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Abstract

We recently hypothesized that a mixture of low-dosed dioxin, polychlorobiphenyl, phthalate and bisphenol may induce estrogeno-mimetic activities in a model of lifelong-exposed female mice. Herein, we evaluated the impact of this mixture in estrogen deficiency conditions. Based on the protective effects of estrogens against metabolic disorders, we reasoned that exposure to pollutants should attenuate the deleterious metabolic effects induced by ovariectomy. In line with the hypothesis, exposure to pollutants was found to reduce the impact of ovariectomy on glucose intolerance and insulin resistance, to enhance the expression levels of the hepatic estrogen receptor α and to attenuate the ovariectomy-induced enhancement of the chemokine MCP-1/CCL2 considered as an indicator of estrogen signalling. Because of the very low doses of pollutants used in mixture, these findings may have strong implications in terms of understanding the potential role of environmental contaminants in the development of metabolic diseases, specifically in females during menopausal transition.

Key words: endocrine disruptors, metabolic disorders, ovariectomy, estrogeno-mimetic compound

Abbreviation: EDC: endocrine disrupting chemical; PCB: polychlorobiphenyl; TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin; BPA: bisphenol A; ER: estrogen receptor; EST: estrogen sulfotransferase; HFHS: high-fat high-sucrose; DEHP: Di-[2-ethylhexyl]-phthalate; GTT: glucose tolerance test; TDI: tolerable daily intake; OVX: ovariectomy; FFA: free fatty acids; AT: adipose tissue; MCP-1: monocyte chemoattractant protein-1; Ccl5: Chemokine (C-C motif) ligand 5; IL6, IL1: interleukin 6, interleukin 1; Gusb: b-glucuronidase; HPRT: hypoxanthine ribosyl transferase; TBP:TATA-Box Binding Protein; PPAR: peroxisome proliferator-activated receptor; SREBP1: sterol response element binding protein 1; CPT1:carnitine palmitoyl transferase

1. Introduction

Convincing evidences have demonstrated that environmental pollutants contribute to the aetiology of obesity and associated metabolic disorders including diabetes and cardiovascular diseases (WHO, 2016, Casals-Casas and Desvergne, 2011, Gore et al., 2015, Lee et al., 2014). These multifactorial diseases have risen dramatically these last few decades and they constitute a true challenge for Public Health in terms of quality of life and life expectancy but also because of the heavy economic cost for the society (Trasande et al., 2016). The World Health Organization (WHO) update (WHO, 2016) reported a doubling of the worldwide prevalence of obesity between 1980 and 2014 so that 39% of adults were overweight in 2014 and the global prevalence of diabetes has also risen during this period of time to affect 8.5% of adults.

It has been suggested that mechanisms by which chemicals interfere with energy homeostasis could be related to their hormono-mimetic properties. An endocrine disrupting chemical (EDC) is an exogenous substance or mixture of chemicals that interfere with any aspect of hormone action (Zoeller et al., 2012). About a thousand of chemicals could display ED activities and a subset was identified as metabolic disruptors (Heindel et al., 2017, Nadal et al., 2017, Casals-Casas and Desvergne, 2011, Janesick and Blumberg, 2016). Indeed, it is well documented that energy balance is a highly controlled process depending on a multitude of hormonal inputs and signalling molecules interconnecting central and peripheral organs to meet energy demands and preserve homeostasis (Mauvais-Jarvis et al., 2013). Identified chemicals concerned dioxins which are by-products issued from industrial processes or polychlorobiphenyls (PCBs) produced for their fire-resistant characteristics. Although dioxin production is limited and PCBs forbidden, their persistent properties made them still present in the environment. Other EDCs may have short half-life (e.g., phthalates, bisphenols). However because they are massively produced by industry for everyday plastic objects or

medical equipment among others, everyone is continuously exposed as reflected by the constant detectable levels of several of them in blood, urine or hair (Casals-Casas and Desvergne, 2011, Lee et al., 2014, Appenzeller et al., 2017, Vandenberg et al., 2012). Importantly, these chemicals have been identified as bearing estrogeno-mimetic, anti-estrogenic or anti-androgenic properties which explain that EDCs were first identified as altering the reproductive function and fertility (Vandenberg et al., 2012, Zoeller et al., 2012). The challenge of EDCs resides in that they may act at low and environmental doses in a manner possibly depending on the tissue considered, the sex of the individual and the timing of exposure, all in a context of multi-exposure (Vandenberg et al., 2012, Zoeller et al., 2012, Casals-Casas and Desvergne, 2011, Gore et al., 2015, Le Magueresse-Battistoni, 2017, Heindel et al., 2017, Barouki et al., 2012).

To enhance our understanding on the metabolic impact of EDCs in mixture, we have developed in the laboratory a model of lifelong exposure to a mixture made of 4 well-spread chemicals including a dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin [TCDD]), PCB153, diethylhexylphthalate (DEHP) and bisphenol A (BPA) at doses in the range of the Tolerable Daily Intake (TDI), thus without expected adverse health effects (Dorne, 2010). Further, these pollutants could be found in a Western-type of diet and are archetypal examples of EDCs suspected to be involved in the aetiology of metabolic disturbances (Lee et al., 2014, Heindel et al., 2017, Le Magueresse-Battistoni, 2017, Le Magueresse-Battistoni et al., 2015, Nadal et al., 2017). Importantly, using this model we reported an aggravation of glucose intolerance and hepatic insulin resistance in adult female mice but not in the sibling males. Specifically, we found reduced expression levels of the estrogen receptor α (ERα) and induction of the estrogen sulfotransferase (EST) that inactivates estrogens, all consistent with reduced hepatic estrogen signalling (Naville et al., 2013). Conversely, in immature female mice characterized by low circulating levels of estrogens, lifelong exposure to the pollutant mixture induced

