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## **Release and toxicity of adipose tissue-stored TCDD: Direct evidence from a xenografted fat model**

Nolwenn Joffin, Philippe Noirez, Jean-Philippe Antignac, Min-Ji Kim,  
Philippe Marchand, Marion Falabrègue, Bruno Le Bizec, Claude Forest,  
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3 **1. Title page**  
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5 **2 Manuscript title: Release and toxicity of adipose tissue-stored TCDD: direct evidence from a**  
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11 **5 Names of the authors:** Nolwenn Joffin<sup>1,2\*</sup>, Philippe Noirez<sup>1,2,3,4,5\*</sup>, Jean-Philippe Antignac<sup>6</sup>, Min-ji  
12 Kim<sup>1,6</sup>, Philippe Marchand<sup>7</sup>, Marion Falabregue<sup>2,3,4</sup>, Bruno Le Bizec<sup>7</sup>, Claude Forest<sup>1,2</sup>, Claude  
13 Emond<sup>5,8,9</sup>, Robert Barouki<sup>1,2</sup>, Xavier Coumoul<sup>1,2</sup>  
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15

16 **8 Equal contributions (\*) :** Philippe Noirez and Nolwenn Joffin  
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18  
19 **9 Affiliations of all authors** (department, institution, city, state/province, and country)  
20

21 <sup>1</sup> INSERM UMR-S1124, Toxicologie Pharmacologie et Signalisation cellulaire

22 <sup>2</sup> Université Paris Descartes, 45 rue des Saints-Pères, 75006 Paris, Sorbonne Paris Cité, Paris,  
23 France  
24

25 <sup>3</sup> IRMES, EA 7329, Institut de Recherche bioMédicale et d'Epidémiologie du Sport, Paris, France

26 <sup>4</sup> Institut National du Sport, de l'Expertise et de la Performance (INSEP), Paris, France

27 <sup>5</sup> Université du Québec à Montréal (UQAM), Montreal, Qc, Canada

28 <sup>6</sup> Université Paris 13, Sorbonne Paris Cité, Bobigny, France

29 <sup>7</sup> Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA), UMR 1329 Oniris-  
30 INRA, Nantes, France  
31

32 <sup>8</sup> BioSimulation Consulting Inc, Newark, DE, USA, 19713

33 <sup>9</sup> Université de Montréal, Montreal, Qc, Canada  
34  
35

36  
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38  
39 **22 Name of and contact information for corresponding authors:** X. Coumoul<sup>1,2</sup>; phone:  
40 +33142863359; fax: +33142863868; email: [xavier.coumoul@parisdescartes.fr](mailto:xavier.coumoul@parisdescartes.fr); R. Barouki<sup>1,2</sup>;  
41 phone: +33142862075; fax: +33142863868; email: [robert.barouki@parisdescartes.fr](mailto:robert.barouki@parisdescartes.fr)  
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44 **25 Running title:** PBPK model describing POPs release by fat tissue  
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**List of abbreviations:** alpha-SMA: alpha-smooth muscle actin; AhR: aryl hydrocarbon receptor; AQP: aquaporin; AT: adipose tissue; ATGL: adipose triglyceride lipase; bw: body weight; CD: cluster of differentiation; COL1A1: collagen 1A1; CPT1B: carnitine palmitoyl-transferase 1B; CYP: cytochrome P450; FABP4: fatty acid binding protein 4; FAS: fatty acid synthase; G6Pase: glucose-6 phosphatase; HSL: hormone-sensitive lipase; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; NOS2: NO Synthase 2; PAI-1: plasminogen activator inhibitor-1; PBPK: Physiologically based pharmacokinetic; PC: pyruvate carboxylase; PCB: polychlorinated biphenyl; PDK: pyruvate dehydrogenase kinase; PECK: phosphoenolpyruvate carboxykinase; PGC-1 alpha: PPARγ-coactivator 1 alpha; POPs: Persistent Organic Pollutants; PPARα: peroxisome proliferator-activated receptor-alpha; PPARγ2: PPAR-gamma2; RT-qPCR: real-time quantitative PCR; SD: Standard Deviation; TNF: Tumor-Necrosis Factor; UCP1: uncoupling protein 1; VLCAD: Very long-chain acyl-CoA dehydrogenase; PBPK: Physiologically Based Pharmacokinetic.

### Highlights

- A new model to study the release and distribution of POPs
- Introduction of a new PBPK model to study the effects of POPs
- Internal release of TCDD activates signatures of inflammation & fibrosis.
- Internal stores of POPs could play a significant role in long term toxicity.

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53 **2. Abstract.**

54 **Background:** Persistent organic pollutants (POPs) are known to accumulate in adipose tissues (AT).  
55 This storage may be beneficial by diverting POPs from other sensitive tissues or detrimental because  
56 of chronic release of pollutants as indirectly suggested during weight loss. The aim is to study the  
57 biological and/or toxic effects that chronic POP release from previously contaminated grafted AT could  
58 exert in a naïve mouse. **Methods:** C57BL/6J male mice were exposed intraperitoneally to 2,3,7,8-  
59 tetrachlorodibenzo-p-dioxin (TCDD); their epididymal fat pads were collected and grafted on the back  
60 skin of uncontaminated recipient mice whose brain, liver, and epididymal ATs were analyzed (TCDD  
61 concentration, relevant gene expression). Kinetics of release and redistribution were modeled using  
62 Physiologically Based Pharmacokinetics (PBPK). **Results:** The grafts released TCDD over a period of 10  
63 weeks with different kinetics of distribution in the three organs studied. A PBPK model was used to  
64 simulate the AT releasing process and the incorporation of TCDD into the major organs. At three weeks  
65 post-graft, we observed significant changes in gene expression in the liver and the host AT with  
66 signatures reminiscent of inflammation, gluconeogenesis and fibrosis as compared to the control.  
67 **Conclusions:** This study confirms that AT-stored TCDD can be released and distributed to the organs  
68 of the recipient hence leading to distinct changes in gene expression. This original model provides  
69 direct evidence of the potential toxic-relevant effects when endogenous sources of contamination are  
70 present.

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72 **Keywords:** adipose tissue, graft, internal release, dioxin, TCDD, PBPK, fibrosis

