234 to initiate downstream activation signals among which the estrogen receptors, the aryl
235 hydrocarbon receptor (AhR) but also the glucocorticoid (GR) and the mineralocorticoid (MR)
236 receptors (Swedenborg et al., 2009, Lee et al., 2018). In addition to analyzing these receptors
237 by RT-qPCR, we also studied *Hsd11b1* which encodes the 11 β -hydroxysteroid
238 dehydrogenase1 (11 β HSD1). Indeed in the adipose tissue, 11 β HSD1 catalyzes the conversion
239 of the inactive cortisone into the active corticosterone thus locally regulating availability of
240 GCs (Sandeep and Walker, 2001). Two-way Anova analysis indicated that the “Pollutant”
241 factor had significant effects on all the genes studied, excepted for *Esr1* and *Gper1* encoding
242 ER α and GPR30, respectively. The “Diet” factor had significant effects on all the genes
243 studied, but not on *Nr3c2* encoding MR. A trend was observed for *Esr1* (Fig. 2, insert). *Post*
244 *Hoc* analysis determined that pollutant exposure significantly down-regulated *Nr3c1* encoding
245 GR, *Nr3c2*, *Hsd11b1* and *Esr2* (encoding ER β) in ST-fed but not in HF-fed nutritional
246 conditions. A trend was observed for *Ahr* (Fig. 2A). Western-blotting analysis of VAT
247 proteins confirmed decreased protein levels of GR in females exposed to the mixture of
248 pollutants (Fig. 2B).

249 3.3. Expression of various nuclear receptor/transcription factors and of 11 β HSD1 in
250 the subcutaneous adipose tissue of female mice fed a ST or a HF diet and exposed to the
251 mixture of pollutants

252 Contrary to the results observed in VAT, exposure to pollutants in SAT did not affect the
253 levels of expression of the glucocorticoid and estrogen receptors, of 11 β HSD1 and of AHR

254 (Fig. 3). In addition, the mRNA levels of *Nr3c1* and *Nr3c2* remained constant in the SAT of
255 females fed a HF diet (Fig. 3). Two-way Anova analysis indicated that the “Diet” factor had
256 significant effects on *Hsd11b1* and the estrogen receptors, and a trend was observed for *Ahr*
257 (Fig. 3, insert). Specifically, as in VAT (Fig. 2), the expression levels of *Hsd11b1* and the
258 estrogen receptors *Esr1* and *Esr2* were significantly decreased and the mRNA levels of *Gper1*
259 were significantly increased in the SAT (Fig. 3).

260 3.4. Low-dosed pollutant exposure targeted leptin gene expression in females

261 To decipher mechanisms underlying changes in glucocorticoid signaling observed in the
262 adipose tissues of standard-fed females, plasma leptin was measured. Indeed, leptin is an
263 adipokine involved in satiety mechanisms which gene is regulated by glucocorticoids (Zhang
264 and Chua, 2017). We observed significant changes in leptin plasma levels (- 55%; $p < 0.05$)
265 together with a small but significant increase in food intake (Fig. 4), despite no changes in the
266 weight of SAT and VAT (Fig. 1). Gene expression analysis showed a significant reduction in
267 *Lep* mRNA levels in VAT but not in SAT. Adiponectin is another hormone produced by
268 adipocytes. Exposure to pollutants had no effects on adiponectin levels measured in plasma
269 or on the mRNA levels of the adipose tissue depots (Fig. 4). To determine if other markers of
270 adipogenesis displayed altered expression following pollutant exposure, we quantified the
271 mRNA of *Nr1c3* encoding PPAR γ and other target genes including *Fabp4* encoding fatty acid
272 binding protein 4 (FABP4) and *Cidec* encoding fat protein specific 27 (FSP27). Expression
273 mRNA levels were similar to controls in both adipose tissues (Supplementary Fig. 1). We also
274 quantified inflammatory cytokines in VAT and SAT. Pollutants did not affect *Tnfa*, *Ccl2*,
275 *Ccl5*, and *Il1b* mRNA levels in SAT and VAT (Supplementary Fig.2).

276 3.5. Pollutant exposure caused changes in the expression levels of glucocorticoid-induced
277 genes including circadian clock genes in the adipose tissues

278 It is well demonstrated that leptin displays a circadian cycling pattern (Fu et al., 2005) and that
279 adrenalectomy results in changes in leptin secretion (Yilmaz et al., 2002) and in circadian
280 gene expression in VAT (Sotak et al., 2016). Therefore, we quantified a glucocorticoid- and
281 clock controlled gene namely *Tsc22d3* encoding the glucocorticoid induced leucine zipper
282 (GILZ) protein (Ayyar et al., 2015) and also various core clock genes including *Clock*, *Arntl*,
283 *Per1*, *Per2* and *Nr1d1* encoding CLOCK, BMAL1, PER proteins and NR1D1, respectively
284 (Reppert and Weaver, 2002). Quantification of these genes by RT-qPCR revealed a
285 significant down-regulation of *Per1* and *Per2* and a trend for *Tsc22d3* ($p=0.07$) in VAT but
286 not in SAT from pollutant-exposed females. *Clock* and *Arntl* mRNA levels remained in the
287 control range (Fig. 5A-B).

