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

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Abstract

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

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

Highlights

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

1. Introduction

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

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

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

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

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

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

respectively (Diamanti-Kandarakis et al., 2009, Casals-Casas and Desvergne, 2011, Pavek, 2016).

Using the exposure model to a mixture of BPA, DEHP, PCB153 and TCDD, we previously demonstrated that the metabolic adverse impact in mice was strongly sex-dependent altering estrogen signaling in the liver of exposed females and hepatic cholesterol metabolism in males (Naville et al., 2013). In addition, the mixture of pollutants affected other metabolic tissues including the adipose tissues and involved other nuclear receptors than the estrogen and xenobiotic receptors (Julien et al., 2018, Julien et al., 2019, Naville et al., 2015, Naville et al., 2019). The purpose of the current study was to address the implication of glucocorticoid signaling in mediating the effects of the pollutants in female mice. We also looked for sexual dimorphism benefiting from male samples taken in a previous study (Naville et al., 2019).

Glucocorticoids (GC) are steroid hormones with strong anti-inflammatory properties. They are produced by the adrenal glands under the negative feed-back involving the hypothalamic-pituitary-adrenal (HPA) axis. They exert vital roles on behavior, immunity and metabolism through binding to corticoid receptors in target cells including the liver and adipocytes (Vegiopoulos and Herzig, 2007). Recent reports highlighted that xenobiotics could bind to GR (Gulliver, 2017), and that BPA and phthalates had a weak GR agonist activity in adipocytes (Gulliver, 2017, Le Magueresse-Battistoni et al., 2017, Casals-Casas and Desvergne, 2011, Sargis et al., 2010).

2. Materials and Methods

2.1.Animals, diet and experimental design

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

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

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

2.2.Tissue collection and blood and plasma analyses

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

2.3. RNA extraction and real-time PCR analyses

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

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 (dilution1/10,000, A5060, Sigma). Blots were revealed using Luminata Classico Western
211 HRP substrate (Milllipore, 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 ± 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).

3.2. Expression of various nuclear receptor/transcription factors and of 11β-hydroxysteroid dehydrogenase1 (11βHSD1) in the visceral adipose tissues of female mice fed a ST or a HF diet and exposed to the mixture of pollutants

Adipose tissues express several nuclear receptor/transcription factors that pollutants can bind to initiate downstream activation signals among which the estrogen receptors, the aryl hydrocarbon receptor (AhR) but also the glucocorticoid (GR) and the mineralocorticoid (MR) receptors (Swedenborg et al., 2009, Lee et al., 2018). In addition to analyzing these receptors by RT-qPCR, we also studied *Hsd11b1* which encodes the 11β-hydroxysteroid dehydrogenase1 (11βHSD1). Indeed in the adipose tissue, 11βHSD1 catalyzes the conversion of the inactive cortisone into the active corticosterone thus locally regulating availability of GCs (Sandeep and Walker, 2001). Two-way Anova analysis indicated that the "Pollutant" factor had significant effects on all the genes studied, excepted for *Esr1* and *Gper1* encoding ERα and GPR30, respectively. The "Diet" factor had significant effects on all the genes studied, but not on *Nr3c2* encoding MR. A trend was observed for *Esr1* (Fig. 2, insert). *Post Hoc* analysis determined that pollutant exposure significantly down-regulated *Nr3c1* encoding GR, *Nr3c2*, *Hsd11b1*and *Esr2* (encoding ERβ) in ST-fed but not in HF-fed nutritional conditions. A trend was observed for *Ahr* (Fig. 2A). Western-blotting analysis of VAT proteins confirmed decreased protein levels of GR in females exposed to the mixture of pollutants (Fig. 2B).