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180 **74 3. Introduction**  
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182 75 Persistent organic pollutants (POPs) are xenobiotics of major concern for environmental health  
183 76 (Bonde et al., 2016; Mostafalou, 2016; Smarr et al., 2016). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)  
184 77 is one of the most potent compounds among the dioxin class of substances and its toxicity equivalence  
185 78 factor is set to a reference value of 1. TCDD binds to the Aryl hydrocarbon Receptor (AhR), a protein of  
186 79 the basic Helix-Loop-Helix / Per - ARNT - Sim (bHLH/PAS) family, which transcriptionally activates the  
187 80 expression of genes which code for xenobiotic metabolizing enzymes (XME) as well as that of other  
188 81 genes (Guyot *et al.* 2013). However, in spite of the induction of these detoxifying enzymes, the TCDD  
189 82 and other POPs are very poorly metabolized and, therefore, not readily eliminated. This, as well as  
190 83 their lipophilicity, accounts for their accumulation in adipose tissue (AT) (Lee *et al.* 2017).  
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192 84 The storage of POPs in AT is believed to have complex implications and consequences (Kim *et*  
193 85 *al.* 2011, 2012). It may be protective to a certain extent since it diverts POPs from critical target organs  
194 86 such as the brain. However, it is also assumed that contaminated AT progressively delivers POPs that  
195 87 would eventually lead to a chronic low-rating release resulting in chronic toxicity. Indeed, we and  
196 88 others observed an increase in blood POPs following weight loss (Chevrier *et al.* 2000, Imbeault *et al.*  
197 89 2002, Kim *et al.* 2011) but such increase could also be the result of the dynamic switch between  
198 90 lipolysis and lipogenesis that occurs during fasting/feeding process, independent of weight loss. The  
199 91 evidence for both scenarios, *i.e.* protection and long-term toxicity, is indirect at this stage.  
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201 92 Long-term exposure to TCDD has been associated with many pathologies including cancer,  
202 93 infertility or metabolic diseases (White and Birnbaum, 2009). Its carcinogenic effects have been  
203 94 established by the World Health Organization (WHO) and the US National Toxicology Program (NTP)  
204 95 despite some conflicting results in humans (Boffetta *et al.* 2011). Regarding metabolic diseases, a  
205 96 recent meta-analysis pointed to a likely link between TCDD exposure and type 2 diabetes (Goodman  
206 97 *et al.* 2015), while *in vitro* and *in vivo* experiments showed that TCDD reduced glucose uptake by the  
207 98 liver and AT, a process that occurs with decreases in insulin production and secretion by beta-  
208 99 pancreatic cells (Novelli *et al.* 2005). TCDD is also suspected to elicit metabolic diseases through either  
209 100 the induction of local inflammation of the AT (Kim *et al.* 2012), the regulation of endogenous  
210 101 metabolizing enzymes (carbohydrate and lipid pathways) (Ambolet-Camoit *et al.* 2015) or epigenetic  
211 102 mechanisms. Such TCDD effects are consistent with the suspected obesogen effects of several POPs  
212 103 (*i.e.* organochlorinated pesticides) (La Merrill *et al.* 2013; Lee *et al.* 2014).  
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229 104 While the long-term toxicity of TCDD (and other POPs) is now established, there is still no direct  
230 105 evidence that its release from AT is involved in such long-term toxicity although such effects are being  
231 106 suspected (Kim *et al.* 2011). In all experimental protocols, animals are either fed or injected with TCDD,  
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107 resulting in single (or repetitive) exposures. There are several possible mechanisms for long-term  
108 toxicity. Such toxicity may be due to the direct effect of ingested TCDD following exposure but could  
109 also be additionally due to TCDD output from internal compartments like AT. Because of the lack of an  
110 experimental model assessing the role of AT-stored TCDD, it has not been possible yet to assess those  
111 hypotheses and to clearly identify the kinetic and dynamic behaviors of TCDD discharged from internal  
112 storage sites.

113 The aims of the present study were 1) to develop an experimental model based on a surgical  
114 graft procedure; 2) to describe the fate and effects of AT-stored TCDD then specifically investigate  
115 whether this compound can be discharged from the AT and display activity on other tissues, and 3) to  
116 build a physiologically based pharmacokinetic (PBPK) model to simulate the distribution of TCDD by  
117 gavage and the donor, and then at the second time simulating a chronic endogenous POP release from  
118 the graft implant in the host, and 4) to characterize the effects of this release on the expression of  
119 several biomarkers linked to pathologies. To achieve this goal, we have developed a unique, new and  
120 original experimental model allowing such investigation. We have grafted TCDD-contaminated AT to  
121 uncontaminated mice and monitored the action that sub-chronic release of this POP could exert in  
122 three host tissues, AT, liver and brain; such novel bioengineered mice allowed the generation of a PBPK  
123 model simulating a chronic POP output from a grafted implant. We characterized the effects of this  
124 release on the expression of several biomarkers linked to the above-described pathologies - including  
125 endogenous metabolic enzymes or inflammatory cytokines - in recipient AT and liver, two organs that  
126 are critically involved in metabolic regulations.

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## 128 4. Materials and Methods

### 129 4.1 Mice

130 The European Communities Council Directive 2010/63/EU on the protection of the animals  
131 was followed for the experiments using animals. All procedures were approved by the ethical  
132 committee for animal research of Paris Descartes university (CEEA.34, number: 12-132). C57BL/6J  
133 male mice (12-week-old) with body weight (bw) about 25 g were obtained from Janvier and allowed 1  
134 week to acclimate at the Paris Descartes university animal facility before the study. Animals were  
135 maintained on a 12-hr light/dark cycle at the ambient temperature of a  $22 \pm 1^\circ\text{C}$  and relative humidity  
136 of  $55 \pm 5\%$  and were provided with Purina 5001 rodent Chow (Safe) and tap water ad libitum.

### 138 4.2 Primary POPs exposure

139 The mice donor C57BL/6J received a single intraperitoneal injection with either dose of 0, 1,  
140 10 or 25  $\mu\text{g}$  TCDD/kg of bw in corn oil. Mice were sacrificed 48h after injection. For each mouse, both  
141 epididymal fat pads were collected: one was used for TCDD quantification and the other for the graft  
142 to a donor mouse.

### 144 4.3 Graft of epididymal AT contaminated with POPs

145 Each epididymal fat pad was grafted into the subcutaneous plane on the back of the mice.  
146 Mice were sacrificed at 0.5, 1, 2, 3, 4 or 10 weeks following the graft. The graft, brain, liver, and  
147 epididymal AT were removed for quantification of TCDD and gene analyses. We observed that the  
148 grafts were revascularized between 4 and 10 weeks following the surgical procedure (data not shown).

### 150 4.4 POPs internal exposure levels quantification

151 The methodology that was applied to isolate, detect, and quantify TCDD had its origin in a  
152 more global previously described method used for measuring dioxins and PCBs (Costera et al. 2006).  
153 Briefly,  $^{13}\text{C}$ -labelled TCDD was added to each sample for quantification according to the isotopic  
154 dilution method. Samples of AT (50 to 250 mg), liver (0.150 to 1.750 g) and brain (150 to 550 mg) were  
155 first submitted to an accelerated solvent extraction at high temperature and high pressure (ASE  
156 Dionex, Sunnyvale, CA). The resulting extracts were weighed to evaluate fat content by a gravimetric  
157 method and were reconstituted in hexane for further sample clean up through three purification steps  
158 using successively acid silica, florisil, and celite/carbon columns. TCDD concentrations were assessed  
159 using gas chromatography (Agilent 7890A) coupled to high-resolution mass spectrometry (GC-HRMS)  
160 on electromagnetic sector instrument (JEOL MS 800D), operating at 10,000 resolutions and in the  
161 single ion monitoring (SIM) acquisition mode. This method was validated according to current

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162 European criteria in the field of regular control of foodstuff of animal origin and accredited according  
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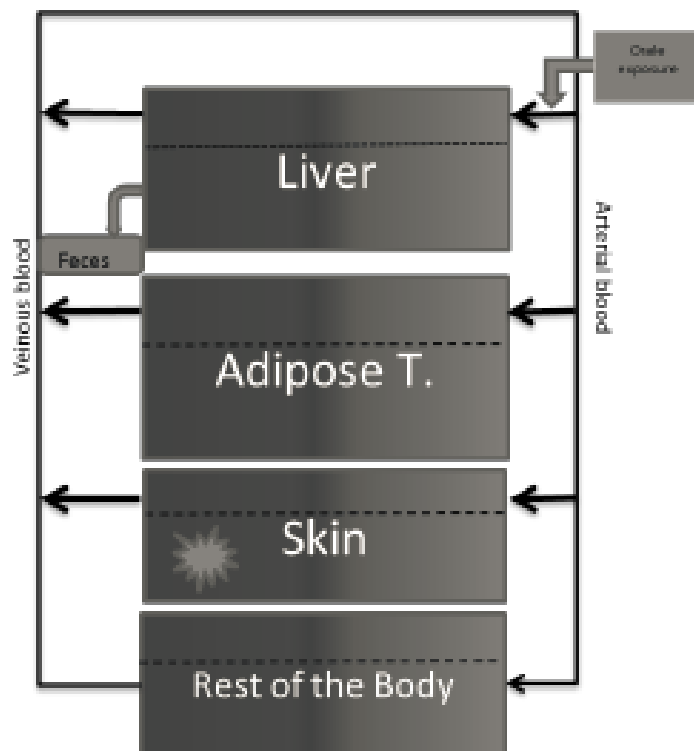
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165 **4.5 PBPK modeling**

