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PII: S0300-483X(17)30223-8
DOI: http://dx.doi.org/doi:10.1016/j.tox.2017.07.017
Reference: TOX 51923

To appear in: *Toxicology*

Received date: 12-5-2017
Revised date: 24-7-2017
Accepted date: 31-7-2017

Please cite this article as: Brulport, Axelle, Le Corre, Ludovic, Chagnon, Marie-Christine, Chronic exposure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces an obesogenic effect in C57BL/6J mice fed a high fat diet.Toxicology http://dx.doi.org/10.1016/j.tox.2017.07.017

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Chronic exposure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces an obesogenic effect in C57BL/6J mice fed a high fat diet

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Abstract

Contaminant involvement in the pathophysiology of obesity is widely recognized. It has been shown that low dose and chronic exposure to endocrine disruptor compounds (EDCs) potentiated diet- induced obesity. High and acute exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a persistent organic pollutant (POP) and an EDC with anti-estrogenic property, causes wasting syndrome . However at lower doses, the TCDD metabolic effects remain poorly understood. We investigated the obesogenic effect during chronic exposure of TCDD at 1 µg/kg body weight (bw)/week in adult C57BL/6J mice fed with a high fat diet (HFD) and exposed from 10 to 42 weeks old to TCDD or equal volume of vehicle by intragastric gavage. Under these conditions, TCDD was obesogenic in adult mice (7 % in males and 8 % in females), which was linked to fat mass. A sex effect was observed in the fat mass distribution in adipose tissue and in the hepatic triglyceride content evolution. In visceral fat pad weight, we observed a decrease (11 %) in males and an increase (14 %) in females. The hepatic triglyceride content increase (41 %) in females only. TCDD failed to induce any change in plasma parameters regarding glucose and lipid homeostasis. Messenger

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ribonucleic acid (mRNA) levels involved in adipose tissue and hepatic metabolism, inflammation, xenobiotic metabolism and endocrine disruption were differently regulated between males and females. In conclusion, these results provide new evidence that dioxin, a POP and EDC can be obesogenic for adult mice with multi-organ effects.

Keywords: TCDD; chronic exposure; obesogen

1. Introduction

Dioxins are a family of compounds including 210 congeners. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exhibits the greatest toxicity of the class. The toxicity of TCDD relates to high concentration acute exposure which was notably demonstrated after the Seveso industrial accident which occurred in Italy (1976). However, TCDD is currently ubiquitous at low concentrations in industrialized countries, mainly due to anthropogenic activities (Conesa *et al.*, 2008; Roeder *et al.*, 1998; Umbreit *et al.*, 1986; Wilson *et al.*, 2008). Since the reduction of industrial processes that use chlorination, open combustion (waste, fires, and volcanic eruptions) has been the major dioxin emitter. Since the signing of the Stockholm convention in 2001 and the introduction of dioxin emission reduction policy in Europe, sediment analysis shown the efficiency of these measures on dioxin level, but local disparities remain present (Van Metre *et al.*, 2015).

The half-life of TCDD is about 8.3 days in air, 0.5 years in water, and 100 years in soil and sediments (Sinkkonen and Paasivirta, 2000). This leads to food-chain bioaccumulation; wildlife constituting a TCDD reservoir. Currently, over 90% of human dioxin exposure comes from diet via contaminated fat sources (dairy products, meat and fish notably) due to the lipophilic nature of dioxins (Roeder *et al.*, 1998). In a recent study, TCDD levels in food ranged from 1 pg/kg for vegetables/fruits/pulses and cereals to 21 pg/kg for fish and seafood with intermediate values for meat (4 pg/kg) and dairy products (6 pg/kg) (Perello *et al.*, 2012). Population are now faced with low dose and chronic exposure to TCDD.

Bioavailability of TCDD may depend on the macronutrient composition as well as methods used to prepare food (Shen *et al.*, 2016; Zhang *et al.*, 2013). In 1998, the World Health Organization (WHO) established a tolerable daily intake (TDI) of 1-4 pg/kg body weight (bw)/day. In 2001, the Joint Food and Agriculture Organization (FAO)/WHO Expert Committee on Food Additives (JECFA) defined the Provisional Tolerable Monthly Intake

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(PTMI) at 70 pg/kg bw/month. Recently, in 2012, the U.S Environmental Protection Agency (EPA) proposes a reference dose for chronic oral exposure of 0.7 pg/kg bw/day. Recent studies suggest that dietary exposure largely exceeds the current recommendations for dioxin exposure with an estimation of 1.3 ± 0.4 pg/kg bw/day in French women with similar levels in U.S children and adolescents (Charnley and Doull, 2005; Danjou *et al.*, 2015).

TCDD exhibits biological adverse effects as it disrupts homeostasis via its actions on the immune and nervous system, reproductive function, as well as altering functions of many organs including skin, liver, pancreas and adipose tissue. Furthermore, TCDD has been classified as carcinogenic for humans by the International Agency for Research on Cancer (IARC). Concerning energy metabolism, environmental substances such as endocrine disruptors (EDCs) have been implicated in obesity development. Indeed, they are obesogenic substances that inappropriately regulate lipid metabolism and adipogenesis to potentiate obesity (Baillie-Hamilton, 2002; Grun and Blumberg, 2006). TCDD has been shown to be an EDC with anti-estrogenic effect *in vivo* (Franczak *et al.*, 2006; Mocarelli *et al.*, 2008; Safe and Wormke, 2003; Shi *et al.*, 2007) and *in vitro* (Göttel et al., 2014; Matthews and Gustafsson, 2006; Swedenborg and Pongratz, 2010).

TCDD is also an energy metabolism disruptor. High and acute TCDD exposure causes a « wasting syndrome » characterized by a loss of body weight accompanied by a decrease in adipose tissue mass in rodents (Brewster and Matsumura, 1988; Chapman and Schiller, 1985; Gasiewicz and Neal, 1979). However little data are available regarding the effects of low dose chronic exposure on metabolism. Only in the French E3N cohort, it has been shown that the body mass index (BMI) is associated with dioxin exposure (Danjou *et al.*, 2015). Notably the proportion of women with a BMI<25 kg/m^2 was greater in the upper quartile of dietary dioxin exposure (> 1.52 pg/kg body weight (bw)/day) than those in the lower quartile (<0.98 pg/kg bw/day) wherein there were more women with a BMI≥25 kg/m^2 (Danjou *et al.*, 2015). At low doses, most of the already known obesogenic EDCs do not directly induce obesity but rather act to potentiate obesity, as observed in rodents when they are fed with an high fat diet (HFD) (Ivry Del Moral *et al.*, 2016; Mackay *et al.*, 2013).

So, the aim of this present study was to check if TCDD could induce an obesogenic effect after chronic exposure at 1 µg/kg bw/d in adult mice fed with a HFD. C57BL/6 strain is an established model for diet-induced obesity. C57BL/6 mice will develop severe obesity, hyperglycemia, and insulin resistance if weaned onto a high-fat diet (Surwit *et al.*, 1995; Rossmeisl *et al.*, 2003).