improvement of glucose tolerance, enhanced lean mass, and reduced inflammation of the adipose tissue consistent with enhanced estrogen signalling (Naville et al., 2015). We therefore put forward the hypothesis that the mixture of pollutants could exert estrogeno-mimetic effects. Indeed, it is well established that estrogens protect females against metabolic disorders and diabetes for concentrations which stay within physiological range values (Mauvais-Jarvis, 2015), and that the metabolic protective effect of estrogens requires intact hepatic estrogen signalling through its receptor α (ERα) (Zhu et al., 2013). To give further insight to the hypothesis, we have evaluated in the present study the impact of the mixture of pollutants in conditions of estrogen deficiency. We reasoned that if the hypothesis still holds true, then the mixture of pollutants should attenuate the deleterious metabolic effects induced by ovariectomy.

2. Materials and Methods

2.1. Animals, diets and experimental design

All procedures were performed with the approval of the Regional Committee of Ethics for Animal Experiments and the French Ministry for Higher Education and Research. After one week acclimatization, five week-old C57Bl6 female mice (Envigo, Gannat, France) were fed a high fat-high sucrose diet (HFHS) (from Envigo) containing (HFp) or not (HF0) a mixture of pollutants. The diet was given 5 weeks before mating with standard-chow adult C57Bl6 males and continued during gestation and lactation. At weaning, the offspring (F1 mice) received the same diet than their dams. By 5 weeks, half of the F1 females were ovariectomized (OVX) and the other half was sham-operated, generating 4 groups of 6-9 animals per group: HF0-OVX and HF0-sham not exposed to the pollutant mixture and HFp-OVX and HFp-sham exposed to the pollutant mixture. Surgeries were performed under

anaesthesia using a mixture of ketamine (100µg/g) and xylazine (10µg/g) injected intraperitoneally. Ovaries were removed (OVX) or not (sham-operated) after two small flank incisions. The analgesic meloxicam was added to the water during the first days after surgery.

With this protocol, HFp dams underwent normal gestation. All parameters (litter size, sex-ratio and pup weight) were in the normal range, consistently with the previous data of the laboratory (Naville et al., 2013). Body weight and food intake were recorded weekly throughout the protocol until mice were 12-weeks of age. Validation of the surgery was assessed at the time of sacrifice with the observation of uterine atrophy in the OVX females. We also quantified 17ß estradiol levels and found them below the detection threshold of the assay (Interchim, Cayman EIA kit, Montluçon, France) both in HF0- and HFp-OVX mice.

The pollutant mixture was made of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, CAS n° 1746-01-6), 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 from Sigma-Aldrich, Saint-Quentin Fallavier, France). Each pollutant was used at a dose close to its tolerable daily intake (TDI) reference dose of either the pollutant itself (DEHP, BPA) or representative congeners (TCDD, PCB153) (EFSA, 2015, European Food Safety Authority Panel on Food Additives, 2005, WHO, 2003, van Leeuwen et al., 2000). Pollutants dissolved in dimethylsulfoxide (DMSO) were diluted in corn oil to facilitate uniform homogenization in the food. Pollutant-free diet contained volumes of DMSO and corn oil identical to the pollutant-containing food. The composition of the diets is given in the supplemental data (Supplementary table 1). Thus, it resulted in a daily exposure to 2pg/kg bw/d of TCDD, 80 ng/kg bw/d of PCB153, 50µg/ kg bw/d of DEHP and 5 µg/ kg bw/d of BPA, as previously described (Naville et al., 2015, Naville et al., 2013). An additional pellet of pollutant-free HFSD diet was provided to animals to ensure they were fed ad libitum.

2.2. Metabolic tests

After 16 hours of fasting, mice were injected intraperitoneally with glucose 1 mg/g of body weight for the glucose tolerance test (GTT). Blood glucose was measured using OneTouchUltra glucometer (Lifescan, Issy-Les-Moulineaux, France). At times 0 and 15 min of the GTT, about 20µl of blood was collected from tail using heparinized glass capillary tubes. Recovered plasma was used for the measurement of insulin by ELISA (Mouse Ultrasensitive ELISA, Eurobio, Courtaboeuf, France).

2.3. Blood and tissue collection

Mice were fasted six hours and blood was collected by retro-orbital sampling. Mice were euthanized by cervical dislocation and liver, gastronecmius muscle, subcutaneous and visceral (periovarian+parametrial) adipose tissue were quickly dissected and snap-frozen in nitrogen liquid. We measured blood concentration of glucose (glucometer), plasma levels of insulin (Mouse Ultrasensitive ELISA, Eurobio), free fatty acids (FFA) (Sigma-Aldrich), triglycerides (Biolabo, Maizy, France), and leptin (Crystal Chem, Zaandam, Netherlands). Triglycerides were also measured in the liver after extraction of lipids from frozen liver samples using a lipid Extraction kit chloroform-free (Clinisciences, Nanterre, France).

2.4. Real time PCR analyses

Total RNA extracted from frozen liver and adipose tissue samples was reverse-transcribed and analyzed by real-time PCR as described (Naville et al., 2011) using a set of specific primers (Supplementary Table 2). Data were normalized relatively to house-keeping genes which were Gusb (encoding b-glucuronidase) for liver, HPRT (encoding hypoxanthine ribosyl transferase) for subcutaneous adipose tissue and TBP (encoding TATA-Box Binding Protein) for visceral adipose tissue.