3.3. Expression of various nuclear receptor/transcription factors and of 11βHSD1 in the subcutaneous adipose tissue of female mice fed a ST or a HF diet and exposed to the mixture of pollutants

Contrary to the results observed in VAT, exposure to pollutants in SAT did not affect the levels of expression of the glucocorticoid and estrogen receptors, of 11βHSD1 and of AHR

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

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

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

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

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

3.6 Analysis of the response to pollutant mixture in male mice

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

4. Discussion

Glucocorticoids exert a strong effect on lipid metabolism via binding to the steroid receptor GR in the adipose tissues and the liver (Lee et al., 2018, Woods et al., 2015) in which 11βHSD1 has predominantly reductase activities allowing local amplification of glucocorticoid action in the absence of changes in plasma corticosterone levels (Lee and Fried, 2014). Although weakly expressed, MR mediates as well metabolic effects related to glucocorticoid binding rather than aldosterone binding in liver and ATs (Kuhn and Lombes, 2013). Based on findings presented herein showing coordinated and reduced expression of *Nr3c1*, *Nr3c2* and *Hsd11b1* mRNA levels and decreased GR protein level in VAT of females fed a standard diet; we concluded that pollutant exposure caused a defect in glucocorticoid signaling. Importantly, these effects exhibited tissue-specificity because they were not observed in female SAT or in liver of both sexes. They also exhibited sex-specificity. Indeed, while the levels of *Nr3c1* were down-regulated in VAT as shown in ST-females, the levels of *Hsd11b1* did not change in VAT and the levels of *Nr3c2* were up-regulated in the SAT of ST-males exposed to pollutants, unlike females.

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

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

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

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

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

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

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

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

Consistent with the definition that endocrine disruptors may influence any hormonal action to mediate their disruptive effects, we also identified that the mixture of pollutants targeted 11βHSD1 in addition to corticoid receptors. These findings extend previous reports that endocrine disrupters can affect enzymes involved in hormone metabolism including the steroidogenic acute regulatory protein (STAR) (Guyot et al., 2004), the estrogen sulfotransferase (SULT1E1) (Naville et al., 2013), aromatase and others (Beausoleil et al., 2018) (Zoeller et al., 2012, Nadal et al., 2017). Noteworthy, pollutants used in the mixture were incorporated at a dose lower than their no-observed adverse effect level (NoAEL) and just above their tolerable daily intake (TDI) dose. The TDI is supposedly the dose without adverse effect in humans and it is derived from the NoAELs determined through experimental studies applying corrective factors to take into account inter- and intra-species variations (Dorne, 2010) of 100 to 1000 (Supp. Table 1). Therefore, this study gave further evidences that the risk assessment procedure which is based on NoAELS for single chemicals may not be protective enough. In addition, comparison of metabolic disturbances elicited by the mixture versus pollutants individually will allow determining if the observed effect resulted from the action of one or several pollutants or from a cocktail effect.

5. Conclusions

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

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

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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FIGURE LEGENDS