166 Several models have been published to mimic the pharmacokinetics of TCDD on different  
167 species (Emond, 2004; Kissel and Robarge, 1998; Leung et al., 1990, 1988). More recently, a PBPK  
168 model for TCDD has been applied to humans and mice (Emond et al., 2010; Emond et al., 2016) to  
169 study the influence of obesity and diabetes on the elimination of TCDD (Emond et al., 2018). A larger  
170 description and assessment work of the PBPK model for mice were performed and peer-reviewed  
171 during the NCEA-USEPA reassessment (USEPA, 2012). This version of the PBPK model was used as a  
172 base for the present work. Briefly, the model is originally composed of 3 compartments (liver, AT and  
173 the rest of the body) connected to the systemic circulation (arterial and venous). The rest of the body  
174 compartment is composed of muscles ( $\approx 70\%$ ) and other non-essential organs for the pharmacokinetics  
175 but is required for the mass balance calculation. The rest of the body is then described as permeability-  
176 limited compartments (composed of extracellular matrix and blood sub-compartments). The  
177 elimination of TCDD from the body is very limited; it happens mostly in the feces and not much in the  
178 urine. In addition, the PBPK model also integrates an enterohepatic cycle; overall, it allows the  
179 description of an elimination rate based on the body burden using a mathematical component function  
180 (Emond et al. 2016; USEPA 2012). One key parameter of this model, which conditions the elimination  
181 rate of TCDD, is the induction of CYP1A2 which is due to AhR activation (CY1A2 is a transcriptional  
182 target of the AhR) which depends on the body burden (Diliberto et al. 1997; Emond *et al.* 2005, 2006).  
183 All the parameters used in this PBPK model, before modification, were previously assessed and  
184 presented in the USEPA 2012 report (in this reference, see Tables 3-8 pages 3-63).

185 To describe the release of TCDD from the AT implanted in the dorsal subcutaneous skin region  
186 (graft), it was necessary to update the original PBPK model by adding a skin compartment and graft  
187 (Figure 1).



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**Figure 1:** Conceptual representation of the PBPK model in mice

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Indeed, we assumed that the graft was an entity added to the skin compartment. Basically, the graft releases the TCDD which diffuses to the skin compartment and is then released in the blood. The blood flow in the skin compartment was described as permeability-limited; all parameters for this additional compartment including the partition coefficient “skin/ blood” (unitless), perfusion of the skin compartment ( $\text{ml}\cdot\text{h}^{-1}$ ), the diffusion permeability of the skin ( $\text{ml}\cdot\text{h}^{-1}$ ) and the volume of skin tissue (ml) were extracted from the literature (Brown *et al.*, 1997; Wang *et al.*, 1997) (Table 1).

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198 **Table 1: physiological parameters used to define both skin and graft compartments used in the PBPK**  
 199 **allografted mice model**

Parameters	Name of the parameters	values	references
$V_{sk}$	Fraction of body weight for skin (unitless)	0.165	Brown <i>et al.</i> , 1997
$V_{skb}$	Fraction of tissue blood volumes for skin (unitless)	0.0159	Wang <i>et al.</i> , 1997
$Q_{skf}$	Tissue blood flow fraction of cardiac output for skin (unitless)	0.057	Brown <i>et al.</i> , 1997
$P_{sk/b}$	Partition coefficient (skin/blood) (unitless)	10	Wang <i>et al.</i> , 1997
KGRAFT	First order parameter of TCDD release in graft to the skin ( $h^{-1}$ )	0.004	Optimized
PASKINF	Diffusion permeability (unitless)	0.09	Optimized
R	Fraction of blood diffusion (PASKIN) from the blood skin to the grafted starting 4 weeks post graft implantation (unitless)	R= 0.0007	Optimized

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 201 The graft sub-compartment is essentially composed of TCDD-delivering AT (see §4.2). This  
 202 delivery was estimated using the concentration of TCDD in the graft at time 0 and the subsequent  
 203 measurements at different time points (using a first order kinetic release of TCDD from the graft to the  
 204 skin). We also consider a functional vascularization of the graft around week 4 (visual observation).  
 205 The parameters used to mimic mathematically the release of TCDD from the graft are presented in  
 206 Table 2:

208 **Table 2: Parameters used to mimic mathematically the release of TCDD from the graft in the**  
 209 **corresponding equations**

Parameters	Description of the parameters
cgraft0	concentration of TCDD in the graft at time 0 ( $pg.g^{-1}$ )
graftmass	mass of the graft (g)
agraftpg	amount of TCDD at time 0 (pg)
kgraft	constant transfer of TCDD from graft to skin ( $h^{-1}$ )
agraftng	amount of TCDD in the graft at time 0 (ng)
agraftnmol	amount of TCDD in the graft at time 0 (nmole)
agraftresnmol	amount of TCDD remaining in the graft at different time points (nmole)

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211 The amount of TCDD which remains in the graft after each time point (dt), also *agraftresnmol*,  
212 was estimated using **equation 1**:

- **Equation 1:** variation of the amount of TCDD (nmole) that remains in the graft after each time interval (dt) in hours

$$\frac{d\text{agraftnmol}(\text{nmol})}{dt(\text{h})} = -k\text{graft} \times \text{agraftresnmol}$$

219 Then, the changes in TCDD concentrations in the grafts were estimated using **equations 2 & 3**.

- **Equation 2** Amount of TCDD (nmoles) remaining in the graft after each time point (dt):

$$\text{agraftresnmol} = \int_{t-1}^t \text{agraftnmol}$$

- **Equation 3:** Concentration of TCDD in pg/g

$$\text{agraftrespgg}\left(\frac{\text{pg}}{\text{g}}\right) = \text{agraftresnmol}(\text{nmole}) \times \frac{MW\left(\frac{\text{ng}}{\text{nmole}}\right) \times 1000\left(\frac{\text{pg}}{\text{ng}}\right)}{\text{graftmass}(\text{g})}$$

228 These simulation parameters were optimized by a first series of experiments carried out to  
229 assess the release of TCDD from the graft during the optimization of the exposure dose of the donor.  
230 Determination of the graft mass and the concentration of TCDD at different time points were  
231 compared to the simulation profile resulting from the model. In addition, simulation of TCDD  
232 generated using the PBPK model was compared to the experimental time points determined  
233 experimentally.

#### 4.6 RNA sampling from tissues

236 AT and liver samples were placed in 1 mL of Qiazol® reagent with 2 stainless steel beads  
237 (Qiagen, France) and were homogenized with a TissueLyser system (RetschMM300, Germany). Total  
238 RNA was prepared using the RNeasy Mini Kit following the manufacturer's instructions (Qiagen,  
239 France). The quality of total RNA was monitored with a Nanodrop ND-1000 spectrophotometer  
240 (Nanodrop products, Wilmington, USA).

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593 **248 4.7 Quantitative real-time PCR**  
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595 **249** Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit  
596 (Life technology, France) according to the manufacturer's directions. Real-time PCR was achieved with  
597 **250**  
598 **251** 20 ng of cDNA, with duplicates for each experiment. STable 1 (Suppl Mat.) gives the gene-specific  
599  
600 **252** primers. The relative amounts of mRNA were estimated using the  $\Delta\Delta C_T$  method with Ribosomal Protein  
601 **253** L13 (RPL13) as the reference gene (Juricek *et al.*, 2014; Pfaffl, 2001).  
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603 **254**