2.5. Western blotting analysis

Proteins prepared from liver were separated on SDS-10% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed as previously described (Naville et al., 2013). Primary antibodies included mouse monoclonal antibodies directed against Esr1 (dilution 1:1000; sc-8005; Santa-Cruz Biotechnology, Cliniscience, Nanterre, France) or α-tubulin (dilution 1:2000; T5168; Sigma-Aldrich). The secondary antibodies were anti-mouse IgG horseradish peroxidase (HRP) conjugate (Bio-Rad, Marnes-la-coquette, France). Blots were revealed using the Luminata Classico Western HRP substrate (Millipore, Molsheim, France) and detection was made using the ChemiDocXRS+imaging system (BioRad). Results were analyzed with Image Lab Software (BioRad). Data were normalized relatively to α -tubulin.

2.6. Statistics

All results are expressed as means ± SEM and differences between means were considered significant at *p*-value <0.05. The normality of each variable was assessed using the Shapiro-Wilk test. To test for statistical significance, two-way ANOVA for non-repeated measures with ovariectomy and pollutants as factors was used. When significant effects without interaction were obtained, differences were tested using Student's *t*-test between the pollutant-exposed group and the non-exposed group. GraphPad Prism 5.0 software was used for all statistical analysis.

3. Results and Discussion

3.1. Pollutants reduced the impact of ovariectomy on glucose intolerance and insulin resistance

Estrogens confer metabolic protection to female mice especially when the animals are exposed to a diabetogenic stress such as a high-fat diet (Riant et al., 2009, Camporez et al., 2013). To examine the plausibility of the hypothesis that the mixture of pollutants exerted estrogeno-mimetic activities, we used a model of estrogen deficiency and investigated if the deleterious metabolic effects of pollutants shown in intact females (Naville et al., 2013) were alleviated in OVX mice. Of note, all females have been fed a HFHS diet and were obese, glucose intolerant and insulin resistant (Naville et al., 2013), ovariectomy resulting in a further gain weight (Fig. 1A and B) with enhanced accumulation of fat mass (Fig. 1D and E) and aggravation of glucose intolerance (Supplementary Figure), in accordance with the literature (Camporez et al., 2013). Pollutants did not alter body weight, weight gain, food intake or the weight of fat pads in either sham-operated or OVX females (Fig. 1). Finally, there were no changes in the weight of the liver or muscle between groups (data not shown).

In line with the hypothesis, we observed on the one hand, that sham-operated females exposed to pollutants consistently displayed an aggravation of glucose intolerance compared to HF0-sham (Fig. 2A). On the other hand, exposure of OVX females to pollutants resulted in an alleviation of glucose tolerance (Fig. 2B). It led to a significant inversion ($p=0.002$) of the AUC profile between the sham and the OVX females lifelong-exposed to pollutants (Fig. 2C). Interestingly, after 16 hours of fasting (time 0 of the GTT) the pollutant-exposed mice exhibited significant lower plasma insulin levels ($p= 0.047$) than the non-exposed mice but only in conditions of estrogen deficiency (Fig. 2D and E). Furthermore, in term of insulin levels, HFp-OVX females responded better than HF0-OVX females to glucose injection and insulin production was around 4-times enhanced (3.94-fold, $p<0.01$) compared to a 2.47-fold enhancement ($p<0.01$) in HF0-OVX females (Fig. 2E). It indicated a greater ability to produce insulin in response to glucose in HFp versus HF0 mice in conditions of estrogen deficiency. In addition, the HOMA-IR calculated at euthanasia was reduced by one third

(p=0.051) in HFp-OVX as compared to HF0-OVX mice (Table 1). To complete the metabolic phenotype, we observed that plasma TG levels were significantly reduced (-15%; p=0.008) in HFp-OVX as compared to HF0-OVX mice (Table 1). Collectively, these data indicated benefit effects of pollutant exposure on insulin sensitivity in conditions of estrogen deficiency induced by ovariectomy.

Notwithstanding, pollutants did not mimic the effects of estrogen replacement on body weight (Fig. 1) (Camporez et al., 2013) or on uterus weight (not shown) which may indicate that the estrogeno-mimetic activity of the mixture was relatively low. Indeed, it is well demonstrated that ovariectomy results in a gain of weight and uteri atrophy, both events being corrected by estrogen replacement in rodent experimental models (Mauvais-Jarvis, 2015). In our experimental protocol, 12-week old OVX females were 9.6% lighter in the HFp group compared to the HF0 group. Nonetheless, they remained significantly heavier than HFp sham-operated mice (Table 1) and leptin levels, which were enhanced in OVX mice consistent with enhanced fat mass, were not normalized by pollutants (Table 1). As well, the ovariectomy-induced elevation of free fatty acids predictive of insulin resistance (Bergman and Ader, 2000) was not corrected by pollutant exposure (Table 1). It could indicate that insulin sensitivity is extremely responsive to the estrogenic activity while normalization of FFA, body weight and leptin levels by estrogen replacement would require a much higher activity. Alternatively, it has been demonstrated that normalization of insulin sensitivity and glucose tolerance preceded body weight and adiposity rescue in mouse models of OVX after E2 replacement (Riant et al., 2009, Camporez et al., 2013). It would therefore be of interest to have a follow-up of the OVX mice during a longer period of time.