2. Materials and Methods

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2.1 Animals and materials

C57Bl/6J mice were purchased from Charles Rivers (L'Arbresle, France). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was provided by Sigma-Aldrich (Saint Quentin Fallavier, France). The high fat diet (HFD) was based on the 4RF25 reproduction diet (Mucedola, Milano, Italia) with the addition of 30 % palm oil (La Vie Saine, Dijon, France) which is rich in saturated fatty acid. Mucedola 4RF25 diet is certified as estrogen free and accurately tested for the detection of estrogenic activities. In this diet, the percentage of phytoestrogens is certified to be less than 4 ppb (parts per billion) according to international standards (U.S. Food and Drug Administration National Center for Toxicological Research Standard No. 2, September 5, 1973). Triglycerides PAP 150™ and Glucose RTU™ kits were obtained from Biomérieux (Marcy l'Etoile, France). Cholesterol FS™ and no esterified fatty acids (NEFA) FS™ kits were purchased from DiaSys (Condom, France). Ultrasensitive™ Mouse Insulin and plasma leptin ELISA kits were provided from Mercodia France SAS (Paris, France) and Phoenix France SAS (Strasbourg, France), respectively.

2.2 Experimental Design

C57Bl/6J mice were housed in a 12 h light-dark cycle at a temperature of 22 °C in one conventional animal house and allowed free access to food and water. The high fat diet was composed on the basis of a 4RF25 reproduction diet added with 30 % palm oil (La Vie Saine, Dijon, France) and with 0.42 g/kg of cholesterol from Sigma Aldrich (Saint-Quentin Fallavier, France) in order that 60 % of energy uptake come from saturated fatty acid. Cages and bottles were made of polypropylene (bisphenol-free). A maximum of five mice were housed in each cage. At 10 weeks old, mice were fed with HFD and divided according to their body weight into two homogeneous groups for each gender (10 mice/group) and exposed from 10 to 42 weeks old to TCDD solubilized in corn oil at 1 µg/kg bw/week by intragastric gavage. For male and female control groups, intragastric gavages were performed with corn oil only. Intragastric gavage allows to control the dose administered to each individual and to be reproducible over time. All mice (fasted 4 h before) were sacrificed at 42-weeks old. Mice were anesthetized with isoflurane, blood was sampled by cardiac puncture and then the mice were euthanized by cervical dislocation. At the sacrifice, liver and visceral adipose tissue were collected and weighed. Animals were treated in the respect of ethic and deontology and the experimental protocol was approved by the ministry and the University of Burgundy's

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ethic committee. Animal experiments have been carried out in accordance with EU Directive 2010/63/EU for animal experiments.

2.3 Body weight and corporal composition

Body weight was monitored weekly. Just before (4 h fasted) sacrifice, body weight, fat and lean masses of 42-week old mice were measured. The fat and lean masses of each mice were determined individually using a quantitative EchoMRI 500T™ (EchoMRI, Houston, USA). Before each measurement, calibration was performed in compliance with the manufacturer's guidelines.

2.4 Food intake measurement

The experiment was performed with 40-week old mice. They were acclimated to individual cages for 48 h. After acclimation, daily food intake was determined by weighing out the difference in the amount of food placed and the amount remaining the following 24 h. To avoid issues of spillage and/or hoarding, mice may be housed in wire-bottomed cages where
spilled food was collected and corrected for.

2.5 Liver triacylglycerol assay

Triacylglycerols were extracted from mouse liver according to Schwartz D.M and Wolins N.E (Danno *et al.*, 1992; Schwartz and Wolins, 2007). In brief, liver (75 up to 190 mg) were crushed in an Omni Bead Ruptor 24 apparatus (Omni International, Kennesaw, USA) with 900 µL of saline and circa twenty 1,4 mm OD zirconium oxide beads (S= 6.95 m/s, T= 30 s, C = 3; D = 10 s). An aliquot equivalent to 10 mg of liver in 200 µl of saline was transferred in glass tubes (13 mm x 100 mm) and further extracted with 2 ml of isopropanol-hexane-water (IHW) (80:20:2, v/v/v) for 30 min, 0.5 ml of hexane-diethyl ether (1:1) for 10 min and finally 1 ml of water for 20 min. An aliquot of 185 mg of organic phase, corresponding to 2.5 mg of liver extract was weighed, evaporated to dryness under vacuum and solubilized with 200 µl of tert-ButOH/Triton-X 100/MeOH (30:10:10). After sonication (5 min), solubilized lipids (10 µL) were twice dispensed in a 96 wells plate. Infinity TG reagent (200 µl, Thermo Fischer Scientific, Asnière sur Seine, France) was then dispensed to each well followed by a 6 min at 37 °C incubation step. Absorbance at 500 nm was read using a microplate reader (MultiskanGo, Thermo-Scientific, France). A calibration curve was

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generated using a triglyceride standard (FS 200 mg/dl, DiaSys-France) to interpolate the mass of TG in liver samples. All reagents of the best analytical grade were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Analysis was carried out in collaboration with lipidomics platform of Université de Bourgogne Franche-Comté (Dr Jean-Paul Pais de Barros, Dijon, France).

2.6 Plasma analysis

Just before sacrifice (i.e. fasted 4 h before), mouse blood samples were collected by an intracardiac puncture using heparinized syringe. After a centrifugation of 10 min at 2000g and 4 °C, the level of total plasma cholesterol, triacylglycerol, NEFA and glucose were measured using respective kits mentioned above. The plasma insulin and leptin levels were determined using separate ELISA kits as mentioned previously. Assays were performed according to the manufacturer's instructions.

2.7 RT-qPCR analysis

Lysis of liver or visceral adipose tissue samples of each mice were performed using Lysing Matrix D™ tubes (MP Biomedical) and TissueLyser LT™ (Qiagen). Total RNA was extracted using Tri-reagent™ (Sigma-Aldrich). cDNA was reverse-transcripted on 3 µg of total RNA samples using the High Capacity cDNA reverse transcription kit™ (Life Technologies). Real time PCR was done with 30 ng of cDNA, 5 µl of Taqman™ Universal Master Mix (Life Technologies), 0.5 µl of Taqman™ gene expression assay (20X ; Life Technologies) for a final volume of 10 µl (qsp H2O). Taqman™ gene expression assay references were Mm01184322_m1 (*pparϒ*), Mm00440939_m1 (*pparα*), Mm00545913_s1 (*socs3*), Mm01211875_m1 (*insr*), Mm00456425_m1 (*adiponectin*), Mm00503040_m1 (*atgl*), Mm00434764_m1 (*lpl*), Mm00495359_m1 (*hsl*), Mm00443258_m1 (*tnfα*), Mm00464228_m1 (*i11β*), Mm01135198_m1 (*cd36*), Mm00662319_m1 (*fasn*), Mm00772290_m1 (*scd1*), Mm00478932_m1 (*ahr*), Mm00442688_m1 (*ar*), Mm00433149_m1 (*esr1*) and Mm99999915-g1 (*gapdh*, reference gene). PCR was run on the Step-One plus™ system (Applied Biosystems) using following conditions: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Gene expression was determined relative to the control gene glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

2.8 Statistical analysis

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All data were expressed as mean ± standard error of mean (SEM). To determine the statistically significant difference between two groups, a Student's t test was used (two-tailed, paired samples for means, and hypothesized difference of 0). Pearson r was used to calculate correlations between fat mass versus body weight. Statistical tests were performed with Graph Pad Prism® software and RStudio® software for Principal Component Analysis (PCA). Results were considered statistically significant at $p<0.05$.