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

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

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

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

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

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

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

Figure 1

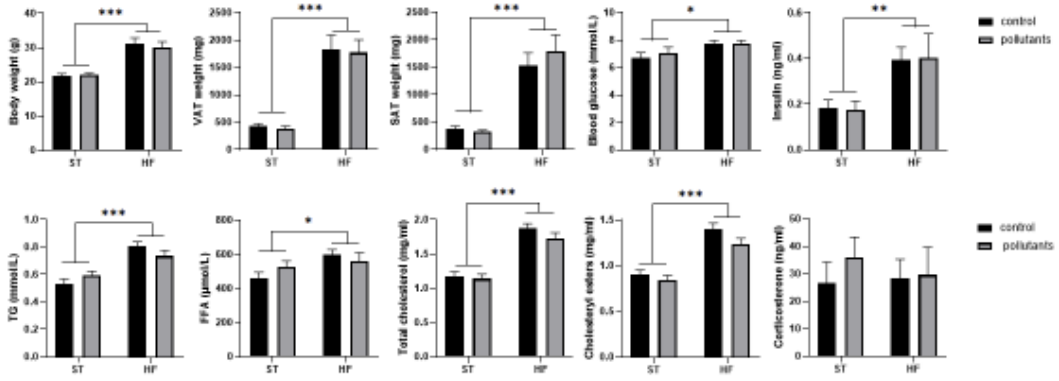

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Figure 2

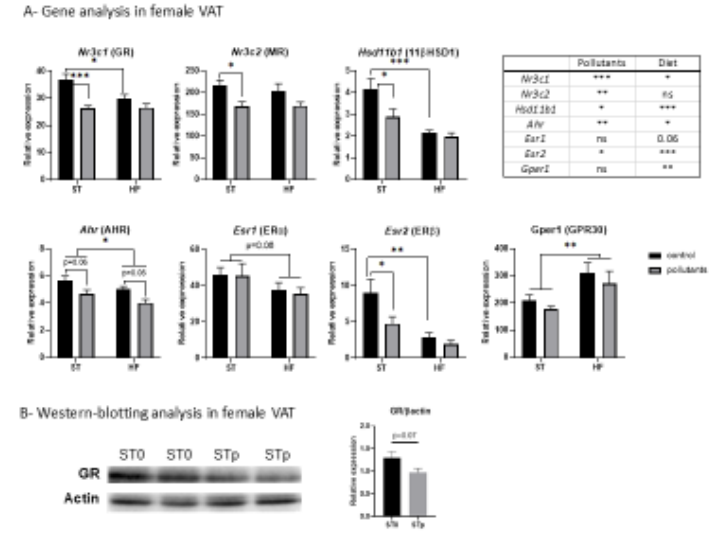

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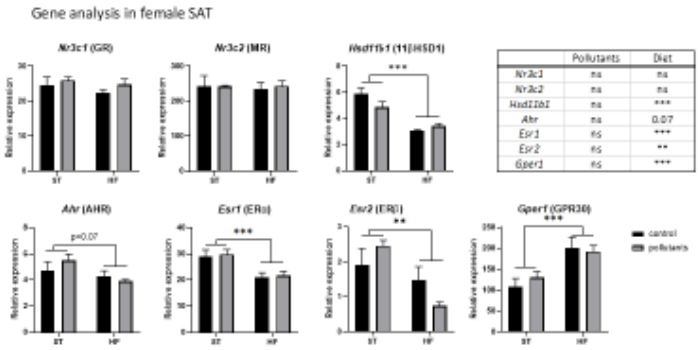
Figure 3



	Pollutants	Diet
Nr3c1	ns	ns
Nr3c2	ns	ns
Hsd11b1	ns	***
Ahr	ns	0.07
Esr1	ns	***
Esr2	ns	**
Gper1	ns	***