3.2. Pollutants reduced the inflammatory impact of ovariectomy on the adipose tissue

Insulin resistance triggers by a high-fat diet is accompanied by enhanced fat mass and low-grade inflammation that largely originates from the adipose tissue (AT) and AT dysfunction is considered as a key initiating factor and a main source of inflammatory signalling in obesity-related metabolic diseases illustrating the interference of inflammatory activation with metabolism (Hotamisligil, 2006). Estrogens well illustrate this interface between inflammation and metabolic disorders because they oppose the ovariectomy-induced metabolic disorders that include enhanced fat mass, insulin resistance, glucose intolerance but also enhanced inflammation (Monteiro et al., 2014, Benedusi et al., 2015). Importantly, monocyte chemoattractant protein-1 (MCP-1 encoded by Ccl2 gene) could play a critical role in inflammation associated with metabolic dysfunction. Indeed, overexpression of MCP-1 in adipocytes triggers adipose tissue inflammation and insulin resistance (Kanda et al., 2006) whereas its deficiency has been reported to ameliorate metabolic parameters of mice fed a high-fat diet (Tateya et al., 2010). Furthermore, MCP-1 level was enhanced in conditions of estrogen deficiency during menopausal transition (Tani et al., 2013) or following ovariectomy (Kim et al., 2013) and the OVX-induced impairment was reversed by estrogen administration (Abu-Taha et al., 2009). Several other studies have identified MCP-1 as being down-regulated by estradiol (Pervin et al., 1998, Arici et al., 1999, Janis et al., 2004) suggesting a role of MCP-1 as an indicator of estrogen change (Tani et al., 2013). All these findings prompted us to determine if Ccl2 gene expression levels changed in the adipose tissue of the OVX females and the impact of pollutants on this endpoint. In agreement with the literature, ovariectomy induced a significant increase of Ccl2 mRNA levels in both the visceral (vAT) and the subcutaneous (scAT) adipose tissues (Fig. 3A; $p<0.05$). Importantly, the enhanced expression level of Ccl2 was partially prevented in OVX mice exposed to pollutants (Fig. 3A; $p<0.05$), in line with the hypothesis of an estrogeno-mimetic activity of the mixture of pollutants. Furthermore, the vAT better responded to the pollutant exposure which halved the 3-fold

enhancement of Ccl2 gene expression levels linked to OVX (Fig. 3A). The investigation of other chemokines and cytokines indicated that these results were restricted to Ccl2 in the vAT (Fig. 3B). OVX resulted in the enhancement of Ccl5 gene expression levels but exposure to pollutants did not affect its expression level. IL6 gene expression levels did not fluctuate between groups. For IL1β, we also found an effect linked to the OVX operation but no effect of the pollutants in the OVX conditions (Fig. 3B). If considering that among the circulating cytokines and chemokines including MCP-1, IL6 and IL1β, only MCP-1 was found to fluctuate with estrogen plasma levels during the menopausal transition (Tani et al., 2013), it argues that the mixture of pollutants could have exerted some estrogeno-mimetic activity preventing the OVX-induced enhancement of Ccl2. However, prevention would not have achieved sufficient levels to impact as well the expression levels of genes encoding IL1β and Ccl5 (Fig. 3). In addition, while MCP-1 has been described as attracting immune cells in the AT (Kanda et al., 2006), we found no changes in the gene expression levels of CD68 used as an indicator of macrophage infiltration in the HFp- compared to the HF0-OVX mice (not shown).

Collectively, the data indicate that the mixture displayed some estrogeno-mimetic activity. It has been demonstrated that metabolic indicators of OVX mice worsened with time along with enhanced inflammatory markers (Benedusi et al., 2015). Although one could again comment on the interest to have a follow-up of the OVX mice during a longer period of time, our data point to insulin sensitivity and Ccl2 gene expression levels as very responsive end-points to estrogen activity.

The AT is not the only source of inflammatory mediators in obesity-associated metabolic disturbances along with the insulin-resistant state. The liver and the muscle could also contribute to these metabolic alterations (Biddinger and Kahn, 2006). Concerning the skeletal muscle, the major organ of glucose uptake and utilization (Biddinger and Kahn, 2006), we

found no differences in the response profile during the insulin-sensitivity tests between the HF0-OVX and HFp-OVX groups (data not shown) eluding the possibility that insulin resistance linked to pollutant exposure in conditions of estrogen deficiency originated from the muscles. In addition, there were no differences in the muscle weight between the 2 OVX groups (data not shown).Therefore, we next focused on the liver.

3.3. Pollutants enhanced E2 signalling in the liver in conditions of estrogen deficiency

The liver orchestrates glucose and lipid metabolisms as well as drug disposition, all pathways shown to be targeted by the mixture of pollutants in lifelong exposed intact adult females (Naville et al., 2013). In addition, in the liver, estrogens are known to enhance insulin sensitivity through interacting with the estrogen receptor α encoded by the Esr1 gene, the only estrogen receptor in the liver of rodents (Leiter and Chapman, 1994, Mauvais-Jarvis, 2011, Zhu et al., 2013). Previously, we postulated that the observed aggravation of glucose intolerance in intact females lifelong exposed to the mixture of pollutants may have originated from a decreased Esr1 expression coupled to an enhanced expression of Sult1e1 which could have reduced estrogen bioavaibility (Naville et al., 2013). We thus measured these two genes in the HF0- and HFp-OVX females. Interestingly, we found enhanced Esr1 mRNA expression levels ($p<0.05$) and a slight trend for the corresponding 66 kDa protein in HFp-OVX which may have contributed to the alleviation of glucose tolerance (Fig. 4A). However, EST mRNA levels were reduced to their nadir after ovariectomy with no change in the pollutant-exposed mice along with circulating estrogen levels reduced to low levels (data not shown). These findings are consistent with the literature indicating that EST gene expression depends on estrogen levels and that its involvement in the regulation of estrogen bioavaibility is lost in ovariectomized animals (Cho et al., 2012).