Gene	Forward primer	Reverse primer
AhR	ACAGTAAAGCCCATCCCC	AGCACAAAGCCATTTCAGC
CYP1A1	ATGAGTTTGGGGAGGTTACTG	AATGAGGCTGTCTGTGATGTC
CYP1B1	GAGGATGTGCCTGCCACTA	CTGTGGCTGCTCATCCTCTT
IL-1 $\beta$	GCCACCTTTTGACAGTGATG	TCTCCACAGCCACAATGAG
IL-6	CTTCCATCCAGTTGCCTTCTTG	AATTAAGCCTCCGACTTGTGAAG
TNF alpha	TCATCCATTCTCTACCCAGC	GTCCCAGCATCTTGTGTTTC
MCP-1	AGCAGCAGGTGTCCCAAAGA	ACGGGTCAACTTCACATTCAA
NOS2	TGAGGGGACTGGACTTTTAG	CTGTGACTTTGTGCTTCTGC
F4/80	TTTCTCGCCTGCCTCTTC	CCCCGTCTCTGTATTCAACC
COL1A1	TCATCGTGGCTTCTCTG	CGTTGAGTCCGTCTTTG
Alpha-SMA	CCTGGTGTGCGACAATG	TGCTCTGGGCTTCATCC
Glycogen synthase 2	CCAGCTTGACAAGTTCGACA	ATCAGGCTTCTCTTCAGCA
PC	CGGCAGGGCGGAGCTAACAT	TTTGGGGAGGCAACAGGGGC
PEPCK-c	AGTGCCCCATTATTGACC	TCTTGCCCTTGTGTTCTG
G6Pase	CCAAGGGAGGAAGGATG	AGGTGACAGGGAAGTCT
PGC-1 alpha	TGCCCTGCCAGTCACAGGA	GCTCAGCCGAGGACACGAGG
RPL13	GGATCCCTCCACCCTATGACA	CTGGTACTTCCACCCGACCTC
CD68	CCAATTCAGGGTGAAGAAA	CTCGGGCTCTGATGTAGGTC
AQP7	GAAGTCAAGGCTTGGTCTGCT	CATGTGAGCCACGGAACCAA
ATGL	CCAACGCCACTCACATC	CAGAGGACCCAGGAACC
HSL	ACTGAGATTGAGGTGCTGTC	AAGGCAGGTGAGATGGTAAC
CPT1B	TTCCGACAAACCCTGAAGCT	TAAGATCTGGGCTATCTGTGTC
VLCAD	TGAATGACCCTGCCAAG	CCACAATCTCTGCCAAGC
CD36	CATGAGAATGCCTCCAAACAC	GGAAGTGTGGGCTCATTGC
GLYCOGEN KINASE	ATCCGCTGGCTAAGAGACAACC	TGCACTGGGCTCCCAATAAGG
PDK4	TGTGATGTGGTAGCAGTAGTC	ATGTGGTGAAGGTGTGAAG
FABP4	AACACCGAGATTTCTT	ACACATTCCACCACCAG
PPAR $\alpha$	AAGCCATCTTCACGATGCTG	TCAGAGGTCCCTGAACAGTG
FAS	TCCTGGGAGGAATGTAAACAGC	CACAAATTCATTACTGCAGCC
UCP1	CCGCGACTTCGGACTCCTGC	TAACGGGTCTCCTGCCCG
PPAR $\gamma$ 2	TTATGCTGTTATGGGTGAAA	CAAAGGAATGCGAGTGGTC

644 **255**  
645 **256** **Supplementary table S1: list of primers used for quantitative RT-PCR experiments**  
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**4.8 Statistical analyses**

Unless otherwise specified, two-group and multiple-group comparisons were made using Mann-Whitney's U-test (nonparametric comparison of two independent series) or Kruskal-Wallis test followed by a pair-wise Dunn's post hoc test (nonparametric comparison of multiple independent series). A p-value < 0.05 was considered statistically significant (\*\* p<0.01, \* p<0.05). The values are expressed as the mean ± standard deviation.

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711 264 **5. Results**

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713 265 **5.1 Xenografted-adipose tissue releases TCDD**

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715 266 Vehicle (control) or three different doses of TCDD (1, 10 or 25  $\mu\text{g}\cdot\text{kg}^{-1}$  bw; corn oil as a control)  
716 267 were intraperitoneally injected to C57BL6/J male mice that were sacrificed 48h later. TCDD  
717 268 concentration was determined in the epididymal AT of control (corn oil) and exposed mice, showing a  
718 269 proportional increase in the TCDD levels in line with the injected doses (STable 2, Suppl. Mat.).

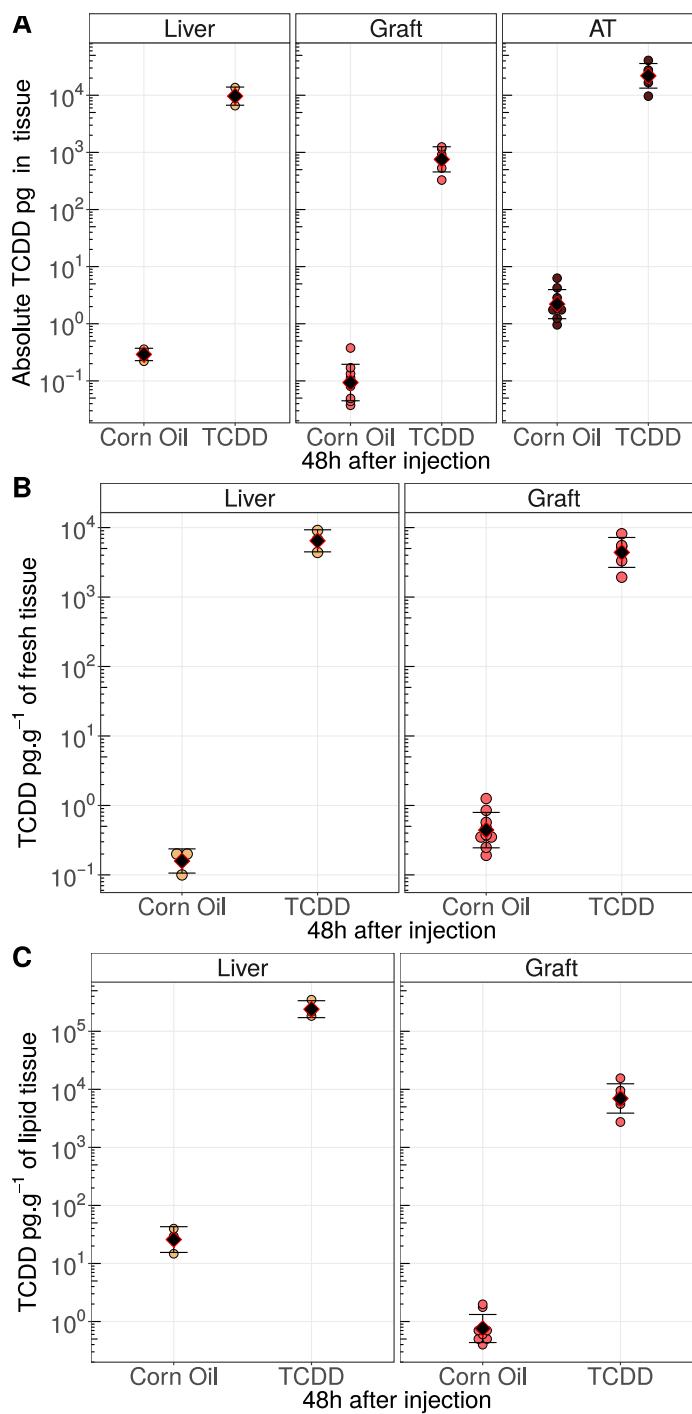
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TCDD dose ( $\mu\text{g}\cdot\text{kg}^{-1}$ bw)	Levels of TCDD in the AT 48h later ( $\text{pg}\cdot\text{g}^{-1}$ fresh tissue)	Relative fold increase in the AT
0	6	-
1	2 335	1
10	21 623	9
25	56 608	24

733 270  
734 271 **Supplementary table S2: comparison of TCDD-injected doses ( $\mu\text{g}\cdot\text{kg}^{-1}$  bw) in C57BL/6J male**  
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736 272 **mice and its levels in the AT ( $\text{pg}\cdot\text{g}^{-1}$  fresh tissue) 48h after injection.**