741

Figure 4

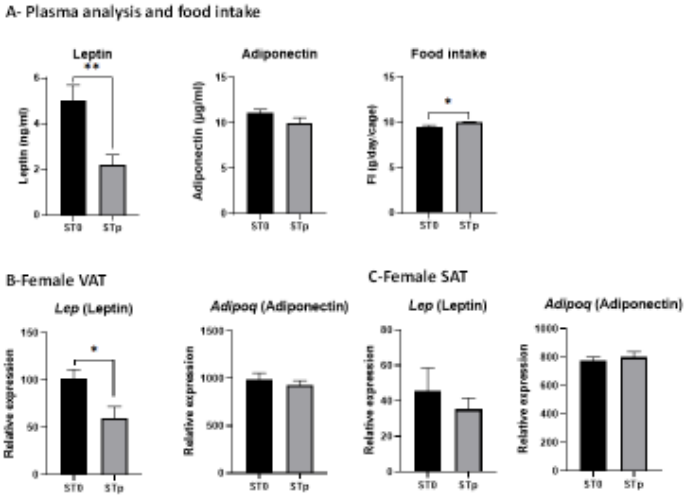


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Figure 5

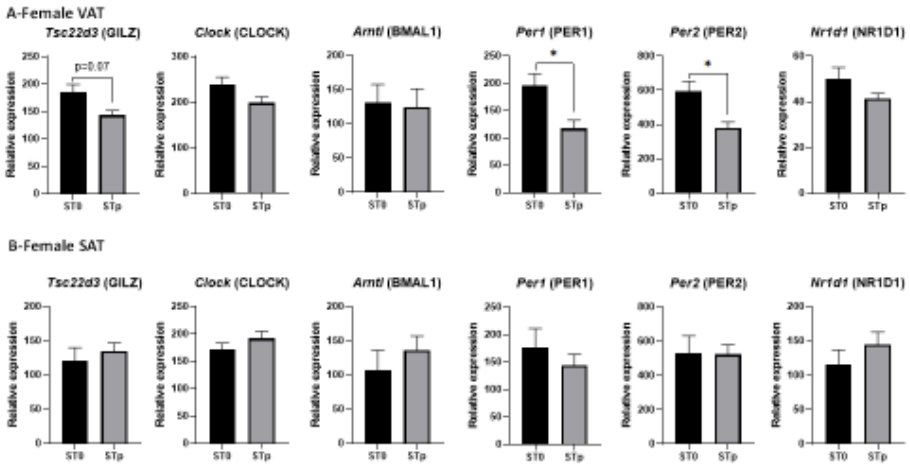

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Figure 6

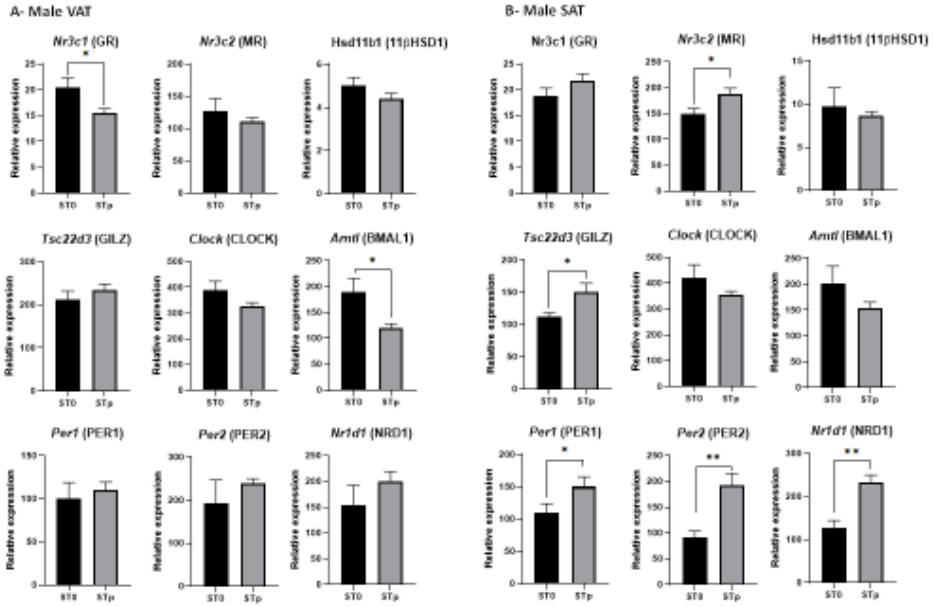

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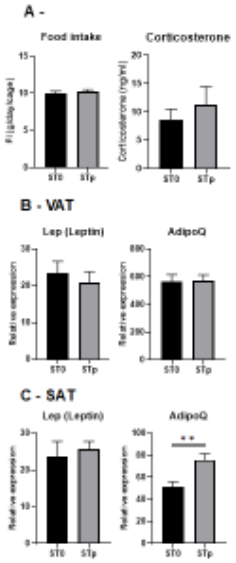
Figure 7


A -
Food intake
Corticosterone
B - VAT
Lep (Leptin)
AdipoQ
C - SAT
Lep (Leptin)
AdipoQ
STO
STp

745

Supplementary File

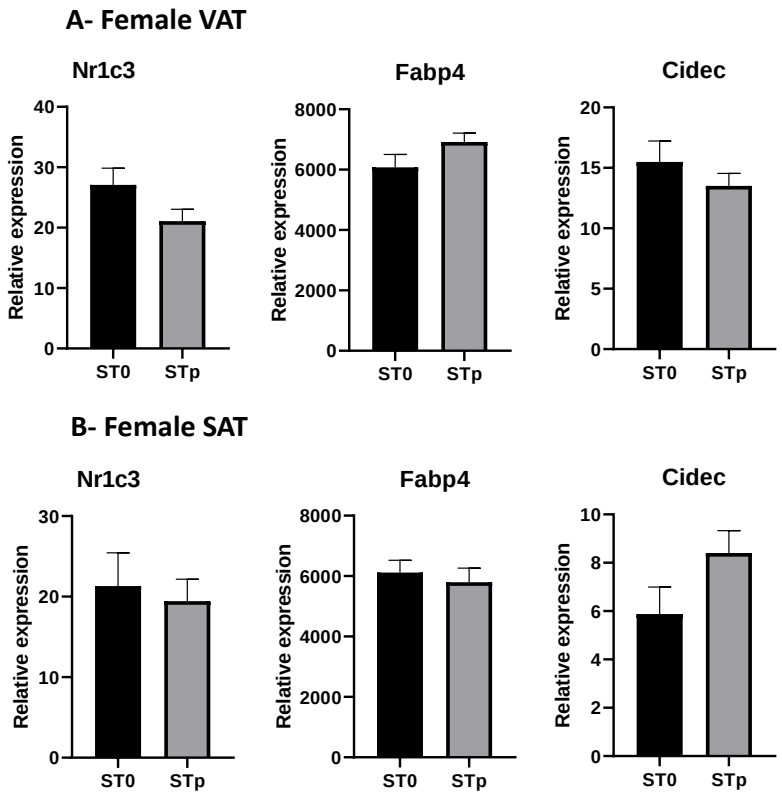
Supplementary Table 1: References doses of the pollutants present in the mixture and doses used in the diets (all per kg and per day) (From Naville et al., 2019).