We also quantified a series of genes by RT-qPCR to determine which pathway(s) could be impacted by pollutants in conditions of estrogen-deficiency that will explain differences in glucose tolerance and insulin sensitivity. However, the expression of genes encoding proteins involved in ß-oxidation or in glucose metabolism was not found altered in the liver of OVX females exposed to pollutants. Genes involved in lipogenesis were not modified either, and this is consistent with the lack of effect of the mixture of pollutants on the hepatic TG levels in the OVX mice (Fig. 4 and Table 1). Specifically, the genes surveyed included the nuclear receptor Nr1c1 encoding peroxisome proliferator-activated receptor-α (PPARα); the transcription factor SREBP1, sterol response element binding protein 1c, encoded by SrebF1; and the carnitine palmitoyl transferase, CPT1α, involved in mitochondrial fatty acid import for β-oxidation. Finally, we also investigated the hepatic gene expression levels of Ccl2 but found no OVX-induced enhancement of Ccl2 (Fig. 4) which may indicate that the liver could cope better than vAT with lower levels of circulating estrogens.

Taken together, we found that the hepatic expression of Esr1 was impacted by pollutants in conditions of estrogen deficiency but not the expression levels of genes involved in fatty acid metabolism or inflammation. Importantly, it has been demonstrated that in mice with a specific deletion of Esr1 in the liver, estrogen replacement in OVX females limited adiposity but failed to improve insulin sensitivity while both adiposity and insulin sensitivity are prevented in mice totally deficient for Esr1 (Zhu et al., 2013). Thus, these findings indicate that hepatic estrogen signalling is required to improve insulin sensitivity in OVX mice. Consequently, as for Ccl2 in AT, the enhanced Esr1 mRNA expression levels detected in the HFp-OVX mice compared to the HF0-OVX mice may well result from the estrogeno-mimetic activity of the mixture of pollutants to which mice have been exposed.

4. Conclusions

In conclusion, we brought here several pieces of evidences suggesting that the mixture of low-dose pollutants made of TCDD, PCB153, BPA and DEHP may exhibit estrogeno-mimetic activity supplying a potential explanation for their benefit effects including enhanced insulin sensitivity and reduced glucose intolerance in a model of ovariectomized mice fed a high-fat high-sucrose diet. Mechanisms could involve, among others, decreased Ccl2 gene expression levels in vAT and enhanced Esr1 gene expression levels in the liver. Further studies are warranted to better understand how exposure to the mixture of pollutants at low doses could trigger estrogeno-mimetic activity and if such activity is the result of additive, antagonist or synergistic effects of one or several of the pollutants present in the mixture. Importantly, these data extend our previous findings demonstrating that exposure to a mixture at doses around the Tolerable Daily Intake range have metabolic effects in a rodent model and that the effects in females are dependent on the estrogen endocrine milieu. Because of the very low doses of pollutants used in mixture, these findings may have strong implications in terms of understanding the potential role of environmental contaminants in the development of metabolic diseases, specifically in females during the menopausal transition.

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Ethics approval and consent to participate:

The protocol for handling mice has been approved by the Regional Committee of Ethics for Animal Experiments and the French Ministry for Higher Education and Research.

Competing interests:

The authors declared that they have no competing interests exist.

Authors' contribution:

Experimental design: BJ, CP, NV, EL, DN, BLMB; Data acquisition and analysis: BJ, DN, BLMB; Writing the manuscript: BLMB, DN and HV. All authors read and approved the final manuscript

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Legends of the figures

Fig. 1: Phenotype of the female mice fed HFSD (HF0-sham and HF0-OVX) or HFSD containing the mixture of pollutants (HFp-sham and HFp-OVX). **A)** Body weight curves before (3 to 5 weeks of age) and after ovariectomy. **B)** Cumulative gain of weight after surgery. **C)** Average of food intake calculated during the four last weeks of life. Weight of visceral (**D**) and subcutaneous adipose tissue (**E**). Results are means ± SE (n=7 to 9). For **A)** and **B)**, a: $p<0.05$ HF0-OVX *vs* HF0-sham; b: $p<0.05$ HFp-OVX *vs* HFp-sham. For **D)**, #: significant effect of the ovariectomy operation tested by two-way ANOVA.

Fig. 2: Glucose tolerance tests (GTT). Blood glucose measurement during GTT for HF0-sham *vs* HFp-sham mice (**A**) and HF0-OVX *vs* HFp-OVX (**B**). **C)** Differential AUC (Δ AUC) between HF0 and HFp, calculated from GTT curves obtained for sham *vs* OVX mice in both groups. Plasma insulin measurement before (time 0) and 15 min after glucose injection for HF0-sham *vs* HFp-sham mice (**D**) and HF0-OVX *vs* HFp-OVX (**E**). Results are means ± SE (n=6 to 9). * $p<0.05$ between time 15 and corresponding time 0. **c**: $p<0.05$ HFp-OVX vs HF0-OVX.

Fig.3. (**A**) mRNA expression of *Ccl2* in the subcutaneous adipose tissue (scAT) and the visceral adipose tissue (vAT). (**B**) expression of genes encoding the inflammatory markers *Ccl5, IL6, IL1b* in the vAT. Results are expressed as mean ± *S.E* (n=7 to 9); **a**: $p<0.05$ HF0-OVX *vs* HF0-sham; **b**: $p<0.05$ HFp-OVX *vs* HFp-sham; **c**: $p<0.05$ HFp-OVX vs HF0-OVX; #: significant effect of the ovariectomy operation tested by two-way ANOVA.