288 3.6 Analysis of the response to pollutant mixture in male mice

289 To determine if changes in glucocorticoid signaling also occurred in males in response to the
290 pollutant mixture in ST-fed conditions, we screened all the genes investigated in females,
291 including *Nr3c1*, *Nr3c2*, *Hsd11b1* as well as *Tsc22d3*, *Clock*, *Arntl*, *Per1*, *Per2* and *Nr1d1* in
292 the VAT and the SAT of males fed a standard diet and exposed to the mixture of pollutants. In
293 the VAT of ST-fed males, we observed a significant decrease of *Nr3c1* mRNA levels and of
294 *Arntl* while in the SAT, several genes were significantly up-regulated including *Nr3c2*,
295 *Tsc22d3*, *Per1*, *Per2* and *Nr1d1* (Fig. 6). Finally, plasma corticosterone levels, food intake,
296 and expression of *Lep* and *Adipoq* were analyzed in males (Fig. 7). Plasma corticosterone
297 levels did not change. Contrary to females, food intake and *Lep* mRNA levels did not change
298 in males but *Adipoq* mRNA levels were enhanced in the SAT (Fig. 7). The mRNA levels of
299 *Nr3c1*, *Nr3c2* and *Hsd11b1* were not affected by exposure to pollutants in the liver, in both
300 sexes (Supplementary Fig. 3).

301

302 **4. Discussion**

303 Glucocorticoids exert a strong effect on lipid metabolism via binding to the steroid
304 receptor GR in the adipose tissues and the liver (Lee et al., 2018, Woods et al., 2015) in which
305 11 β HSD1 has predominantly reductase activities allowing local amplification of
306 glucocorticoid action in the absence of changes in plasma corticosterone levels (Lee and
307 Fried, 2014). Although weakly expressed, MR mediates as well metabolic effects related to
308 glucocorticoid binding rather than aldosterone binding in liver and ATs (Kuhn and Lombes,
309 2013). Based on findings presented herein showing coordinated and reduced expression of
310 *Nr3c1*, *Nr3c2* and *Hsd11b1* mRNA levels and decreased GR protein level in VAT of females
311 fed a standard diet; we concluded that pollutant exposure caused a defect in glucocorticoid
312 signaling. Importantly, these effects exhibited tissue-specificity because they were not
313 observed in female SAT or in liver of both sexes. They also exhibited sex-specificity. Indeed,
314 while the levels of *Nr3c1* were down-regulated in VAT as shown in ST-females, the levels of
315 *Hsd11b1* did not change in VAT and the levels of *Nr3c2* were up-regulated in the SAT of ST-
316 males exposed to pollutants, unlike females.

317 The origin of the sex-dimorphism probably stems from differences in sexual steroids,
318 estrogens and androgens. It is well described that manifestation of metabolic syndrome caused
319 by GC excess depend on interactions between glucocorticoids and sex hormones (Alemany,
320 2012, Quinn et al., 2014). For example, a high-fat feeding resulted in enhanced corticosterone
321 plasma levels in males (not shown) but not in females (the present study). Origin of the sex-
322 dimorphism could as well stem from cross-interactions of AHR, glucocorticoids and estrogen
323 signaling as reviewed (Mauvais-Jarvis, 2018, Swedenborg et al., 2009, Casals-Casas and
324 Desvergne, 2011, Leblanc et al., 2019), raising the hypothesis that a mixture of pollutants
325 could locally contribute to the alteration of GC signaling in females without impacting
326 corticosterone plasma levels. Consistently, we detected mRNA changes in *Ahr* and *Esr2* in the

327 VAT of pollutant-exposed ST-females in addition to the decreased expression of the
328 glucocorticoid receptors. These effects were restricted to the VAT in pollutant-exposed
329 females and distinct from those observed in males [(present study and (Naville et al., 2019)],
330 suggesting that changes in GC but also in estrogen and AHR signaling in response to
331 exposure to the mixture of pollutants may cause the overall metabolic changes in females,
332 described in the present study. Certain effects caused by pollutant exposure overlapped those
333 described following a high-fat feeding as previously reported (Labaronne et al., 2017). It
334 included decreased of *Nr3c1*, *Hsd11b1*, *AhR* mRNA levels but also of the estrogen receptor
335 *Esr2* (a trend was observed for *Esr1*) and enhanced expression of *Gper1*. This may explain
336 why exposure to pollutants did not cause changes in the VAT of females fed a high-fat diet.
337 Noteworthy, *Gper1* increased in SAT and VAT in both females (present study) and males
338 (Naville et al., 2019) in response to a high-fat diet indicating that this estrogen receptor was
339 not regulated in a sex-dependent way.