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

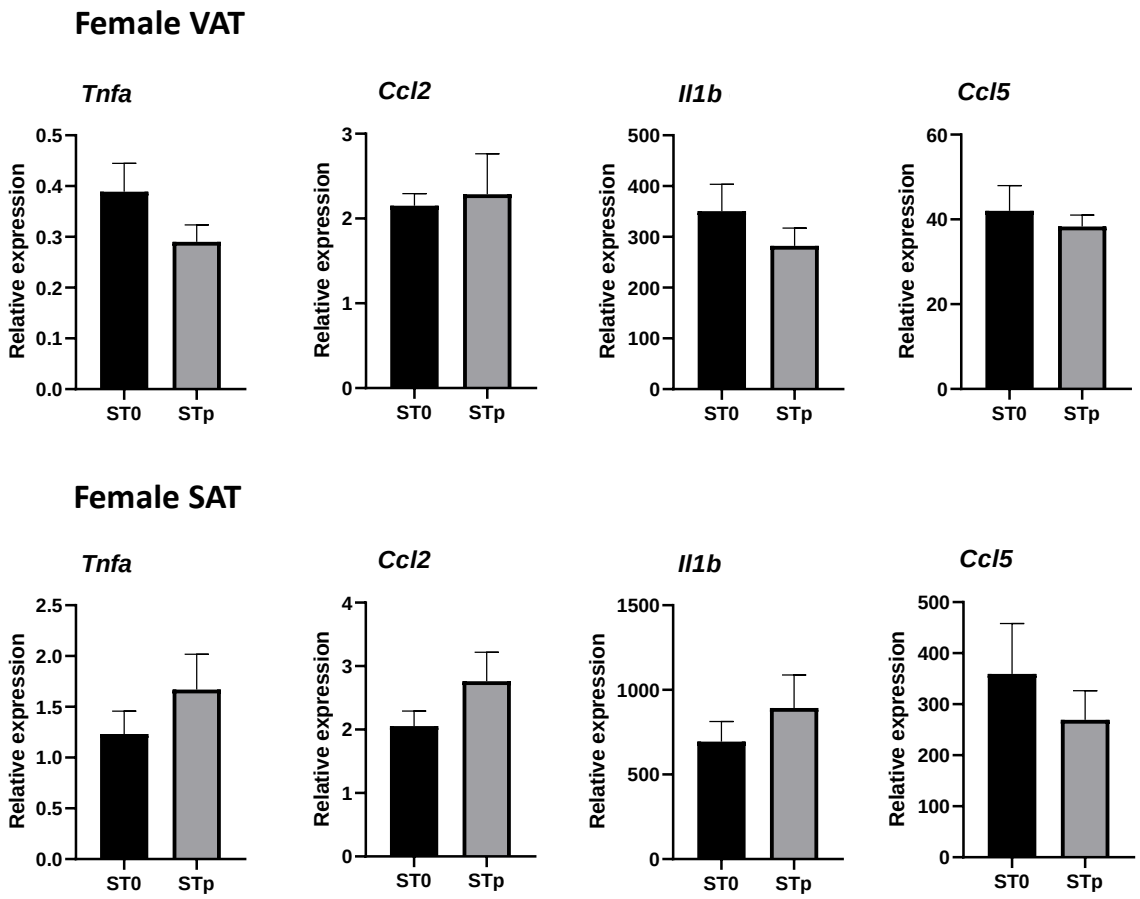
Supplementary Table 2: List of the primers used in RT-qPCR