Fig.4. (**A**) mRNA and protein expression of ERα (*Esr1*) in the liver of estrogen deficient females. (**B**) Hepatic expression of genes involved in lipogenesis (*SrebF1*), beta-oxydation (*Nr1c1, Cpt1*) or encoding the inflammatory marker MCP-1 (*Ccl2).* Results are expressed as mean ± *S.E* (n=5 to 9); **c**: $p<0.05$ HFp-OVX vs HF0-OVX.

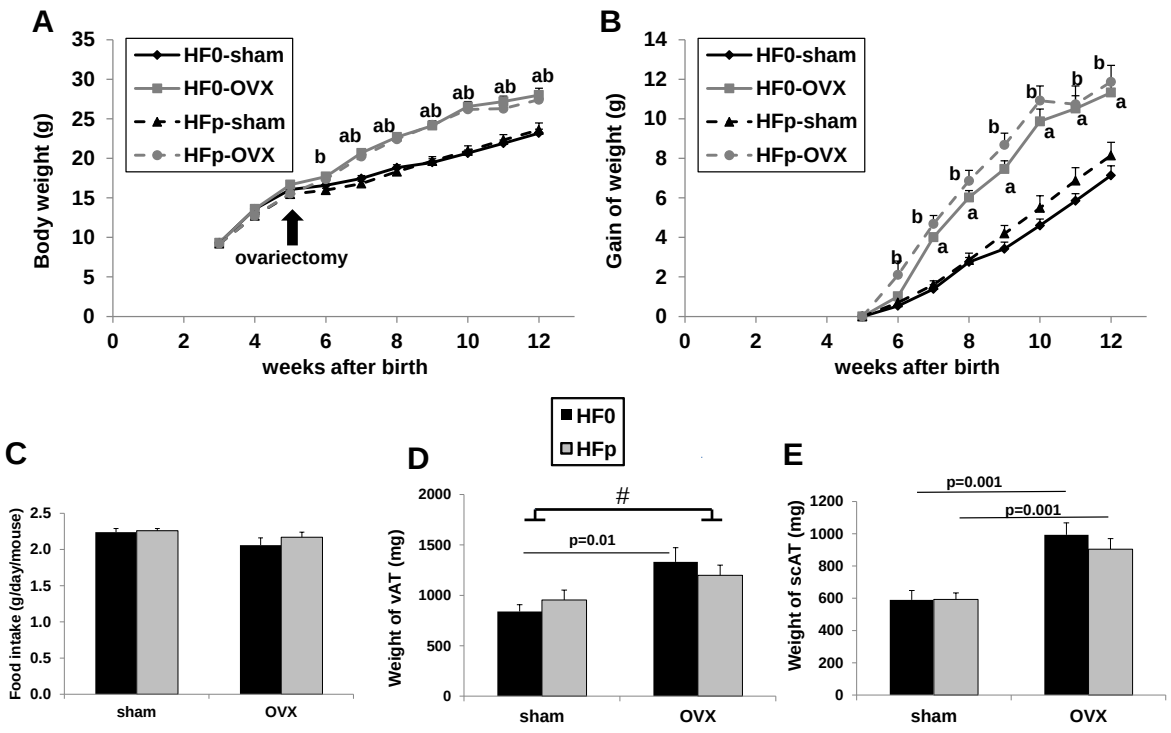
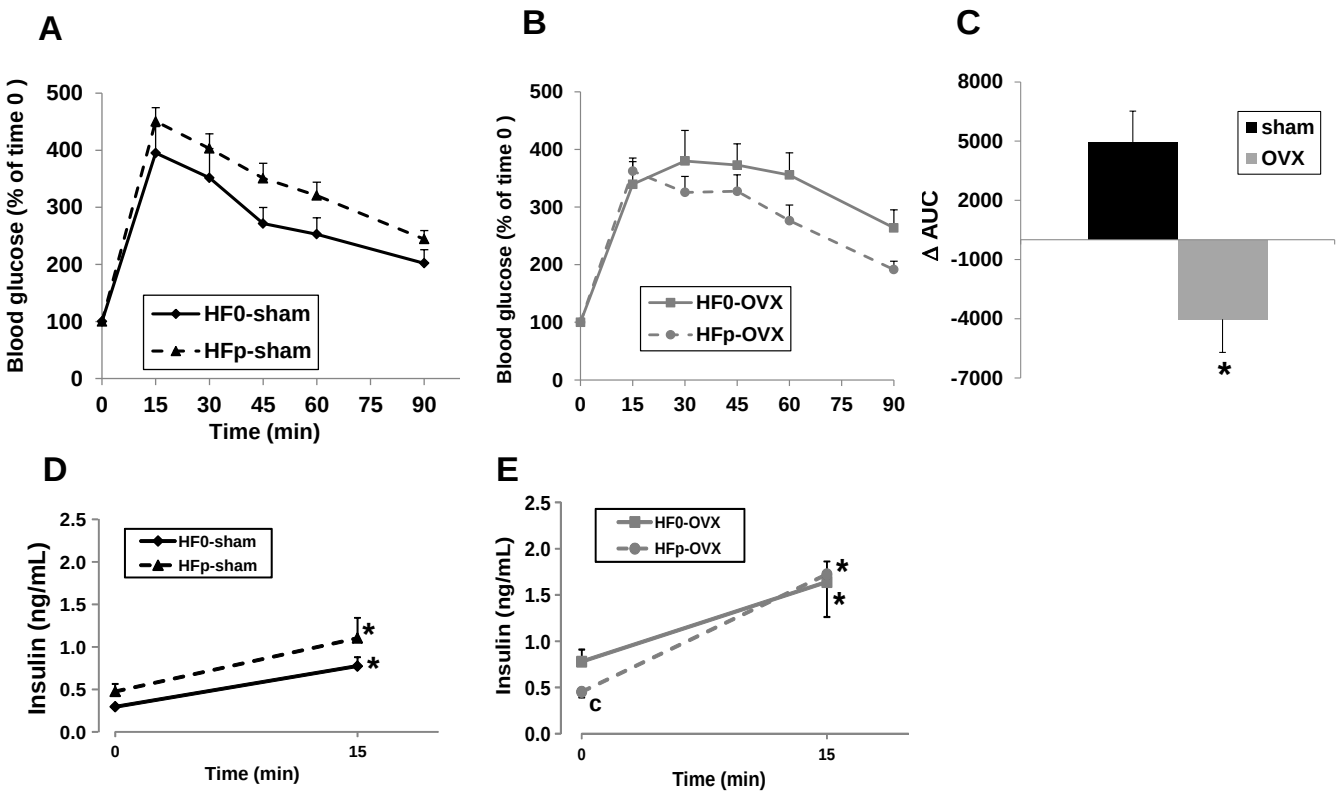
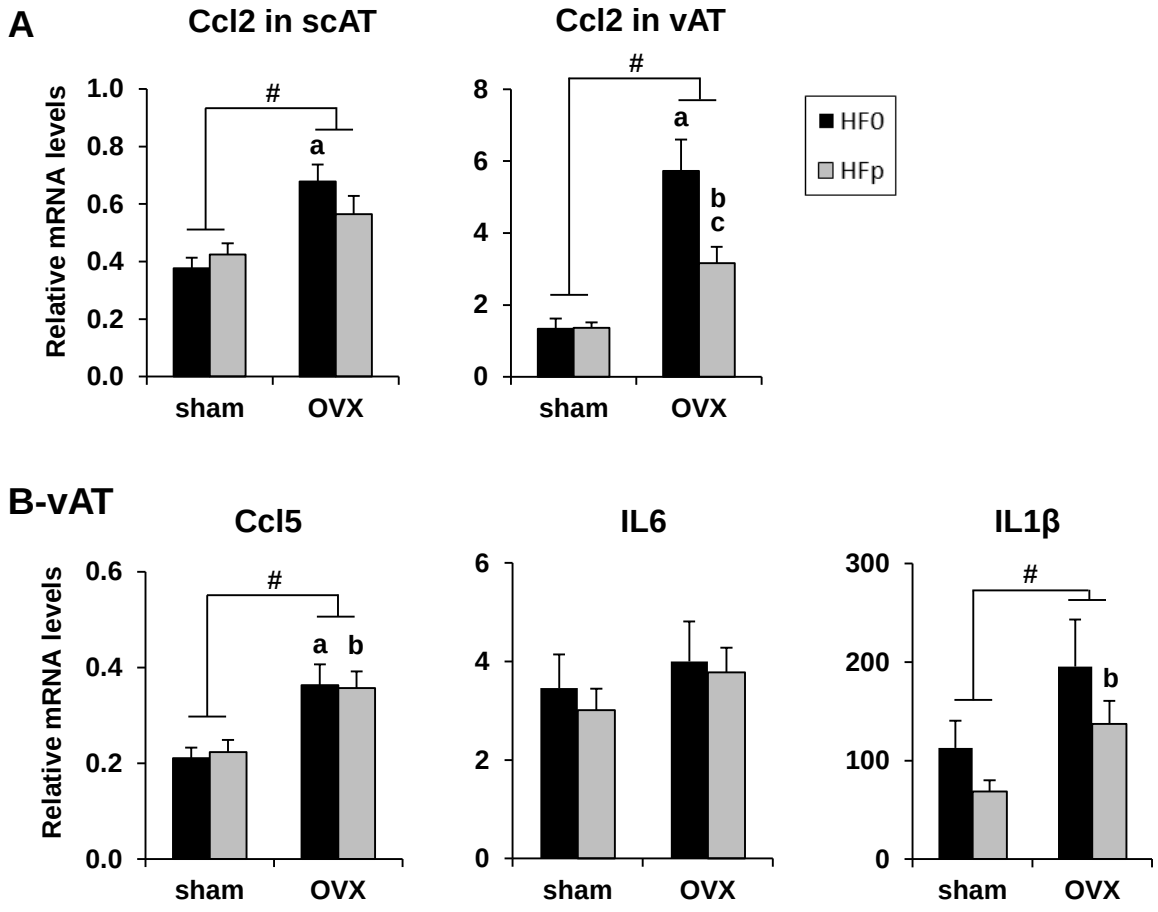