3. Results

3.1 TCDD-induced overweight is sex-dependent and related to visceral adipose tissue and plasma leptin level

In mice fed with a high fat diet during 32 weeks, TCDD induced a significant body weight gain in males and in females in the same range (7 % ± 1 and 8 % ± 1, respectively) (Fig. 1A). Interestingly, we showed an offset in time between males and females on the body weight gain kinetic. The overweight induced by TCDD occurred in 23-week old males and 35- week old females (Fig. 1C). In order to investigate whether this overweight is related to an obesogenic effect of TCDD, we evaluated the mouse fat mass. To quantify the whole body fat (adipose tissue and ectopic fat depots), we used a quantitative ECHO-MRI-based technology analyzer. Mouse fat mass significantly increased regardless of sex after TCDD exposure (13 % ± 1 in male and 11 % ± 1 in female) (Fig. 1B). Concerning correlation between fat mass and body weight in control and TCDD male and female mice, total body fat mass showed a strong positive correlation ($r^2 = 0.89$ in control males ; $r^2 = 0.84$ in TCDD males; $r^2 = 0.69$ in control females and $r^2 = 0.75$ in TCDD females) with body weight (Fig. 1D). Then, we weighed visceral adipose tissue (VAT) fat pad and measured blood parameters related to energy metabolism at sacrifice. In males, TCDD induced a significant decrease of visceral fat pad weight (11 % ± 1) (Fig. 1E). In contrast, TCDD significantly increased the VAT mass in females (14 % ± 1) (Fig. 1E). However, TCDD failed to induce any change in most of the plasma parameters (glucose, insulin, no esterified fatty acids (NEFA), cholesterol, triglycerides) whatever the sex (Table 1). In contrast, leptin levels, a key hormone in adiposity regulation, was significantly decreased in females exposed to TCDD (Table 1).

3.2 TCDD induced a sex-dependent regulation of genes involved in visceral adipose tissue lipolysis, insulin resistance and inflammation

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Regarding the VAT mass changes, we investigated the mRNA expression of enzymes involved in lipolysis such as adipose triglyceride lipase (*atgl*) which is the rate-limiting enzyme (Zimmermann *et al.*, 2004). Males exhibited a significant up-regulation of *atgl* mRNA expression (126 % ± 2) (Fig. 2A), whereas a significant down-regulation in females was observed (47 % ± 1) (Fig. 2A). No change in the hormone sensitive lipase (*hsl*) mRNA expression was observed whatever the sex (Supplementary Fig. S1). Concerning mRNA levels of genes involved in lipid capturing and storage in adipocytes, we didn't observe any significant changes for lipoprotein lipase (*lpl*), cd36 or pparϒ (Supplementary Fig. S1). Regards to insulin-sensitivity marker genes, we assessed the mRNA expression of suppressor of cytokine signaling 3 (*socs3*), insulin receptor (*insr*) and *adiponectin*. TCDD induced a significant decrease of the *socs3* mRNA expression in males (41 % ± 1) (Fig. 2B). In contrast, *insr* and *adiponectin* mRNA levels were not modified whatever the sex (Supplementary Fig. S1). For the inflammatory state assessment, an endocrine feature of adipose tissue in obesity, we analyzed tumor necrosis factor α (*tnfα*) and interleukin 1β (*il1β*) mRNA expression. In contrast to females, TCDD induced a significant increase of the *tnfα* mRNA expression (104 % ± 2) (Fig. 2C) and *il1β* mRNA level (275 % ± 4) in male mice (Fig. 2D).

3.3 TCDD exposure induced increases of normalized liver weight in males and hepatic triglycerides content in females

Obesity is associated with an increased risk of developing liver disease such as steatosis lesions. Furthermore, it's already known that visceral adipose tissue expansion is involved in non-alcoholic fatty liver disease (NAFLD) development (Cohen *et al.*, 2011). At sacrifice, we weighed mouse livers and evaluated the hepatic triglyceride content. In TCDD exposed males, liver weights normalized to body weights were significantly increased (23 % ± 1) (Fig. 3A) without any difference in hepatic triglycerides content (Fig. 3B). In TCDD exposed females, liver weight to body weight ratios tended to increase without statistical significance (22 % ± 1) (Fig. 3A). In contrast, hepatic triglyceride content increased significantly in females (41 % ± 1) (Fig. 3B).

3.4 TCDD effect on hepatic triglyceride content was related to scd1 overexpression and with a nuclear receptor expression pattern sex-dependent

Due to the hepatic triglyceride content in TCDD exposed females, we investigated the mRNA level of stearoyl-coA desaturase-1 (*scd1*) and fatty acid synthase (*fasn*), two enzymes involved in "*de novo*" fatty acid synthesis in liver. TCDD induced a significant increase of

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scd1 mRNA level (56 % ± 2) in male (Fig. 4A). This increase was more pronounced in females (92 % ± 2) (Fig. 4A). The *fasn* mRNA level was not modified in either sex (Supplementary Fig. S2). Furthermore, concerning metabolism, there was no statistical difference in *cd36*, *pparϒ* and *pparα* mRNA levels, involved in lipid capturing, storage and oxidation, respectively (Supplementary Fig. S2). Interestingly, in contrast to what we observed in adipose tissue, we did not show any change concerning insulin sensitivity and inflammation in male liver. Regards to *socs3* and *tnfα* mRNA expression, TCDD induced a decrease in males (45 % ± 1 and 18 % ± 1, respectively) and an increase in females (70 % ± 1.7 and 49 % ± 1, respectively) without reaching a statistical significance (Figs. 4B and 4C). TCDD is the strongest agonist of the aryl hydrocarbon receptor (*ahr*), a xenobiotic receptor. Xenobiotics are also able to activate hormone receptors (estrogen receptor 1 (*esr1*) and androgen receptor (*ar*)) and can lead to endocrine and metabolic disruption. In females, TCDD significantly up-regulated both *ahr* and *ar* mRNA expression in a similar range (36 % ± 1 and 32 % ± 1, respectively) (Figs. 4D and 4E). No changes were observed with males (Figs. 4D and 4E). There was no difference in mRNA expression, whatever the sex for *esr1* (Fig. 4F).