340 Glucocorticoids regulate almost every aspect of adipose tissue biology metabolism
341 including adipogenesis, adipocyte metabolism, inflammation and adipokine production
342 preferentially targeting visceral adipose tissue as exemplified in Cushing's syndrome with the
343 development of visceral obesity at the expense of the subcutaneous adipose tissue (Lee and
344 Fried, 2014). Importantly, the pollutant-induced effects described herein in female mice
345 occurred in the absence of changes in the weight of SAT and VAT excluding impact of
346 pollutant exposure on adipogenesis. Accordingly, adipogenic markers remained in the control
347 range. To validate glucocorticoid-signaling disturbances in pollutant-exposed females, we
348 analyzed the mRNA levels of one primary GR target gene presenting glucocorticoid response
349 elements (GREs) in the promoter, *Tsc22d3* encoding GILZ for glucocorticoid induced leucine
350 zipper (Ayroldi and Riccardi, 2009). *Tsc22d3* mRNA levels decreased in VAT (a trend) but
351 no changes were observed in SAT in females, thus suggesting pollutants interfered with

352 glucocorticoid signaling in VAT, only. In addition, as pollutants may have pro-inflammatory
353 actions (Naville et al., 2015, Kim et al., 2012, Pardo et al., 2018, Rebourcet et al., 2010), we
354 measured mRNA levels of several cytokines in VAT (and SAT) but they remained in the
355 control range. Eventually, we demonstrated that leptin plasma levels and *Lep* mRNA levels
356 significantly decreased in VAT of the pollutant-exposed females (not in SAT) while
357 adiponectin plasma levels and *Adipoq* mRNA levels in VAT and SAT remained in the control
358 range. Interestingly, males exposed to the mixture of pollutants exhibited enhanced mRNA
359 levels of several adipogenic markers including *Adipoq* in SAT but not in VAT and no changes
360 in *Lep* mRNA levels or leptin plasma levels [the present study and (Naville et al., 2019)],
361 again highlighting sex-differences in the response to pollutant-exposure in metabolically-
362 active tissues. Further studies will aim at understanding the coordinated regulation of *Nr3c2*
363 and *Tsc22d3* mRNA levels in the SAT of exposed males together with the enhanced
364 expression of several adipogenic markers including *Nr1c3*, *Fabp4* and *Cidec* described
365 elsewhere (Naville et al., 2019).

366 Leptin level was the only significant metabolic phenotype difference measured in
367 females in response to exposure to the mixture of pollutants. Leptin is an adipokine that exerts
368 satiety effects and reduced leptin fits with enhanced food intake as we observed in females but
369 not in males. Leptin is expressed as a function of adipose tissue mass and is more expressed in
370 females than in males (Zhang and Chua, 2017); thus, reduced leptin levels in basal conditions
371 of feeding without gain of weight despite enhanced food intake could indicate enhanced
372 energy expenditure and thermogenesis. Since we did not measure these parameters in the
373 present study, validation of the hypothesis will await further studies. A second hypothesis to
374 explain the reduction of glucocorticoid signaling and leptin expression in females exposed to
375 pollutants could be a possible dysregulated peripheral clock in VAT. There are several clues
376 in the literature to support this hypothesis. Glucocorticoids are secreted in circadian cycles to

377 coordinate the circadian rhythm with energy balance and leptin synthesis (So et al., 2009).
378 Adrenalectomy diversely affected peripheral circadian clocks including GILZ expression in
379 the adipose tissue (Sotak et al., 2016) and led to decreased serum leptin (Yilmaz et al., 2002).
380 The powerful GR agonist, dexamethasone led to the disappearance of clock gene rhythmicity
381 in adipose tissues (Gomez-Abellan et al., 2012).

382 The circadian clock regulates metabolism and energy homeostasis in peripheral tissues
383 including the adipose tissues and synchronization with the master clock in the hypothalamic
384 suprachiasmatic nuclei (SCN) would be in part orchestrated by glucocorticoids to keep in step
385 diurnal functions with the environmental cycles of light and nutrition (Dickmeis, 2009,
386 Balsalobre et al., 2000). Circadian rhythms are generated by feedback loops in core clock
387 gene expression (Reinke and Asher, 2019). Among core clock components investigated, we
388 demonstrated herein significant reduction of *Per1* and *Per2* mRNA levels in VAT but not in
389 SAT in response to pollutant exposure in females, and no effect on *Clock* and *Arntl*.
390 Interestingly, both *Per1* and *Per2* mRNA levels enhanced in the SAT (not in VAT) of males
391 exposed to the mixture of pollutants. These findings are consistent with the presence of GRE
392 elements in the promoters of *Per1* and *Per2* genes (Koyanagi et al., 2006, So et al., 2009).
393 They also validate changes in GC signaling in the VAT of exposed females and in the SAT of
394 exposed males. Overall, these findings pointed that several peripheral circadian clock-related
395 genes were diversely affected by exposure to a mixture of pollutants in a sex- and tissue-
396 dependent way (at least at the mRNA level in the ATs) extending previous reports (Labaronne
397 et al., 2017) (Shen et al., 2019, Kopp et al., 2017). Further studies are needed to explore why
398 VAT in females and SAT in males were differentially regulated in response to the mixture of
399 pollutants in a context of standard diet feeding, and why SAT in females was not affected. It
400 may explain the mild metabolic phenotype described in females (the present study). Indeed,
401 circadian metabolic disturbances may be early events and exposure to pollutants greater than

402 15 weeks could have resulted in more harmful phenotypes than what was described herein.
403 This hypothesis merits further attention.