A
Body weight (g)
HF0-sham
HF0-OVX
HFp-sham
HFp-OVX
ovariectomy
weeks after birth
B
Gain of weight (g)
weeks after birth
C
Food intake (g/day/mouse)
sham
OVX
D
HF0
HFp
Weight of vAT (mg)
p=0.01
#
E
Weight of scAT (mg)
p=0.001
p=0.001

Figure 1


A
Blood glucose (% of time 0)
500
400
300
200
100
0
0 15 30 45 60 75 90
Time (min)
HF0-sham
HFp-sham
B
Blood glucose (% of time 0)
500
400
300
200
100
0
0 15 30 45 60 75 90
HF0-OVX
HFp-OVX
C
Δ AUC
8000
5000
2000
-1000
-4000
-7000
sham
OVX
*
D
Insulin (ng/mL)
2.5
2.0
1.5
1.0
0.5
0.0
0 15
Time (min)
HF0-sham
HFp-sham
*
*
E
Insulin (ng/mL)
2.5
2.0
1.5
1.0
0.5
0.0
0 15
Time (min)
HF0-OVX
HFp-OVX
*
*
C


A
Ccl2 in scAT
Ccl2 in vAT
Relative mRNA levels
1.0
0.8
0.6
0.4
0.2
0.0
8
6
4
2
0
#
a
b
c
sham
OVX
HF0
HFp
B-vAT
Ccl5
IL6
IL1β
0.6
0.4
0.2
0.0
6
4
2
0
300
200
100
0

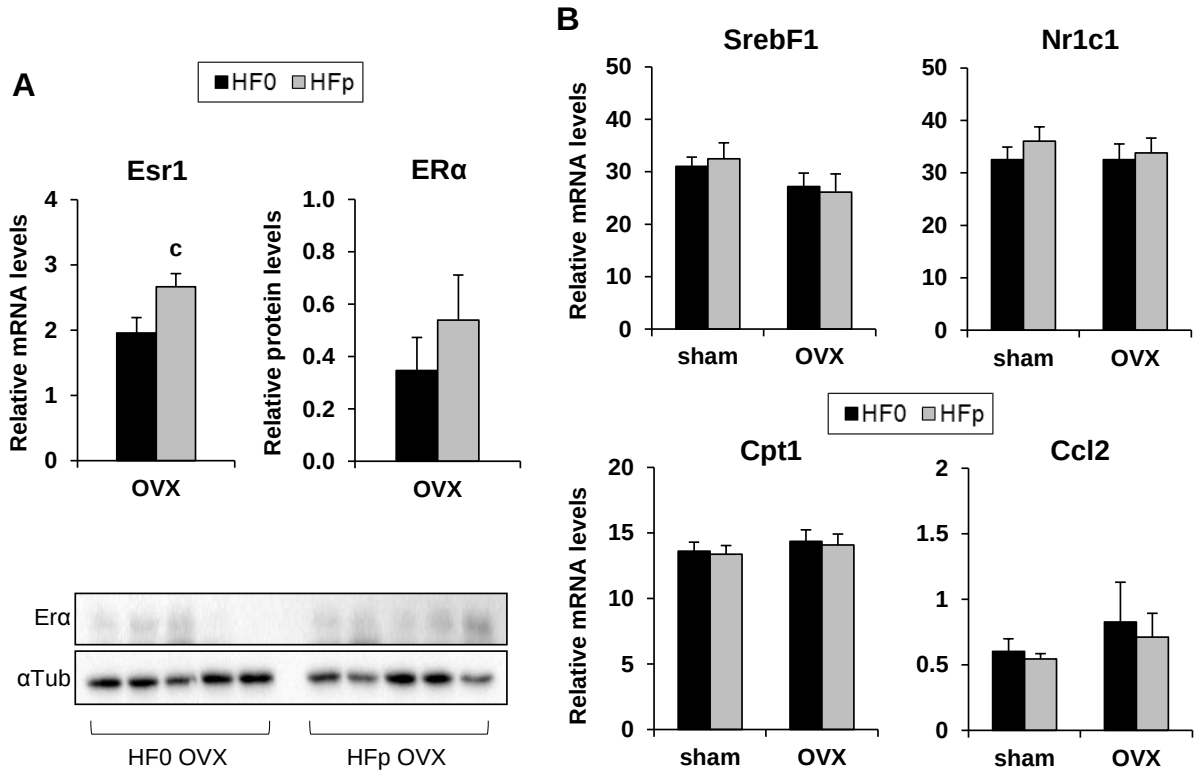

A
HF0 HFp
Esr1
ERα
Relative mRNA levels
Relative protein levels
c
OVX
OVX
Erα
αTub
HF0 OVX
HFp OVX
B
SrebF1
Nr1c1
Relative mRNA levels
sham
OVX
sham
OVX
HF0 HFp
Cpt1
Ccl2
Relative mRNA levels
sham
OVX
sham
OVX

Table 1: Body weight and biochemical characterization of mice. Results are means ± SE (n=7 to 9). **a**: p<0.05 HF0-OVX vs HF0-sham; **b**: p<0.05 HFp-OVX vs HFp-sham; **c**: p<0.05 HFp-OVX vs HF0-OVX.

	sham		**OVX**	
	HF0	**HFp**	**HF0**	**HFp**
Body weight (g)	23.0 ± 0.5	23.3 ± 0.7	28.0 ± 1.4[a]	27.1 ± 0.7[b]
blood glucose (mM)	10.4 ± 0.7	9.7 ± 0.9	9.6 ± 0.6	9.5 ± 0.6
insulin (ng/ml)	0.26 ± 0.01	0.28 ± 0.03	0.58 ± 0.1[a]	0.50 ± 0.05[b]
HOMA-IR	2.97 ± 0.23	3.01 ± 0.38	7.56 ± 1.17[a]	5.22 ± 0.47[b]
Free Fatty Acids, FFA (µmol/L)	369.1 ± 37.7	341.4 ± 30.7	602.7 ± 81.6[a]	563.0 ± 33.4[b]
plasma triglycerides, TG (mM)	0.78 ± 0.05	0.72 ± 0.05	0.83 ± 0.03	0.71 ± 0.02[c]
leptin (ng/ml)	19.9 ± 4.2	17.2 ± 1.1	34.7 ± 4.2[a]	34.2 ± 2.8[b]
liver triglycerides (nmol/mg liver)	23.2 ± 2.9	22.5 ± 1.6	26.5 ± 2.1	28.9 ± 3.6