3.5 Individual distribution and correlation with physiological parameters and mRNA expression of target genes

As visualized with the principal component analysis (PCA) loading plot, there is a good discrimination of all groups according to the dimension 1 (Fig. 5A and Table 2A). Interestingly, only the treated groups are separated according to the dimension 2 (Table 2A). The PCA inertia was 51 % which helps to explain about 50 % of individual variability (Figs. 5A and 5B).The combined analyses of the variable correlations according to the 1 and 2 dimensions show several trends i) *ar*, *ahr* and *socs3* mRNA expression in liver appeared to play a role whatever the sex ii) *esr1* mRNA level in liver and the VAT mass appeared to play a pivotal role in females iii) and liver weight normalized to body weight appeared to play a central role in males (Fig. 5B and Table 2B).

4. Discussion

In this study and for the first time, TCDD was demonstrated obesogenic after chronic exposure at 1 µg/kg bw/d in adult mice fed with a HFD. Concerning the HFD, we previously described that it was very efficient to induce the obesity development in the same mice strain

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(Ivry Del Moral *et al.*, 2016) and it also was in accordance with study of de Wit *et al* (de Wit *et al.*, 2008).

Firstly, the period of exposure is a critical point to define in order to investigate the effect of endocrine disruptor like TCDD. Indeed, endocrine disruptor exhibit different pattern of effects following exposure period so-called "critical windows" like fetal development or puberty. It is now well established that fetal and newborn developments are particularly sensitive to EDC exposure which can induce adulthood harmful consequences (Gluckman and Hanson, 2004). Exposure in adult mice allowed to discriminate the direct effect on energy metabolism independently of adverse effects occurring during development (hormone regulation of cell proliferation, migration and differentiation in developing organs) (Le Corre et al., 2015; Anderson et al., 2000). Then, we choose to expose mice are 10 week old and until potential obesogen effect of TCDD occurred.

Populations are currently faced with low dose and chronic exposure to TCDD. Human exposure being in the order of pg/kg bw/day (Danjou *et al.*, 2015). TCDD half-life is about 11 days in C57BL/6 mice and about 7-8 years in human adipose tissue (Birnbaum, 1986; Gasiewicz *et al.*, 1983; Kopec *et al.*, 2013; Roeder *et al.*, 1998). TCDD half-life depends on several factors such as percent body fat, species, age and exposure levels with a dose-dependent elimination (Emond *et al.*, 2006; Reitz *et al.*, 1996). In this study, the dose of 1µg/kg bw/week was chosen regards to a physiologically based pharmacokinetic model. Emond *et al*, reported that a single oral dose of 1µg/kg bw in rodents was a dose which had the less effect on the TCDD half-life in rodents, taking into account adiposity and TCDD hepatic metabolism mediated by CYP1A2 (Emond *et al.*, 2006). To extrapolate human exposure to mice, a mathematical model based on physiological data shows that the dose of 1 µg/kg bw appears to be appropriate for a chronic exposure study in mice (Emond *et al.*, 2006). Moreover, the use of this physiologically-based pharmacokinetic model by Duval *et al*, has shown that TCDD concentration is constant in adipose tissue and was 2-fold decreased in liver after 8 days post-injection of 5 µg/kg bw TCDD in C57BL/6 mice (Duval *et al.*, 2017). Taking into account that TCDD elimination is very slow at low exposure, as it has been shown in Human adult at Seveso, weekly injection of 1 µg/kg bw appears to be relevant to study on obesogenic effects (Michalek *et al.*, 2002).

In our study, we showed that TCDD exposure induced a significant excess weight gain which was correlated with the fat mass increase in both male and female mice without any change in food intake (Supplementary Fig. S3). However, differences observed between male

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and female mice in the weight gain kinetics, the VAT mass and normalized liver weights suggest that the underlying mechanisms involved in the weight gain could be sex-dependent.

In males, a reduction in VAT fat pad was observed while the overall fat mass increased significantly. At molecular level, it was associated with *atgl* mRNA overexpression and a significant decrease in *socs3* mRNA level. It's reported that *atgl* is the rate-limiting enzyme in lipolysis (Zimmermann *et al.*, 2004) and *socs3* is a marker gene of local insulin resistance in obesity (Shi *et al.*, 2006). Insulin constitutes the major antilipolytic pathway (Nielsen *et al.*, 2014). This improvement of the local insulin sensitivity may be a physiological adaptation which tends to limit lipid release from VAT in TCDD exposed males.

In contrast, in females, unchanged *socs3* mRNA level coupled with the *atgl* mRNA down-regulation could allow VAT to maintain its triglyceride storage capacity. Then, this is a potential local modulation of insulin sensitivity as the indirect assessment of insulin resistance *via* the HOMA index did not show any systemic variation whatever the sex. These data are consistent with the work of Shi *et al*, who showed that *socs3* expression variation in a tissue had no effect on insulin sensitivity at the systemic level (Shi *et al.*, 2006). These observations support the idea of a local metabolic disturbance of TAV caused by TCDD exposure in male mice.

The obesity is associated with chronic low-grade systemic inflammation which is caused, at least in part, by adipose tissue inflammation (Greenberg *et al.*, 2006). In our study, we investigated the mRNA expression of classical inflammation marker gene *tnfα* and *il1β.* As a result, only an overexpression of *tnfα* and *il1β* were observed in the VAT of TCDD-exposed males. Based on the localization of VAT, we hypothesize that this tissue suffered from a direct toxicity of TCDD which provokes a mass decrease and a local inflammation. This could be mediated by an intestinal hyperpermeability phenomenon, through further investigations are necessary to examine this issue. Indeed, the gut plays a key role in energy metabolism and is on the front line in the case of food contaminants exposure. TCDD has been shown to inhibit the development of mouse intestinal epithelial cells *via* the *ahr* pathway (Park *et al.*, 2016). Moreover, jejunal epithelium whole-genome microarray analysis of TCDD exposed mice showed at low and high doses a common activation of gene clusters that was consistent with the increase of hepatic fat accumulation (Fader *et al.*, 2015).

Concerning the liver, treatment with TCDD had a higher effect on liver weight than body weight gain, independently of sex but this effect was significant only in males. Interestingly, this increase was higher in males but did not appear to be related to hepatic

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triglyceride content, in contrast to females. The causes which contribute to the increased liver weight remain unclear. Several hypothesis are possible such as induction of xenobiotic metabolism enzymes, TCDD being a strong agonist of AhR which regulates the expression of several of them (Mandal *et al.*, 2005). Another hypothesis could be related to the VAT expansion, which is well known to have an important lipolytic activity, may release triglycerides in the hepatic portal vein resulting in an increase of hepatic triglyceride content (Gentile *et al.*, 2015). Then, in males, the decreased VAT mass could contribute to the stabilization of hepatic triglyceride content, in contrast to the female which exhibit a better VAT storage capacity. Indeed, in female *vs* male mice, VAT represents respectively 20 % *vs* 9 % of the total fat mass in control groups (21 % *vs* 7 % in treated groups). Obviously, further studies are necessary to conclude on this issue.