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740 274 The dose of 10  $\mu\text{g}\cdot\text{kg}^{-1}$  bw did not saturate the AT since higher concentrations of TCDD in the  
741 275 AT were obtained following the injection of 25  $\mu\text{g}\cdot\text{kg}^{-1}$  TCDD; thus, this dose (10  $\mu\text{g}\cdot\text{kg}^{-1}$  bw) was  
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743 276 selected for all subsequent experiments (SFigure 1, Suppl Mat.).  
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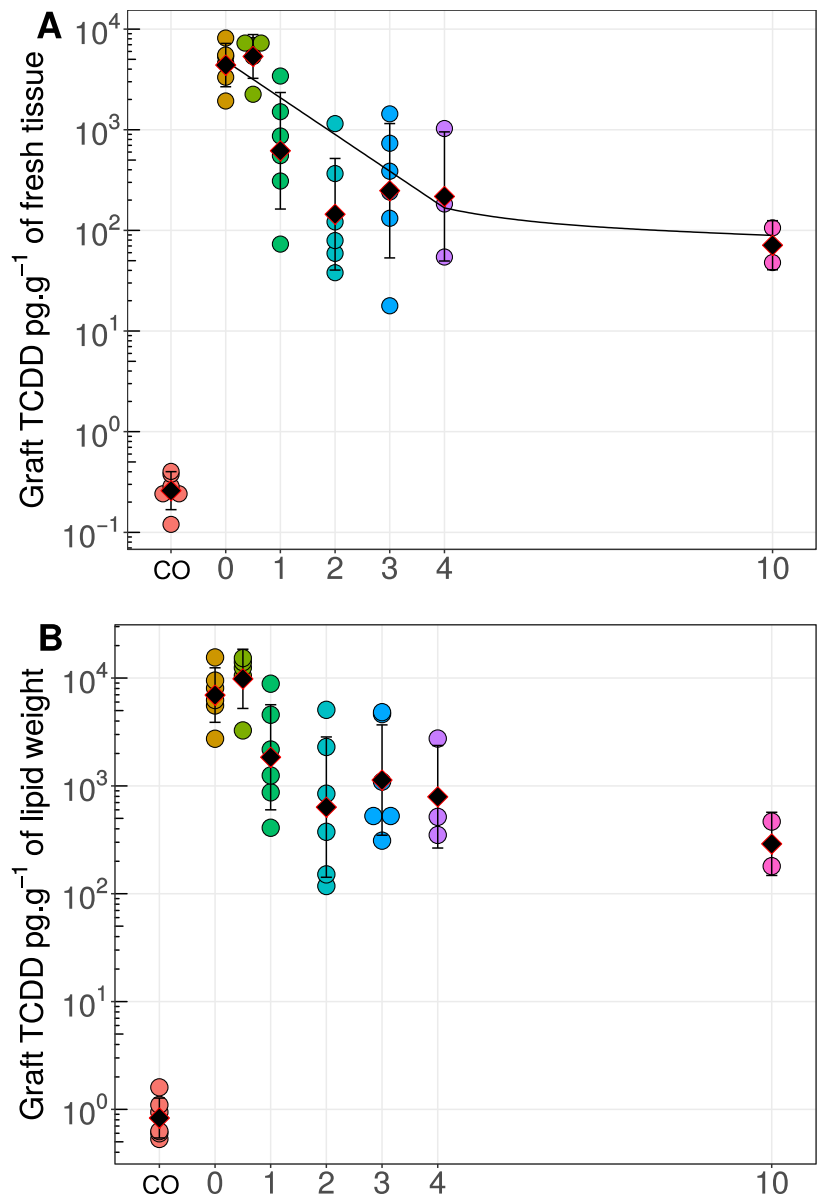


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278 **Figure 1:** Global burden (pg, A) and concentration levels (pg.g<sup>-1</sup> fresh tissue, B and pg.g<sup>-1</sup> lipid tissue,  
279 C) of TCDD determined in the liver, the graft, and/or the whole AT of the mice donors which had  
280 received a single intraperitoneal injection (see §4.2). The levels of TCDD were measured in the liver  
281 and the AT; the upper panel represents the absolute quantity (in pg) of TCDD in the liver, the  
282 epididymal AT (used later as a graft) and the extrapolated whole-body AT; the middle and lower panels  
283 represent the concentration of TCDD (respectively in pg.g<sup>-1</sup> of fresh tissue or pg.g<sup>-1</sup> of lipids). One of  
284 the epididymal AT (the left one) has been used as a graft (weight of the graft: 100-150 mg;  
285 approximated concentration of TCDD: 10<sup>4</sup> pg.g<sup>-1</sup> of lipids).

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286 The contaminated AT (epididymal left AT) were then grafted on uncontaminated host C57BL/6J  
287 mice. Figure 2 shows that TCDD concentration decreases in the TCDD-contaminated grafted tissue in  
288 a time dependent manner.



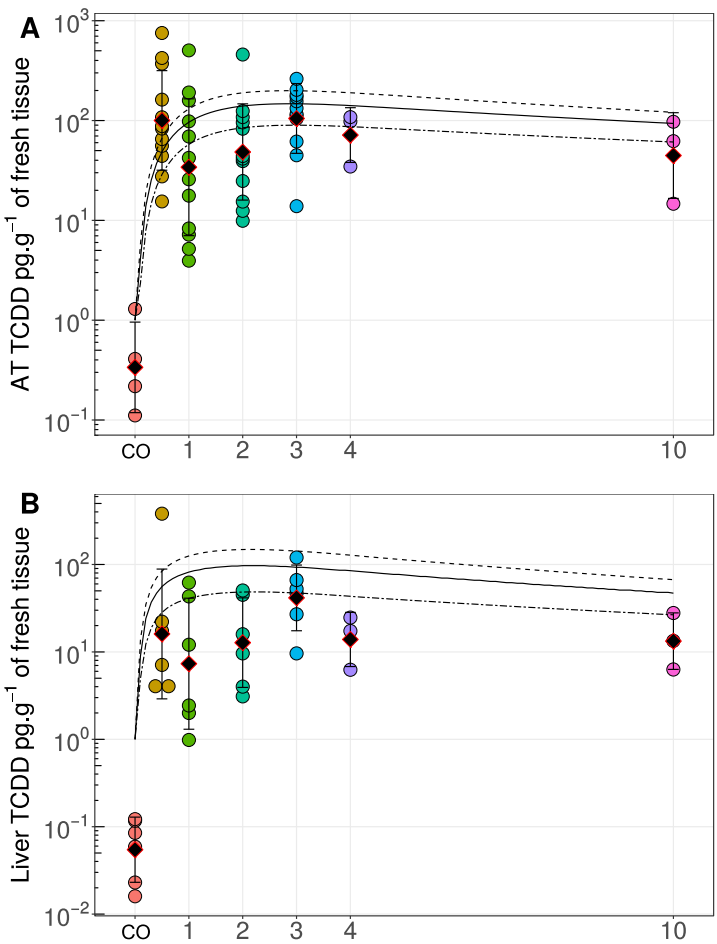
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**Figure 2:** Levels of TCDD (A: pg.g<sup>-1</sup> fresh tissue; B: pg.g<sup>-1</sup> lipid weight) measured in the grafted AT (3-6 mice/condition) at different time points following the graft (in weeks). The corn oil condition (CO) designates the level of TCDD present in a graft from mice injected with corn oil. In panel A, the line represents the profiled simulation of TCDD concentrations (expressed in pg TCDD.g<sup>-1</sup> fresh tissue) from the graft implanted in dorsal subcutaneous for 10 weeks.



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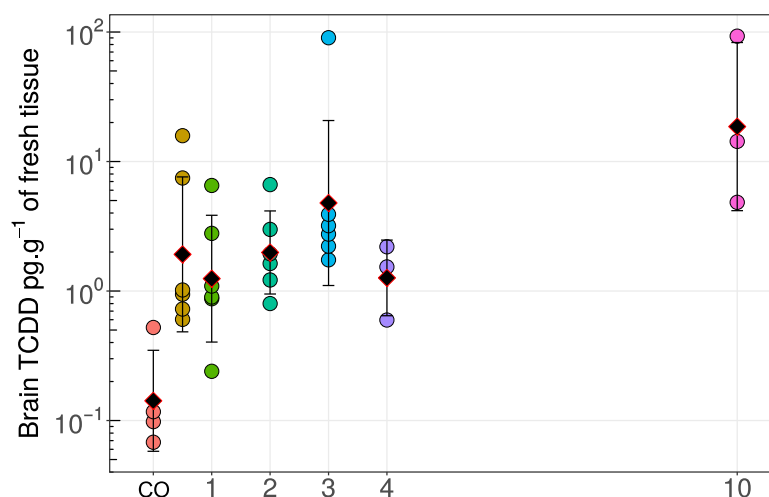
292 **5.2 TCDD redistributes to other tissues**

293 Subsequently, we quantified the distribution of TCDD in three organs of the grafted mice,  
294 namely the epididymal AT of the host, liver and brain (respectively figures 3A and 3B, SFigure 2 Suppl.  
295 Mat). The levels of TCDD increased significantly in the host AT (figure 3A, from <1 to >100 pg.g<sup>-1</sup> of  
296 fresh tissue in 3 weeks) during the 3-week-time period and remained then stable over the course of  
297 the experiments. The distribution in the brain was slower than the distribution in the host AT, with a  
298 basal level of 0.1 pg.g<sup>-1</sup> of fresh tissue and a concentration that reached 1 pg.g<sup>-1</sup> of fresh tissue at 1  
299 week and 10 pg.g<sup>-1</sup> after 10 weeks (SFigure 2). The levels of TCDD also increased significantly in the  
300 liver (figure 3B, from <0.1 to >10 pg.g<sup>-1</sup> of fresh tissue in 2 weeks) during the 2-week-time period and  
301 remained then stable over the course of the experiments.