404 Consistent with the definition that endocrine disruptors may influence any hormonal action to
405 mediate their disruptive effects, we also identified that the mixture of pollutants targeted
406 11 β HSD1 in addition to corticoid receptors. These findings extend previous reports that
407 endocrine disruptors can affect enzymes involved in hormone metabolism including the
408 steroidogenic acute regulatory protein (STAR) (Guyot et al., 2004), the estrogen
409 sulfotransferase (SULT1E1) (Naville et al., 2013), aromatase and others (Beausoleil et al.,
410 2018) (Zoeller et al., 2012, Nadal et al., 2017). Noteworthy, pollutants used in the mixture
411 were incorporated at a dose lower than their no-observed adverse effect level (NoAEL) and
412 just above their tolerable daily intake (TDI) dose. The TDI is supposedly the dose without
413 adverse effect in humans and it is derived from the NoAELs determined through experimental
414 studies applying corrective factors to take into account inter- and intra-species variations
415 (Dorne, 2010) of 100 to 1000 (Supp. Table 1). Therefore, this study gave further evidences
416 that the risk assessment procedure which is based on NoAELS for single chemicals may not
417 be protective enough. In addition, comparison of metabolic disturbances elicited by the
418 mixture versus pollutants individually will allow determining if the observed effect resulted
419 from the action of one or several pollutants or from a cocktail effect.

420

421 **5. Conclusions**

422 We here demonstrated that exposure to a mixture of low-dosed pollutants caused reduced
423 expression of glucocorticoid receptors in the visceral but not in the subcutaneous adipose
424 tissue or in the liver in female mice and that it was associated with decreased plasma levels of
425 leptin and reduced expression of *Lep* and of various canonical genes related to the circadian

426 clock machinery in visceral adipose tissue. Inasmuch as lowering glucocorticoid signaling
427 following adrenalectomy prevents physiological oscillations of circadian genes and impact
428 adipokine secretion (Sotak et al., 2016, Yilmaz et al., 2002), our data fuel the hypothesis that
429 a mixture of low-dosed chemicals may alter clock genes resulting in metabolic effects at least
430 in the visceral adipose tissue in female mice.

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437

438 **Declaration of competing interest**

439 The authors declare that there are no conflicts of interest.

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700 **FIGURE LEGENDS**

701 **Figure 1:** Metabolic phenotyping of female mice fed a standard (ST) or a high-fat (HF) diet
702 and exposed to pollutants (grey columns) or not (dark columns). Results are expressed as
703 means±SEM of n=7-8 female mice/group. Statistical analysis was performed using a two-way
704 Anova analysis to determine the overall effect of the pollutant mixture and the diet. ns: not
705 significant;*, p<0.05; **p<0.01; ***p<0.001.

706 **Figure 2 :** Effect of the mixture of pollutants and of the high-fat high-sucrose (HF) diet on the
707 expression of genes encoding GR and MR, 11βHSD1, AHR and estrogen receptors in female
708 VAT (A). Values are means ± SEM with n=7-8. Data were analyzed using two-way Anova as
709 presented in the insert followed by *Post hoc* analysis between relevant groups; ns: not
710 significant; *p<0.05 ; ** p<0.01 ; ***p<0.001 ; ST : standard diet. (B) Western-blotting
711 analysis of the effect of pollutant exposure on GR protein expression in female VAT. Values
712 are means ± SEM with n = 6. ST0: standard diet not containing the mixture of pollutants;
713 STp : standard diet containing the mixture of pollutants.

714 **Figure 3 :** Effect of the mixture of pollutants and of the high-fat high-sucrose (HF) diet on the
715 expression of genes encoding GR, MR, 11βHSD1, AHR and estrogen receptors in the SAT of
716 female mice. Values are means ± SEM with n=7-8. Data were analyzed using two-way Anova
717 as presented in the insert; ns: not significant; ** p<0.01 ; ***p<0.001. ST : standard diet.

718 **Figure 4 :** Effect of the mixture of pollutants on plasma leptin and adiponectin levels, food
719 intake (A) and on the expression of *Lep* and *Adipoq* in female VAT (B) and SAT (C). Values
720 are means ± SEM with n = 7-8 except for food intake (n = 4 because mice were 2 per cage).
721 *p>0.05 ; ** p<0.01. ST0 : standard diet not containing the mixture of pollutants ; STp :
722 standard diet containing the mixture of pollutants.

723 **Figure 5** : Effect of the mixture of pollutants on the expression of *Tsc22d3* and the circadian
724 genes *Clock*, *Arntl*, *Per1*, *Per2* and *Nr1d1* in female VAT (A) and SAT (B). Values are
725 means \pm SEM with n=7-8. * $p>0.05$; ** $p<0.01$. ST0 : standard diet not containing the
726 mixture of pollutants ; STp : standard diet containing the mixture of pollutants.