In order to precise this issue about lipid distribution, we investigated lipid plasma parameters. Regardless of the sex, the fat mass increase did not result in hypertriglyceridemia, hypercholesterolemia or elevated plasma NEFA level. Then, it supports the hypothesis that fat mass excess associated to TCDD exposure was distributed between adipose tissue and ectopic deposits in a sex-dependent manner. Bastos-sales *et al*, highlighted *in vitro*, on murine preadipocyte cell line, that TCDD inhibited adipocyte differentiation at nanomolar range (Bastos Sales *et al.*, 2013). It could limit adipose tissue storage as an adipocyte hypertrophy without hyperplasia and promote the development of ectopic deposits.

Adipocyte hypertrophy may also explain the absence of correlation between fat mass and leptin levels in all treated groups (data not shown). Likewise, regards to the plasma leptin decrease only in treated females, it could be due to the fact that sex hormones play a role in the regulation of leptin secretion by adipose cells as described during the menstrual cycle in women (Thomas *et al.*, 2000).

Estrogens play an important role in the maintenance of lipid homeostasis (Jones *et al.*, 2000). As shown in liver-specific *esr1* deficient mice, hepatic *esr1* signaling was required to prevent liver fat accumulation (Zhu *et al.*, 2013). Interestingly, it has also been demonstrated recently that the activation of a non-nuclear estrogen receptor improves hepatic steatosis in female mice (Chambliss *et al.*, 2016). In this study, we did not observe a treatment effect on liver *esr1* mRNA expression, but we did observe a difference between sexes with a significant increase in females compared to males in all groups (Supplementary Fig. S4). As suggested by principal component analysis, liver *esr1* could play a critical role in differences observed between males and females for hepatic parameters.

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We also investigates the mRNA expression of *ahr* which the major target of TCDD effect and also an important nuclear receptor involved in liver homeostasis (Barouki *et al.*, 2012). In our study, hepatic *ahr* mRNA were significantly overexpressed in treated female mice and decrease in treated male. Recently, it has been shown that *ahr* plays a protective role against HFD-induced hepatic steatosis (Wada *et al.*, 2016). Interestingly, in *ahr*-deficient mice, the induction of *socs3* expression (an *ahr* responsive gene) in the liver reversed the HFD-induced hepatic steatosis (Wada *et al.*, 2016). However, in our study, there was a difference between sexes regards to *socs3* mRNA levels which showed a tendency to increase in females and decrease in males in treated groups. So, we can hypothesize that females, in contrast to males, are partially protected against liver fat accumulation by both estrogen and *ahr/socs3* dependent pathways. In the same way, *ar* mRNA overexpression in female could also confer protection against dyslipidemia, insulin sensitivity impairment and limit the liver fat accumulation in treated females. Indeed, it reported that androgen receptor-deficient female mice (and fed with HFD) exhibited obesity, dyslipidemia, insulin resistance phenotype and hepatic triglyceride accumulation (Fagman *et al.*, 2015).

In both sex and TCDD groups, the triglyceride accumulation in the liver appears to be mediated by *scd1* which is reported as an enzyme involved in fatty acid synthesis (Angrish *et al.*, 2011). Furthermore, sixteen putative functional dioxin response elements (DREs) that bind *ahr* were identified within *scd1* genomic loci (Angrish *et al.*, 2011). Consequently, a treatment effect on liver *ahr* mRNA up-regulation could induce *scd1* overexpression in females.

Together, data on triglyceride storage in VAT and liver and the stability of plasma parameters showed that TCDD potentiated the diet-induced obesity without to induce related metabolic disturbances.

5. Conclusions

In this work, we demonstrated for the first time that TCDD is obesogenic at 1µg/kg bw/week after chronic exposure in adult mice fed with HFD. We observed a sex difference in the fat mass distribution in adipose tissue and liver. This could be linked to the anti-estrogenic TCDD property and may be mediated by an *ahr* pathway. By our multi-organ approach, this work contributes to develop an integrative approach in metabolic toxicology. There is a need to advance in the understanding of obesity physiopathology linked to persistent organic pollutant (POPs) such as EDCs. This is a major health and societal issue, as in European

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Union, obesity and associated comorbidity linked to EDCs exposures have a probable cost in the range of € 18-25 billion per year (Legler *et al.*, 2015). Furthermore, in USA, the global disease and dysfunction cost of EDCs is about $ 340 billion, which is 1.56 times higher than in Europe (Attina *et al.*, 2016).

Acknowledgments

This work was funded by AgroSupDijon ; Ministère de l'Enseignement Supérieur et de la Recherche (doctoral fellowship to AB) ; INSERM (Institut National de la Santé et de la Recherche Médical) ; Université de Bourgogne Franche-Comté. We are grateful to Mr. Guillaume Macquard and Ms. Aurélie Dastugue for their technical assistance.

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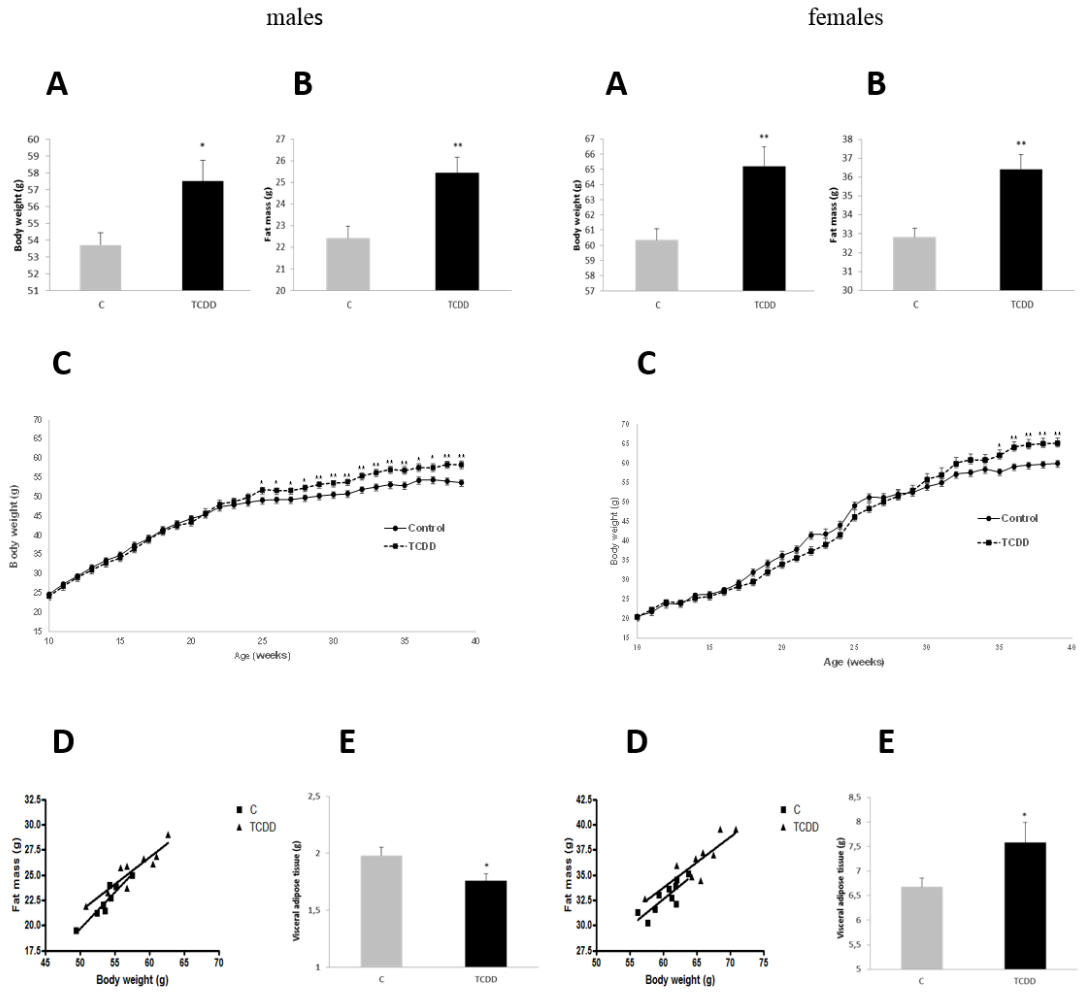