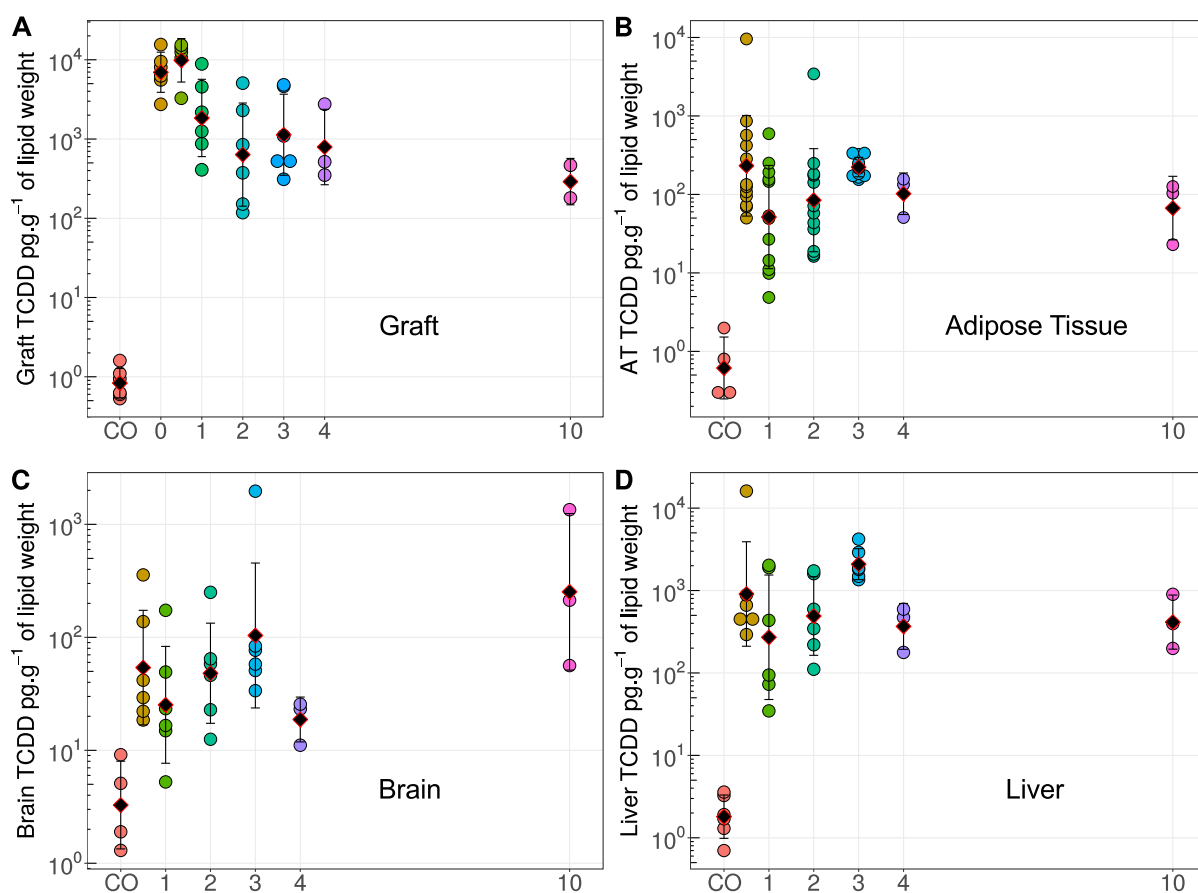
302 **Figure 3:** Levels of TCDD (pg.g<sup>-1</sup> of fresh tissue) in the host AT (A), and the liver (B) (n=6 mice/condition  
303 except weeks 4 and 10) at different time points following the graft (in weeks). For all panels, the  
304 symbols (●) represent the individual measurement and the symbols (◆) represents the mean  
305 measurement and the standard deviation of the TCDD measured (mean ± SD) at different time points  
306 following the sacrifice. (A) simulation curves of the TCDD accumulation and experimental values in the

AT and (B) in the liver issued from the host following subcutaneous grafting. For each of these tissues, we simulate the mean concentration at time 0, and the concentrations corresponding to the mean  $\pm$  SD (see text). The corn oil condition (CO) designates the level of TCDD present in a graft from mice injected with corn oil.



**Figure 2:** Levels of TCDD (pg.g<sup>-1</sup> of fresh tissue) in the brain (n=6 mice/condition except weeks 4 and 10) at different time points following the graft (in weeks). For all panels, the symbols (●) represent the individual measurement and the symbols (◆) represents the mean measurement and the standard deviation of the TCDD measured (mean  $\pm$  SD) at different time points following the sacrifice. The corn oil condition (CO) designates the level of TCDD from mice injected with corn oil.

When the levels of TCDD were represented in pg.g<sup>-1</sup> of lipid weight (SFigure 3, Suppl. Mat.), a similar profile was obtained for the AT, a tissue with a high fat content. The fast and slow accumulations of TCDD respectively in the liver and the brain were also clearly characterized.

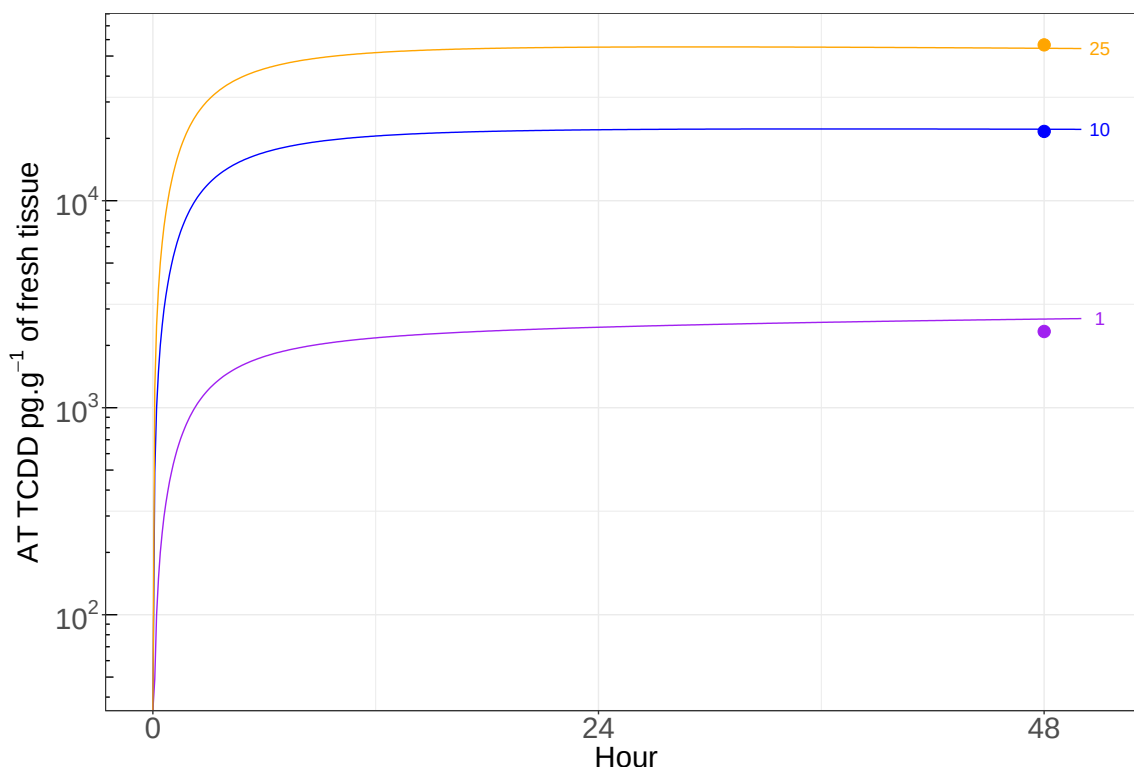


**Figure 3:** Levels of TCDD ( $\text{pg}\cdot\text{g}^{-1}$  lipid weight.) in the graft (A), host AT (B), brain (C) and liver (D) tissues ( $n=3-6$  mice/condition) at different time points following the graft ( $w$ : weeks). The corn oil condition (CO) designates the level of TCDD present in a graft from mice injected with corn oil.