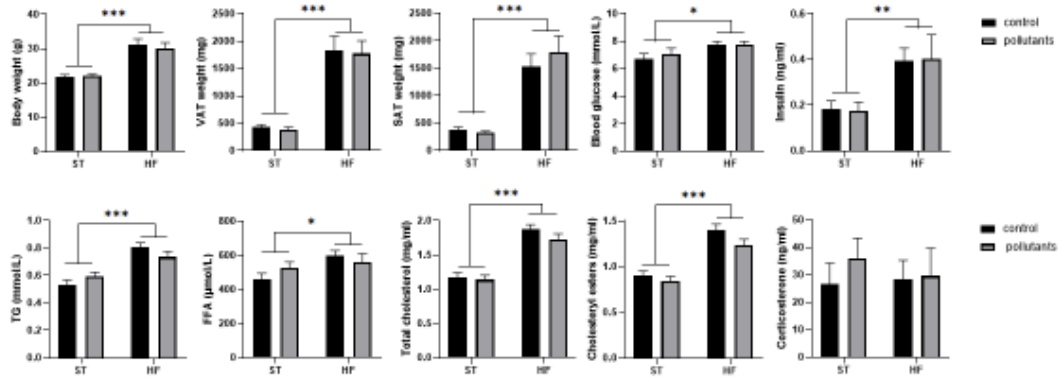
727 **Figure 6**: Effect of the mixture of pollutants on the expression of *Nr3c1*, *Nr3c2*, *Hsd11b1*,
728 *Tsc22d3*, *Clock*, *Arntl*, *Per1*, *Per2* and *Nr1d1* in the VAT (A) and SAT (B) of male mice fed a
729 standard-diet. Values are means \pm SEM with n=8. * $p>0.05$; ** $p<0.01$; *** $p<0.001$. ST0 :
730 standard diet not containing the mixture of pollutants ; STp : standard diet containing the
731 mixture of pollutants.

732 **Figure 7** : Effect of the mixture of pollutants on food intake and corticosterone plasma levels
733 (A), and on the expression of *Lep* and *Adipoq* in male VAT (B) and SAT (C). Values are
734 means \pm SEM with n=8 (n=4 for food intake). * $p>0.05$; ** $p<0.01$. ST0 : standard diet not
735 containing the mixture of pollutants ; STp : standard diet containing the mixture of pollutants.

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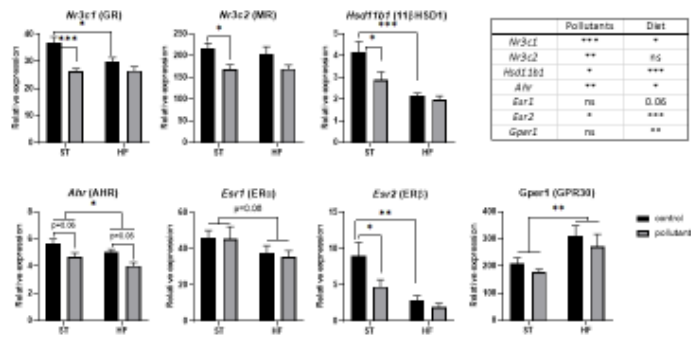
Figure 1



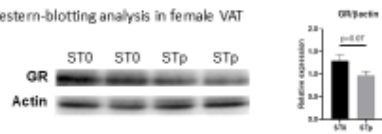
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Figure 2

A- Gene analysis in female VAT



B- Western-blotting analysis in female VAT



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Figure 3

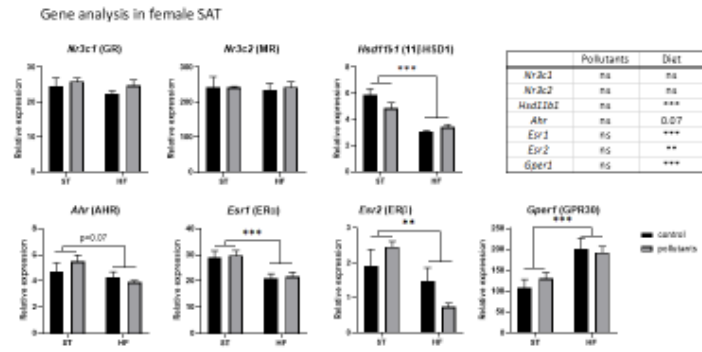
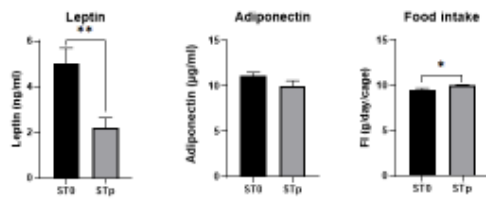
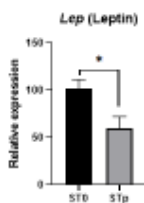