Fig. 1. TCDD effect on body weight, fat and VAT masses in males (left) and females (right). (A) Body weight, (B) fat mass, (C) body weight gain kinetic, (D) correlation between body weight and fat mass and (E) visceral adipose tissue mass in C57Bl/6J mice fed with a high fat diet and after a TCDD exposure or not from 10 to 42 weeks old at 1 µg/kg body weight/week. Except for the weight gain kinetic that was assessed weekly, all other parameters were measured at 42 week of age. Values represent means ± SEM. Paired student *t* test were performed; *p<0.05, **p<0.01 (compared to the control group (c)). Pearson correlation between body weight and fat mass in control (r^2 = 0.89; p = 0.0002) and TCDD treated (r^2 = 0.84; p = 0.0005) males, and in control (r^2 = 0.69; p = 0.003) and TCDD treated (r^2 = 0.75; p = 0.003) females. n=10 per group.

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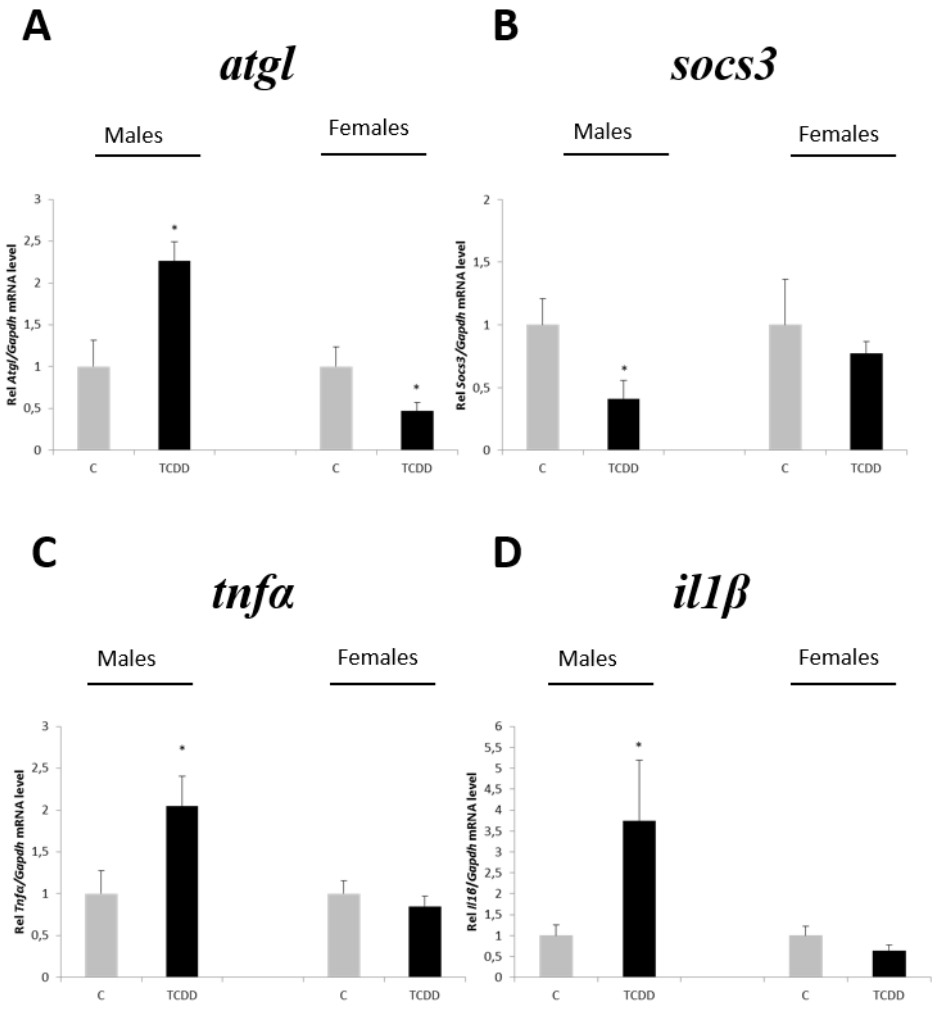

Fig. 2. TCDD effect on mRNA expression of VAT homeostasis marker genes in male and female mice. Adipose tissue mRNA expression of *atgl* (A), *socs3* (B), *tnfα* (C) and *il1β* (D) in C57Bl/6J male and female mice fed with a high fat diet and after a TCDD exposure or not from 10 to 42 weeks-old at 1 µg/kg body weight/week. Measurements are performed at 42 week-old. Values represent means ± SEM. Paired student *t* test were performed; *p<0.05 (compared to the respective control group (c)). n=10 per group.