Overall, our experiments demonstrate that TCDD is quickly released from a contaminated grafted-AT and redistributed in several key organs of the grafted C57BL6 mice albeit with different kinetics.

### 5.3 PBPK modeling - exposure of the donor

The first step consisted to simulate the exposure of donor mice to a single intraperitoneal injection dose at concentrations of 1, 10 and 25  $\mu\text{g}$  TCDD per kg bw (STable 2, Suppl. Mat.). Using the PBPK mouse model, we simulated the profile of TCDD concentrations in the AT (SFigure 4, Suppl. Mat.).



**Figure 4:** TCDD concentration in mouse AT following a single exposure of TCDD intraperitoneal at 1, 10 or 25 µg.kg<sup>-1</sup> body weight. The lines depict simulated concentrations and symbols the experimental data at 48h post exposure (see Supplementary table S2).

The simulation matched the experimental measurements of TCDD with very high accuracy (STable 2, Suppl. Mat. and SFigure 4, Suppl. Mat.) and the ratio of the nominal TCDD doses matched well with that of the measured TCDD concentrations in AT (Eq.4).

**Equation 4: Comparisons between ratio of dose and tissue concentrations:**

$$\frac{\frac{10\mu\text{g}}{\text{kg}}}{1\mu\text{g}/\text{kg}} \cong \frac{\frac{21623\text{pg}}{\text{gtissue}}}{\frac{2335\text{pg}}{\text{gtissue}}} \cong 10 \quad \frac{\frac{25\mu\text{g}}{\text{kg}}}{1\mu\text{g}/\text{kg}} \cong \frac{\frac{56608\text{pg}}{\text{gtissue}}}{\frac{2335\text{pg}}{\text{gtissue}}} \cong 25$$

#### 5.4 PBPK modeling - release of TCDD from the graft / distribution of TCDD in host compartments

The second step consisted of simulating the decrease of TCDD in the graft. Because of the large standard deviations, we used the mean of TCDD concentrations measured in the different assays (4815 pg.g<sup>-1</sup> fresh tissue at time 0). The first-order elimination rate constant was fixed at 0.004 h<sup>-1</sup>. The concentration of TCDD in the graft was about 85 pg.g<sup>-1</sup> fresh tissue at 10 weeks (corresponding to an elimination of 98.23%). The calculated half-life base based on the sequential point was 1.03 weeks on the first part of the biphasic curve (and 5.53 weeks for the second part of the curve).

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1123  
1124 335 We also simulated the accumulation of TCDD concentrations in the host AT and liver  
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1126 336 compartments following insertion of the graft. These compartments concentrate more than 90% of  
1127 337 the TCDD (Carrier, 1995). However, because the standard deviation (SD) of the initial concentration  
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1129 338 reflecting the amount of TCDD in the graft was relatively large, the simulation was performed using 1)  
1130 339 the mean of the concentrations in the graft, 2) this mean + SD or 3) this mean - SD. Thus, for each  
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1132 340 compartment, 2 curves were simulated (Figure 3). The prediction of the model was also accurate as  
1133 341 the experimental data points for the liver and the AT were found between each maximal and minimal  
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1135 342 simulating curve (Figure 3).  
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1137 343 Overall, these two simulations (release and distribution) improved the confidence in the PBPK  
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1139 344 model for mice.  
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### 1141 345 1142 1143 346 **5.5 TCDD release from AT alters the expression of metabolic genes** 1144

1145 347 We next investigated whether the TCDD released from graft-contaminated fat pad could alter  
1146 348 expression of genes coding for TCDD-target genes, *i.e.* key biologic or disease-related functions. We  
1147 349 and others have previously demonstrated that TCDD induced inflammation and fibrosis in the liver of  
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1149 350 exposed mice (Duval et al., 2017). Thus, we studied several biomarkers that could be related to chronic  
1150 351 liver diseases (*i.e.* fibrosis, steatosis). We carried out RT-qPCR experiments using host liver and AT  
1151  
1152 352 samples at 3 weeks after grafting.  
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1155 353 We show that the levels of expression of AhR-target genes in the liver and AT were higher than  
1156 354 at time 0, a result in line with a rapid contamination of these organs (Tables 1 and 2). In addition, the  
1157 355 expression of fibrotic and inflammatory markers augmented also significantly, in line with our former  
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1159 356 studies on TCDD treated mice (Duval et al., 2017). The host AT also displayed an inflammatory profile  
1160 357 (Table 2).  
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Function	Genes	Fold ind.
XME	AhR	2.65
	CYP1A1	8.96**
	CYP1B1	3.62**
Inflammation	IL-1 $\beta$	4.17**
	IL-6	1.25
	TNF alpha	1.94
	MCP-1	5.98**
	NOS2	2.42
	F4/80	3.42**
Fibrosis	COL1A1	3.93**
	Alpha-SMA	4.51**
Metabolism	Glycogen synthase 2	3.17**
	PC	3.01**
	Cytosolic PEPCK	2.94**
	G6Pase	2.39**
	PGC-1 alpha	2.24**

**Table 1: Relative mRNA level of XME, inflammatory, fibrotic and metabolic marker in the liver following a 3-week internal exposure (statistically significant: \*\* p<0.01, 6 animals per condition).**

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Function	Genes	Fold ind.
XME	AhR	0.88
	CYP1A1	2.55*
	PAI-1	15.92*
	IL-6	9.4**
	IL10	2.4
Inflammation	TNF alpha	2.01*
	MCP-1	1.83*
	NOS2	1.35*
	CD68	2.79**
	ATGL	1.67*
	HSL	0.66
	CPT1B	1.34
Metabolism	VLCAD	2.10*
	AQP7	1.23
	CD36	0.49
	Cytosolic PECK	1.24*
	Glycerol Kinase	0.06*
	PK4	3.82*
	PGC-1 alpha	1.18
	FABP4	3.93
	PPAR $\alpha$	0.85
	FAS	1.74
	UCP1	1.99*
PPAR $\gamma$ 2	1.42	

**Table 2: Relative mRNA expression of several XME, inflammatory, fibrotic and metabolic markers in the AT following a 3-week internal exposure (statistically significant: \* p<0.05; \*\* p<0.01, 6 animals per condition).**

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384                    Due to the role of pyruvate carboxylase (PC), cytosolic phosphoenolpyruvate carboxykinase  
385 (PEPCK) and glucose 6 Phosphatase (G6Pase) in maintaining glycemia (potentially linked to type 2  
386 diabetes), we investigated the expression of their genes in the liver and found that their mRNA content  
387 increased from time 0 to time 3-weeks following grafting.