Figure 4

A- Plasma analysis and food intake



B-Female VAT



C-Female SAT

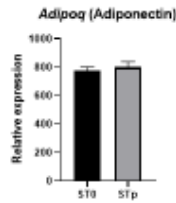
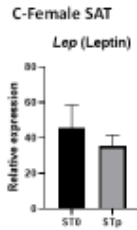
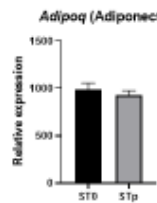
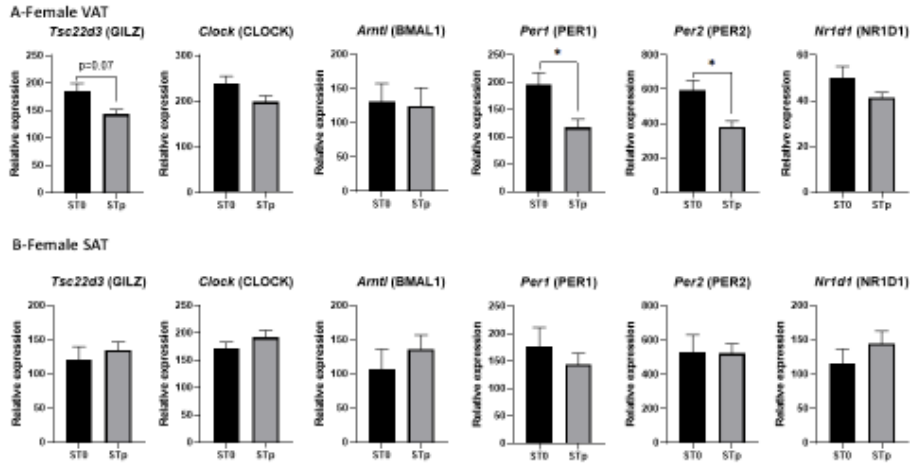
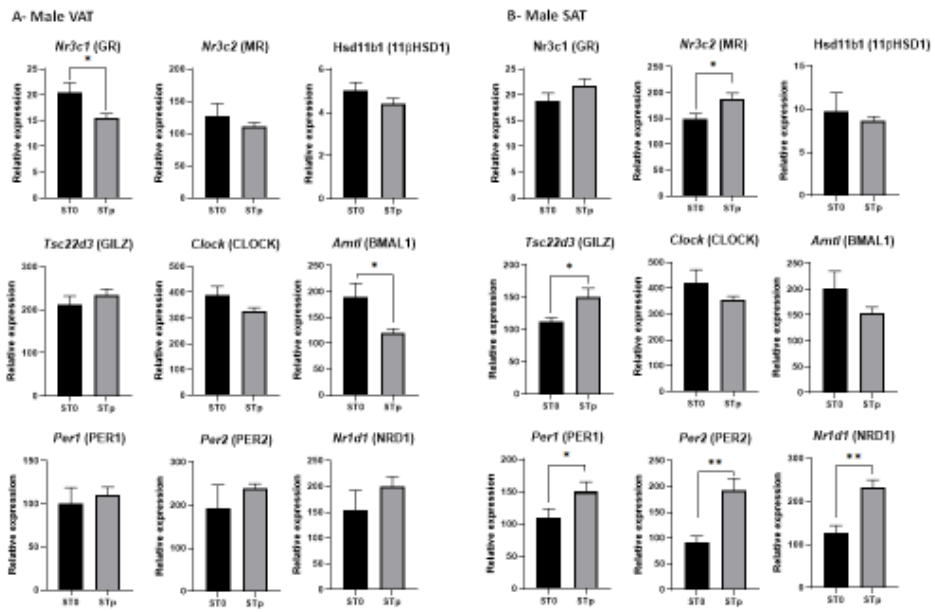


Figure 5



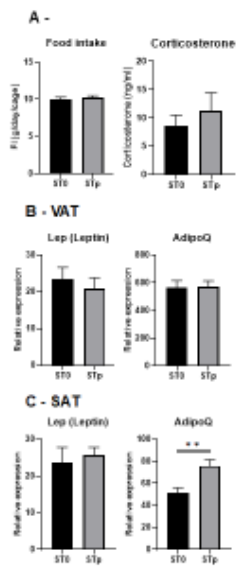
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Figure 6



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Figure 7



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746 **Supplementary File**

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748 **Supplementary Table 1:** References doses of the pollutants present in the mixture and doses
 749 used in the diets (all per kg and per day) (From Naville et al., 2019).

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Pollutants in the mixture	PCB153	BPA	TCDD	751
				DEHP ₇₅₂
NOAEL	40µg	5mg	2 ng	5mg ⁷⁵³
TDI	20ng	4µg	1-4pg	50µg ⁷⁵⁴
doses used	200ng	40µg	20pg	500µg ⁷⁵⁵

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758 **Supplementary Table 2:** List of the primers used in RT-qPCR