		sequences: 5'->3'	
gene	**reference**	**sense**	**antisense**
Adipoq	NM_009605	AGGCCGTGATGGCAGAGATG	CTTCTCCAGGTTCTCCTTTCCTGC
Ahr	NM_013464.4	TCA-TCT-GGT-TTC-CTG-GCA-ATG-AAT	ATA-AGC-TGC-CCT-TTG-GCA-TC
Arntl	NM_007489	CTGCCTGGAAGGAAGTTACA	AAGGTCCACAGGATTTGACT
Ccl2	NM_011333	TGG-AGC-ATC-CAC-GTG-TTG-GC	ACT-ACA-GCT-TCT-TTG-GGA-CA
Ccl5	NM_013653	TCAAGGAGTATTTCTACACCAG	TGGCACACACTTGGCGGTTC
Cidec	NM_178373;NM_001301295	TGG-CAC-AAT-CGT-GGA-GAC-AG	AGA-GGG-TTG-CCT-TCA-CGT-TC
Clock	NM_007715,NM_001289826,NM_001305222	ACAGCAGCTTCCTTCAGTTC	TGCTCTGTTGTAGTGGAAAG
Esr1	NM_000125;NM_001122740;NM_001122741;NM_001122742	TGT-TTG-CTC-CTA-ACT-TGC-TC	CCT-TCT-CTT-CCA-GAG-ACT-TC
Esr2	NM_207707; NM_010157	CTCTTCCCAGCAGCAGTCAGTC	AGCATCTCCAGCAGCAGGT
Fabp4	NM_024406	CAG-AAG-TGG-GAT-GGA-AAG-TCG	CGA-CTG-ACT-ATT-GTA-GTG-TTT-GA
Gper1	NM_029771	AGC-TGA-TCA-GAT-CTA-GGG-AG	GTC-CTG-GGA-GCC-TGT-TAG-TC
Gusb	NM_010368	CTT-CAT-GAC-GAA-CCA-GTC-AC	GCA-ATC-CTC-CAG-TAT-CTC-TC
Hsd11b1	NM_181755, NM_005525,NM_001206741	TTGCCCATGCTGAAGCAGAGC	CTGTTTCTGTGTCTATGAGGC
Il1b	NM_008361	ACT-GTT-CCT-GAA-CTC-AAC-TG	CTT-GTT-GAT-GTG-CTG-CTG-CG
Lep	NM_008493	CACCAGGATCAATGACATTTC	TGCCAGTGTCTGGTCCATCTTG
Nr1c3	NM_001127330,NM_011146	TCT-CTC-CGT-AAT-GGA-AGA-CC	GCA-TTA-TGA-GAC-ATC-CCC-AC
Nr1d1	NM_021724	GGGAGGTGGTAGAGTTTGCC	CCAGGGAGTTGAGCTTCTCG
Nr3c1	NM_008173	ACACCTGGATGACCAAATGACC	GCAGGGTAGAGTCATTCTCTGC
Nr3c2	NM_001083906	CCAAGTGTCCCAACAGTTCT	CATCTTCCATCACTTCCTGT
Per1	NM_0011065; NM_001159367	TGAGCCAGAGGCCAGATTG	TGTATGGCTGCTCTGACTG
Per2	NM_011066	ATCTCCAGGCGGTGTTGAAG	TGCCTTTCTCCTCACTCTCG
Tbp	NM_013684	TGG-TGT-GCA-CAG-GAG-CCA-AG	TTC-ACA-TCA-CAG-CTC-CCC-AC
Tnfa	NM_013693	CCA-GAC-CCT-CAC-ACT-CAG-ATC	CAC-TTG-GTG-GTT-TGC-TAC-GAC
Tsc22d3	NM_001077364,NM_010286	AACAAGATTGAGCAGGCCAT	TGGAACTTTTCCAGTTGCTC

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 ± SEM with n=7-8. ST0: standard diet not containing the mixture of pollutants; STp: standard diet containing the mixture of pollutants.



Supplementary Figure 2: Effect of the mixture of pollutants on the expression of *Tnfα*, *Ccl2*, *Il1β* and *Ccl5* in female VAT and in female SAT. Values are means ± SEM with n=7-8. ST0: standard diet not containing the mixture of pollutants; STp: standard diet containing the mixture of pollutants.



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