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## 390 6. Discussion

391 In the present study we provide direct experimental evidence for the release of TCDD from  
392 adipose tissue (AT, graft) and its migration into other tissues. We also show that the released pollutant  
393 induces relevant changes in gene expression in the target tissues and we characterize the kinetics of  
394 its distribution. The study was designed to address a knowledge gap and a methodological gap. Indeed,  
395 one of the most challenging issues in environmental toxicity is to understand and model long-  
396 term/low-dose effects (Barouki, 2010). Concerning POPs, such long-term effects are usually explained  
397 by continuous exposure due to the persistence and bioaccumulation of those pollutants in the  
398 environment and by continuous release from internal storage sites such as the AT. It was not clear  
399 whether release from the AT was significant and whether it could induce biological or toxic effects in  
400 other tissues. Evidence for the latter mechanism has been gathered indirectly for example by studying  
401 weight loss in obese humans (Kim et al., 2011). The present study addresses specifically this point and  
402 provides direct evidence for the release of a POP from AT and describes its effects on other tissues.  
403 Thus, release of POPs from AT is likely to play a significant role in long-term effects of pollutants.

404 In order to study the release of POPs from the AT and its properties, we developed an adequate  
405 experimental model based on grafting of AT from a contaminated animal into a non-contaminated  
406 one. It explores the effects of a continuous release from an internal compartment, the AT and reflects  
407 exposures of humans and animals to POPs. Other models of continuous release do exist such as release  
408 capsules which have been designed mostly for pharmacological purposes (i.e. treatment of chronic  
409 pain) or micro-osmotic pumps (Mercadante, 2017; Tauer et al, 2013); however, both models differ  
410 from our model in the nature of the delivery compartment which is biologically relevant in our case  
411 and includes adipocytes, endothelial and immune cells which could influence the release. In that sense,  
412 the model described here is a better representation of the actual internal exposure than artificial  
413 models. However, it has its limitations too in that the graft could undergo different stresses and  
414 outcomes such a fibrosis or altered vascularization which may influence the observed results.

415 This AT graft model is clearly complementary to more traditional exposure models through  
416 ingestion or injection. It is not meant to be an alternative to other models but rather to identify the  
417 contributions of internal sources as compared to external exposures. In that sense it can help in  
418 assessing long-term effects even after withdrawal of external exposures. The model also allows the  
419 derivation of relevant toxicokinetic and toxicodynamic features that are complementary to those  
420 derived from classical drug treatment regimens.

421 Our experimental data improve the confidence in the PBPK model for mice that was developed  
422 previously (USEPA, 2012), and adapted for the present work. The intraperitoneal exposures to 1, 10

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423 and 25  $\mu\text{g.kg}^{-1}$  of body weight were also modeled (SFigure 4, Suppl. Mat.). The model accurately  
424 reproduces the experimental measurements, 48 hours following the single intraperitoneal  
425 administration for all doses, regarding the ratio of doses and the amount measured in tissues (Equation  
426 1). Yet, the model has its limitations since it requires surgery and some of the effects could be biased  
427 by the fate of the graft. Indeed, we observed individual differences in vascularization or fibrosis, which  
428 could impact TCDD release from the graft. These observations could partly explain why between 4 to  
429 10 weeks, the slope of TCDD elimination from the graft, changes (Figure 3); a better characterization  
430 of the vascularization of the graft will be necessary for our on-going projects. We believe that besides  
431 the structural variabilities described previously, metabolic adaptations and heterogenous lipid content  
432 could also influence TCDD release. A better characterization of the graft would improve the description  
433 of the PBPK model. However, at this point, it predicts with accuracy the evolution of TCDD  
434 concentrations in both AT and liver known as the most important depository organs of this pollutant  
435 in mammals. Indeed, our experimental data that match the PBPK model, indicate that despite some  
436 variability in the initial concentrations of TCDD in the graft (mean of  $4815 \pm 2122 \mu\text{g.kg}^{-1}$ ), the POP  
437 diffuses to other key organs such as the host AT, liver or brain following different kinetics. The release  
438 of TCDD from the graft is relatively rapid with a half-life of 1.03 weeks ( $\cong 173$  hours, from weeks 0-4)  
439 and 5.53 weeks (932 hours from week 4), suggesting that after 10 weeks, more than 97% has been  
440 released from the graft. The modeled kinetics of release and distribution is in line with the  
441 experimental data (Figure 3); this simulation represents an exciting predictive tool which also could be  
442 improved in the future using a wider range of POPs and representative mixtures.

443  
444 The release of TCDD influences important biological pathways that are involved in liver and AT  
445 dysfunction. For this part of the work, the focus was first on xenobiotic metabolizing enzymes  
446 (biomarkers of an activated AhR signaling pathway) and inflammatory cytokines/chemokines which  
447 are classically induced upon TCDD exposure. As expected (regarding the distribution of TCDD), the AhR-  
448 target genes (CYP1A1, CYP1B1, PAI-1) were significantly induced at the mRNA levels in the liver and AT  
449 3 weeks following grafting (Tables 1 and 2). Similarly, an inflammatory signature is observed in both  
450 tissues which is in line with the literature and our former studies using traditional modes of exposure  
451 (Duval *et al.* 2017; Kim *et al.* 2011, 2012). Furthermore, a highly significant increase of the expression  
452 of two biomarkers of fibrosis in the liver (alpha-Smooth Muscle Action or alpha-SMA and collagen 1A1  
453 or COL1A1IL) was obtained, confirming our recent observations on the occurrence of liver fibrosis  
454 following exposure to TCDD (Duval *et al.* 2017). Finally, the mRNA expression of the three key  
455 gluconeogenic enzymes, pyruvate carboxylase (PC), cytosolic phosphoenolpyruvate carboxykinase  
456 (PEPCK), glucose 6-phosphatase (G6Pase) showed significant inductions. Such enzymes contribute to  
457 the maintenance of glycemia during fasting, but their induction is also classically described in type 2

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457 diabetes (Beale *et al.*, 2007). These results are different from those of other teams who reported that  
458 expression of PEPCK is strongly repressed by TCDD; in 1993, Stahl *and coll.* demonstrated that TCDD  
459 exposure decreased hepatic gluconeogenesis due to a reduction of PEPCK activity and mRNA  
460 expression (Stahl *et al.*, 1993) which has been also observed in another model by Dunlap *and coll*  
461 (Dunlap *et al.*, 2002). This was also demonstrated for PC following a short treatment in hepatic rat  
462 livers (Ilian *et al.*, 1996). We hypothesize that the difference between those observations and our data  
463 is due to the mode of exposure; most previous experiments were performed using an acute-based  
464 exposure protocol while our model allows a constant delivery of TCDD for several weeks. While we are  
465 not able at this point to explain mechanistically the differences, we think that a sustained low-dose  
466 delivery of TCDD could have a different impact than an acute delivery of TCDD, on the regulation of  
467 gluconeogenesis enzymes.

468 This observation suggests that a continuous exposure of TCDD could disrupt carbohydrate and  
469 lipid metabolism and, more generally, liver functions. Thus, the TCDD released from an internal storage  
470 site can induce gene expression changes in at least two tissues that reflect biological activity of this  
471 pollutant and which is in line with what is known about its toxicity. While it is likely that some of the  
472 effects of TCDD on gene expression are related to the activation of the AhR pathway (such as the  
473 induction of CYP1 genes), other effects may be indirect. For example, TCDD and the AhR activate the  
474 expression of inflammatory cytokines through direct or indirect, genomic or non-genomic pathways  
475 (Kim *et al.* 2012), and those cytokines could also be responsible for the other genomic alterations  
476 observed in the liver and the AT; moreover, as the release of TCDD from the graft is relatively rapid  
477 and then high, these gene expressions could represent an adaptive response to a major environmental  
478 stress (a high dose of TCDD). In order to delineate the actual mechanisms involved in the effects of  
479 contaminated/xenografted-AT on gene expression in distant tissues after such periods of exposure (3  
480 weeks in our case), additional studies would be required. Thus, we can conclude that the release of  
481 POPs from internal storage sites could play a significant role in the toxicity of those pollutants that  
482 needs to be further investigated using a wider range of POPs and representative mixtures.

483  
484 **7. Conclusion**

485 The experimental model described here represents a novel approach to explore exposure to  
486 pollutants from endogenous sources and their long-term toxicities. It is meant to be complementary  
487 to other experimental protocols of exposure through diet, dermal, intraperitoneal injection or gavage  
488 procedures.

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