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gene	reference	sequences: 5'→3'	
		sense	antisense
<i>Adipoq</i>	NM_009605	AGGCCGTGATGGCAGAGATG	CTTCTCCAGGTTCTCCTTTCTGCTGC
<i>Ahr</i>	NM_013464.4	TCA-TCT-GGT-TTC-CTG-GCA-ATG-AAT	ATA-AGC-TGC-CCT-TTG-GCA-TC
<i>Arntl</i>	NM_007489	CTGCCTGGAAGGAAGTTACA	AAGGTCCACAGGATTTGACT
<i>Ccl2</i>	NM_011333	TGG-AGC-ATC-CAC-GTG-TTG-GC	ACT-ACA-GCT-TCT-TTG-GGA-CA
<i>Ccl5</i>	NM_013653	TCAAGGAGTATTTCTACACCAG	TGGCACACACTTGGCGGTTTC
<i>Cidec</i>	NM_178373;NM_001301295	TGG-CAC-AAT-CGT-GGA-GAC-AG	AGA-GGG-TTG-CCT-TCA-CGT-TC
<i>Clock</i>	NM_007715;NM_001289826;NM_001305222	ACAGCAGCTTCCTTCAGTTC	TGCTCTGTTGTAGTGAAAAG
<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	CTCTTCCCAGCAGCAGTCAGTC	AGCATCTCCAGCAGCAGGT
<i>Fabp4</i>	NM_024406	CAG-AAG-TGG-GAT-GGA-AAG-TCG	CGA-CTG-ACT-ATT-GTA-GTG-TTT-GA
<i>Gper1</i>	NM_029771	AGC-TGA-TCA-GAT-CTA-GGG-AG	GTC-CTG-GGA-GCC-TGT-TAG-TC
<i>Gusb</i>	NM_010368	CTT-CAT-GAC-GAA-CCA-GTC-AC	GCA-ATC-CTC-CAG-TAT-CTC-TC
<i>Hsd11b1</i>	NM_181755, NM_005525, NM_001206741	TTGCCCATGCTGAAGCAGAGC	CTGTTTCTGTGTCTATGAGGC
<i>Il1b</i>	NM_008361	ACT-GTT-CCT-GAA-CTC-AAC-TG	CCT-GTT-GAT-GTG-CTG-CTG-CG
<i>Lep</i>	NM_008493	CACCAGGATCAATGACATTTTC	TGCCAGTGTCTGGTCCATCTTG
<i>Nr1c3</i>	NM_001127330, NM_011146	TCT-CTC-CGT-AAT-GGA-AGA-CC	GCA-TTA-TGA-GAC-ATC-CCC-AC
<i>Nr1d1</i>	NM_021724	GGGAGGTGGTAGAGTTTGCC	CCAGGGAGTTGAGCTTCTCG
<i>Nr3c1</i>	NM_008173	ACACCTGGATGACCAATGACC	GCAGGGTAGAGTCATTCTCTGC
<i>Nr3c2</i>	NM_001083906	CCAAGTGTCCCAACAGTTCT	CATCTTCCATCACTTCCTGT
<i>Per1</i>	NM_0011065; NM_001159367	TGAGCCAGAGGCCAGATTG	TGTATGGCTGCTCTGACTG
<i>Per2</i>	NM_011066	ATCTCCAGGCGGTGTTGAAG	TGCCTTTCTCCTCACTCTCG
<i>Tbp</i>	NM_013684	TGG-TGT-GCA-CAG-GAG-CCA-AG	TTC-ACA-TCA-CAG-CTC-CCC-AC
<i>Tnfa</i>	NM_013693	CCA-GAC-CCT-CAC-ACT-CAG-ATC	CAC-TTG-GTG-GTT-TGC-TAC-GAC
<i>Tsc22d3</i>	NM_001077364, NM_010286	AACAAGATTGAGCAGGCCAT	TGGAACTTTTCCAGTTGCTC