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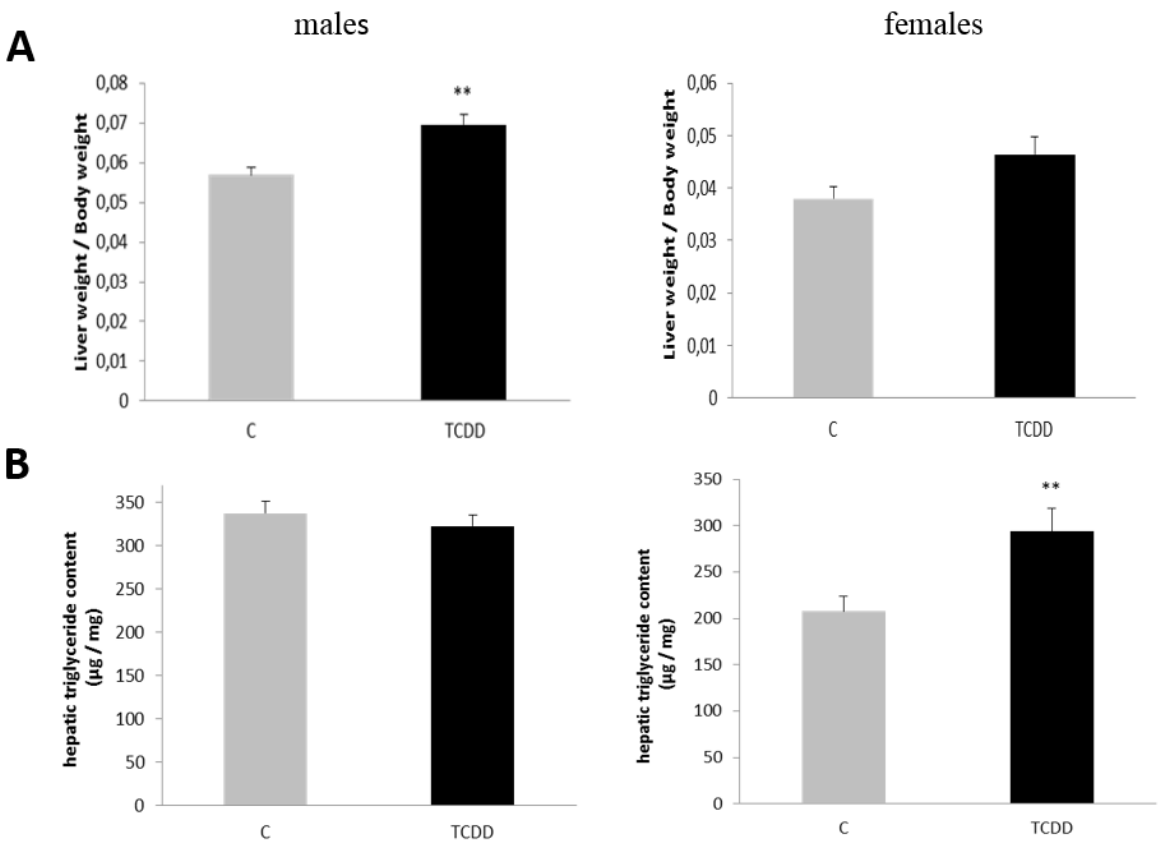

Fig. 3. TCDD effect on liver weight and hepatic triglyceride content in males (left) and females (right). Normalized liver weight (A) and hepatic triglyceride content (B) of C57Bl/6J mice fed with a high fat diet and after a TCDD exposure or not from 10 to 42 weeks-old at 1 µg/kg body weight/week. These parameters were measured at 42 week-old. Values represent means ± SEM. Paired student *t* test were performed; **p<0.01 (compared to the control group (c)). n=10 per group.

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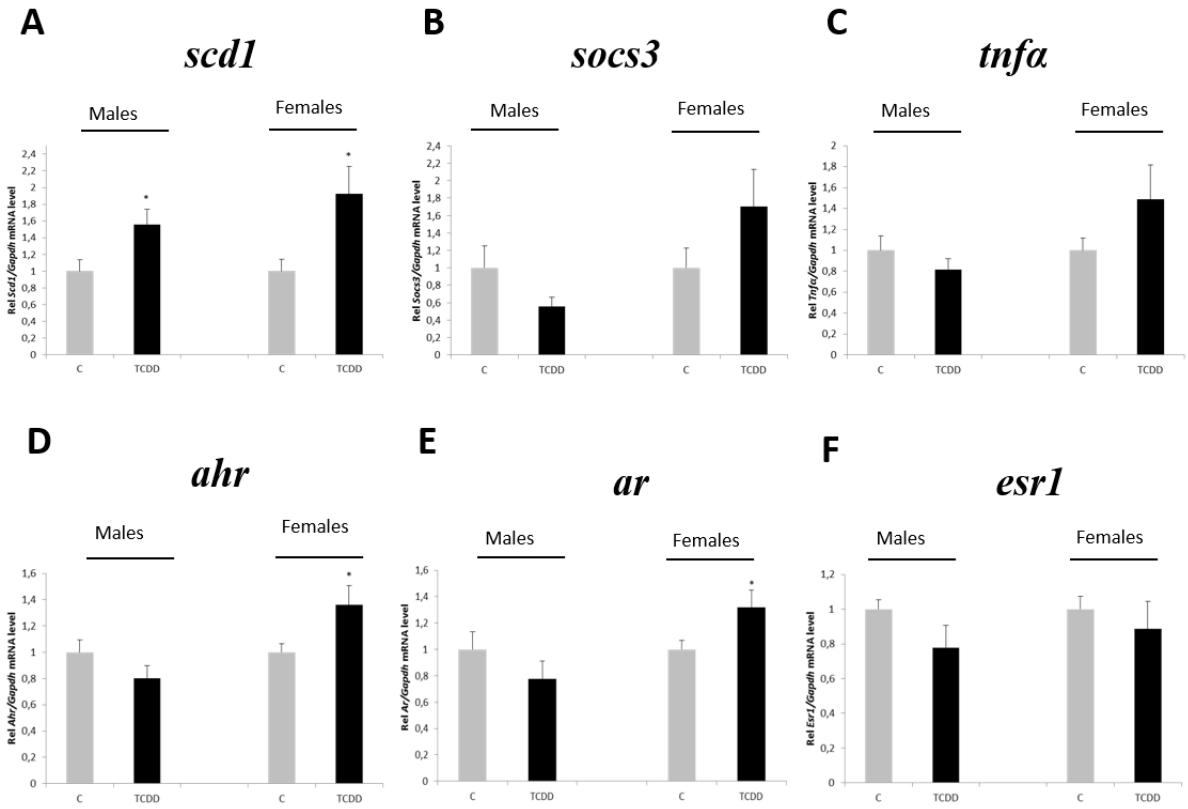

Fig. 4. TCDD effect on mRNA expression of nuclear receptors and marker genes related to hepatic steatosis. Liver mRNA expression of *scd1* (A), *socs3* (B), *tnfα* (C), *ahr* (D), a*r* (E) and *esr1* (F) in C57Bl/6J male and female mice fed with a high fat diet and after a TCDD exposure or not from 10 to 42 weeks-old at 1 µg/kg body weight/week. Measurements are performed at 42 week-old. Values represent means ± SEM. Paired student *t* test were performed; *p<0.05 (compared to the respective control group (c)). n=10 per group.