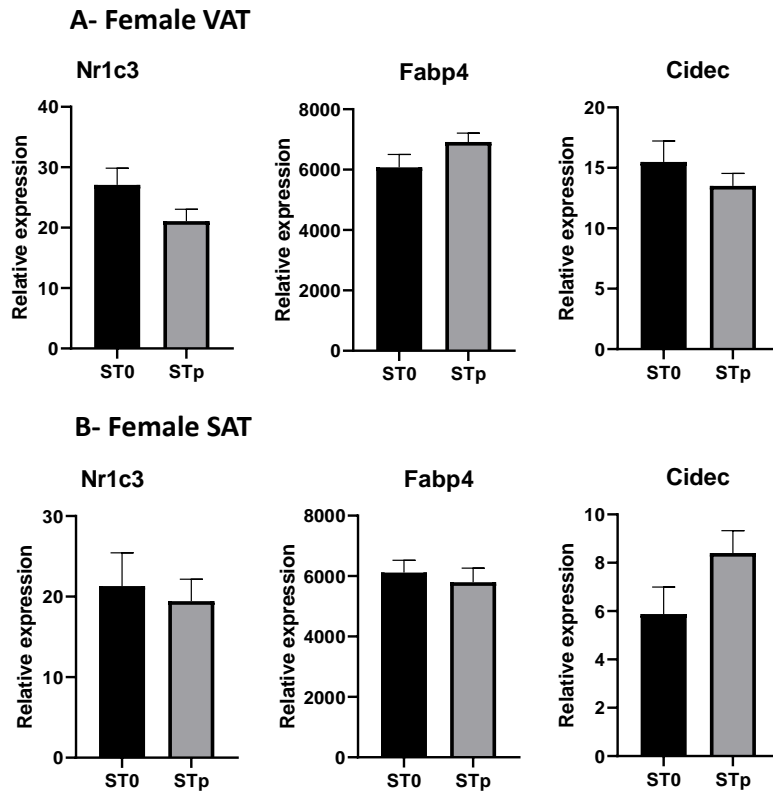
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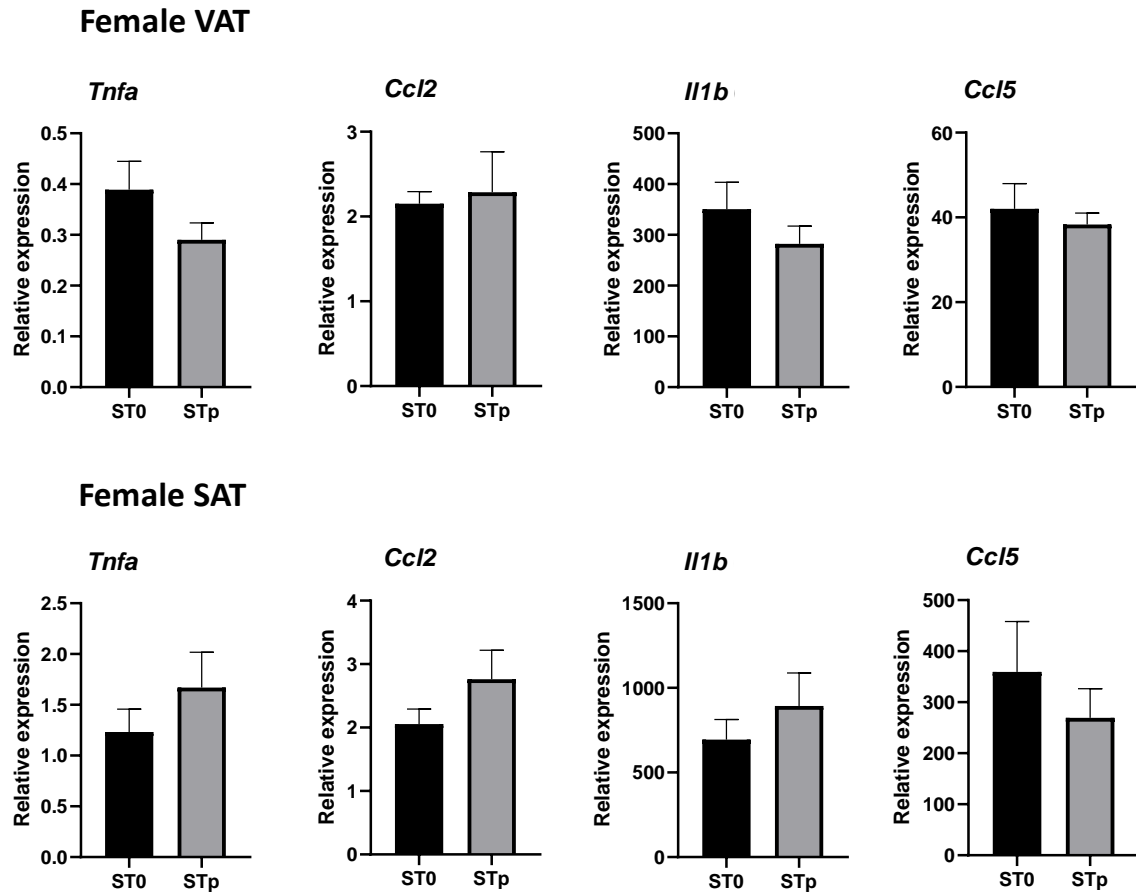
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Supplementary Figure 1: Effect of the mixture of pollutants on the expression of *Nr1c3*, *Fabp4* and *Cidec* in female VAT (A) and in female SAT (B). Values are means \pm SEM with n=7-8. ST0: standard diet not containing the mixture of pollutants; STp: standard diet containing the mixture of pollutants.



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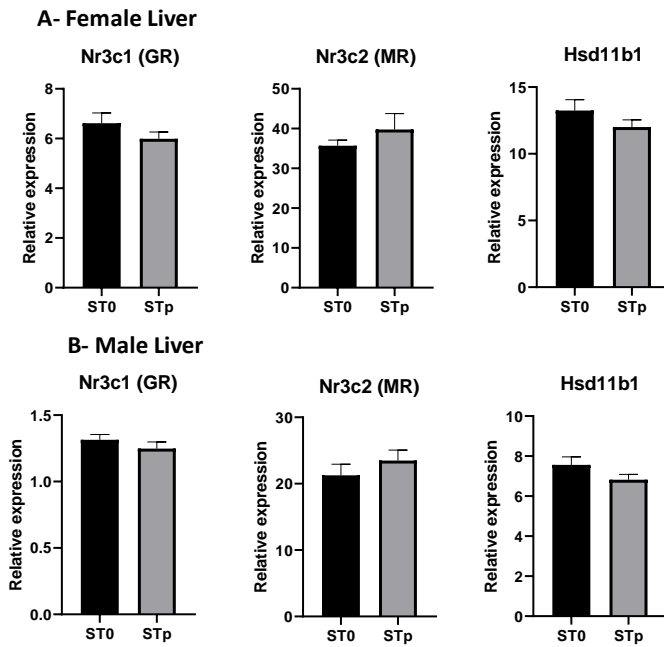
781 **Supplementary Figure 2:** Effect of the mixture of pollutants on the expression of *Tnfa*, *Ccl2*,
782 *Il1b* and *Ccl5* in female VAT and in female SAT. Values are means \pm SEM with n=7-8. ST0:
783 standard diet not containing the mixture of pollutants; STp: standard diet containing the mixture
784 of pollutants.



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786

787 **Supplementary Figure 3:** Effect of the mixture of pollutants on the expression of *Nr3c1*,
788 *Nr3c2* and *Hsd11b1* in female liver (A) and male liver (B). Values are means \pm SEM with
789 $n=7-8$. ST0: standard diet not containing the mixture of pollutants; STp: standard diet
790 containing the mixture of pollutants.
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