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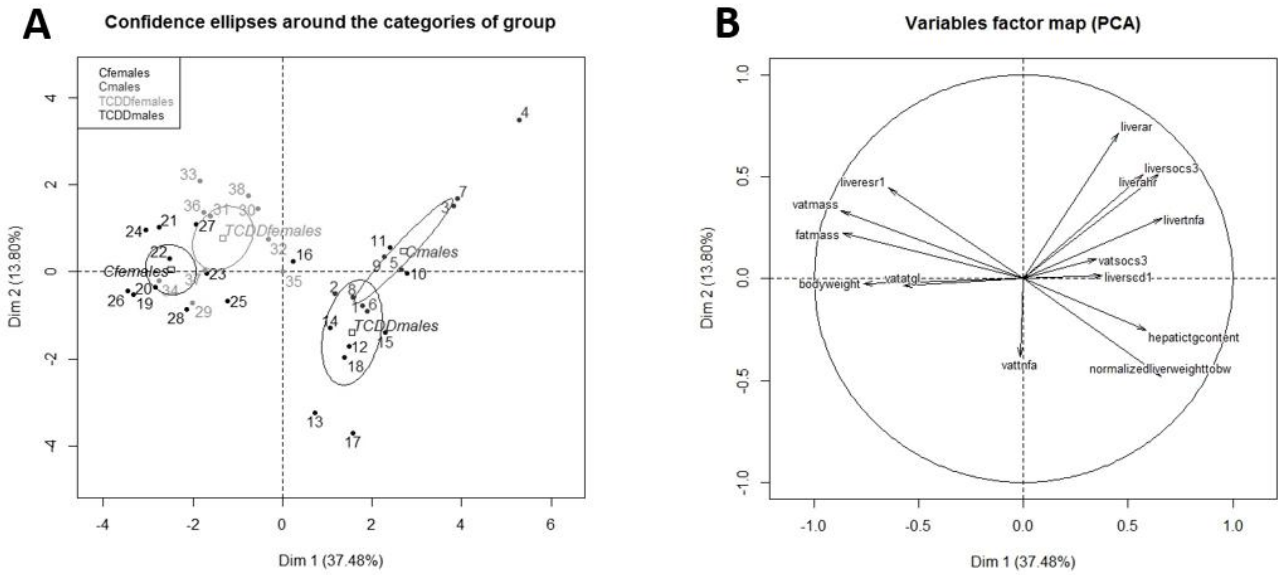

Fig. 5. Individual distribution and correlation with physiological parameters and mRNA expression of target genes analyzed. Principal Component Analysis (PCA): score plot of physiological parameters and mRNA expression of target genes (A) and correlation circle (B). Performed in C57Bl/6J male and female mice fed with a high fat diet and after a TCDD exposure or not from 10 to 42 weeks-old at 1 µg/kg body weight/week. n=10 per group. Cmales: Control males. Liver*tnfα/ahr/socs3/ar/scd1/esr1* refer to the hepatic mRNA expression of specified gene; vat*sosc3/atgl/tnfα* refer to the visceral adipose tissue mRNA expression of specified gene; nz.liverweighttobw refers to the liver weight normalized to the body weight; hepatcitgcontent refers to hepatic triglyceride content; fatmass and vatmass refer to the fat mass and weight of visceral adipose tissue.

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Table 1. TCDD effect on plasma parameters in male (up) and female (bottom) mice.

The homeostatic model assessment of insulin resistance (HOMA-IR) adapted to mice was calculated as ([glucose (mmol/l)] * [insulin (mUI/l)/108.6). NEFA: no esterified fatty acids. Values represent means ± SEM. Paired student *t* test were performed; *p<0.05 (compared to the control group (c)). n=10 per group.

males / Plasma parameters	Control	TCDD
Glucose (g/l)	3 ± 0.1	3 ± 0.2
Insulin (µg/l)	5 ± 0.3	5 ± 0.2
HOMA-IR	17 ± 1	16 ± 2
Leptin (pg/ml)	99 ± 9	104 ± 7
NEFA (mg/dl)	9 ± 0.6	9 ± 1
Cholesterol (g/l)	1 ± 0.07	1 ± 0.07
Triglycerides (g/l)	0.5 ± 0.07	0.5 ± 0.05

females / Plasma parameters	Control	TCDD
Glucose (g/l)	2 ± 0.08	2 ± 0.08
Insulin (µg/l)	5 ± 0.2	5 ± 0.3
HOMA-IR	12 ± 0.6	11 ± 0.7
Leptin (pg/ml)	171 ± 7	150 ± 4 *
NEFA (mg/dl)	10 ± 1	12 ± 1
Cholesterol (g/l)	1 ± 0.09	1 ± 0.09
Triglycerides (g/l)	0.6 ± 0.08	0.5 ± 0.05

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Table 2. Principal Component Analysis (PCA).

Qualitative (groups) (A) and quantitative (variables) (B) statistical analyses. n=10 per group. C males: Control males. Liver*tnfα/ahr/socs3/ar/scd1/esr1* refer to the hepatic mRNA expression of specified gene; vat*sosc3/atgl/tnfα* refer to the visceral adipose tissue mRNA expression of specified gene; nz.liverweighttobw refers to the liver weight normalized to the body weight; hepatcitgcontent refers to hepatic triglyceride content; fatmass and vatmass refer to the fat mass and weight of visceral adipose tissue.

(A)	Dimension 1	
	r^2	p-value
groups	0.8	1.2 e-13
	estimate	p-value
C males	2.6	7.2 e-06
TCDD males	1.4	2^e-02
C females	-2.6	9.8^e-06
TCDD females	-1.4	3.2^e-02
	Dimension 2	
	r^2	p-value
groups	0.4	0.002
	estimate	p-value
TCDD males	-1.4	0.0003
TCDD females	0.8	0.04

(B)	Dimension 1		Dimension 2	
	correlation	p-value	correlation	p-value
liver*tnfα*	0.66	$6.2e^{-06}$		
nz.liverweighttobw	0.65	$7.8e^{-06}$	-0.48	$2.5e^{-03}$
liver*ahr*	0.65	$1.1e^{-05}$	0.51	$9.5e^{-04}$
hepatictgcontent	0.58	$1.2e^{-04}$		
liver*socs3*	0.57	$1.9e^{-04}$	0.51	$1.1e^{-03}$
liver*ar*	0.45	$4.2e^{-03}$	0.71	$4.5e^{-07}$
liver*scd1*	0.37	$2e^{-02}$		
vat*sosc3*	0.35	$3.1e^{-02}$		
vat*atgl*	-0.57	$1.7e^{-04}$		
liver*esr1*	-0.64	$1.3e^{-05}$	0.44	$5.2e^{-03}$
bodyweight	-0.76	$2.7e^{-08}$		
fatmass	-0.86	$3.4e^{-12}$		
vatmass	-0.87	$1.4e^{-12}$	0.33	$4e^{-02}$
vat*tnfα*			-0.38	$1.8e^{-02}$