

## RESEARCH ARTICLE

# Perinatal exposure to a human relevant mixture of persistent organic pollutants: Effects on mammary gland development, ovarian folliculogenesis and liver in CD-1 mice

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## Abstract

The ability of persistent organic pollutants (POPs) with endocrine disrupting properties to interfere with the developing reproductive system is of increasing concern. POPs are transferred from dams to offspring and the high sensitivity of neonates to endocrine disturbances may be caused by underdeveloped systems of metabolism and excretion. The present study aimed to characterize the effect of *in utero* and lactational exposure to a human relevant mixture of POPs on the female mammary gland, ovarian folliculogenesis and liver function in CD-1 offspring mice. Dams were exposed to the mixture through the diet at Control, Low or High doses (representing 0x, 5000x and 100 000x human estimated daily intake levels, respectively) from weaning and throughout mating, gestation, and lactation. Perinatally exposed female offspring exhibited altered mammary gland development and a suppressed ovarian follicle maturation. Increased hepatic cytochrome P450 enzymatic activities indirectly indicated activation of nuclear receptors and potential generation of reactive products. Hepatocellular hypertrophy was observed from weaning until 30 weeks of age and could potentially lead to hepatotoxicity. Further studies should investigate the effects of human relevant mixtures of POPs on several hormones combined with female reproductive ability and liver function.

## Introduction

Persistent organic pollutants (POPs) are of significant concern due to their high resistance to degradation and potential toxicity [1]. This has led to the regulation of production, use and

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release of POPs by the Stockholm Convention [2]. A variety of POPs exhibit endocrine disrupting properties as they interfere with the synthesis, transport, metabolism, or elimination of endogenous hormones. The hormonal system is of vital importance in the fully functioning organism. Thus, there is a growing concern about endocrine disrupting chemicals (EDCs) and their interference with the reproductive, metabolic, neuroendocrine or cardiovascular systems [3, 4]. POPs are known to be transferred from mothers to offspring through the placenta and breast milk [5–8]. As early stages of development are particularly sensitive to disturbances by EDCs, the maternal transfer of POPs may have devastating consequences [9–11].

Mammary gland (MG) development is an intricate process finely regulated by hormones, growth factors and stromal factors, all of which can be disturbed by EDCs [12, 13]. One extensively studied example is the effect of perfluorooctanoic acid (PFOA) on MG development in rodents [14–19]. Other studies have also reported effects in the developing female MG or alterations in ovarian follicle maturation by various EDCs (extensively reviewed in [3] and [4]). Changes in ovarian folliculogenesis can lead to premature ovarian insufficiency and infertility [20, 21]. Furthermore, exposure to EDCs may increase the risk of developing breast, cervical, uterine or ovarian cancer [22].

The high sensitivity of fetuses and neonates to POP exposure, compared to adults, can be partly explained by immature metabolic and excretion systems [23, 24]. The cytochrome P450 (CYP) family of enzymes (included in phase I metabolism) catalyzes the transformation of xenobiotics to more polar derivatives that can be further metabolized or excreted [25–27]. However, transformation by CYP enzymes may also form free radicals (e.g. reactive oxygen species) or activate procarcinogens, causing potentially more deleterious effects than the original compound [28, 29]. The expression of CYP enzymes is regulated by different nuclear receptors such as the aryl hydrocarbon receptor (AHR), the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) [30, 31]. Various POPs have been shown to stimulate these receptors and, consequently, increase the enzymatic activities of CYPs in rodents [32–35]. Furthermore, upregulation of hepatic CYP transcripts at early life stages has been stated as early indicators of liver toxicity and carcinogenicity [36].

Previously, toxicological studies focused on the use of single compounds or technical mixtures of POPs. However, recent attention has shifted to study more realistic exposure scenarios with multiple compounds that may interact, and cause effects not anticipated by dose addition [37, 38]. For the present study, a complex mixture of POPs was designed based on information from Scandinavian food basket surveys [39]. The mixture included polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), brominated flame retardants (BFRs) and perfluoroalkylated substances (PFASs). The present study aimed to investigate how *in utero* and lactational exposure to the mixture of POPs affected the developing female reproductive organs (the MG and ovary) and liver function in CD-1 mice.

## Materials and methods

### Ethical considerations

The study was approved by the Institutional Animal Care and Use Committee at the Norwegian University of Life Sciences (NMBU) and the Norwegian Food Safety Authority (application ID: FOTS 7722). It was conducted in accordance with The Norwegian Regulation on Animal Experimentation at the Section for Experimental Biomedicine, NMBU-Faculty of Veterinary Medicine, in Oslo, Norway. The animals were health-monitored according to recommendations by the Federation of European Laboratory Animal Science Association (FELASA; <http://www.felasa.eu/>) and kept under Specific Pathogen Free (SPF) conditions.

## Feed design

The POP mixture was designed by Berntsen and colleagues [39]. In brief, the composition of PCBs, OCPs, BFRs and PFASs was chosen based on concentrations in Scandinavian food products stated in publications prior to 2012. Human estimated daily intake (hEDI) levels were estimated and adapted to a 25 g mouse consuming 3 g feed/d. The concentrations were upward adjusted to 5000x (Low dose) and 100 000x (High dose) hEDI. All polybrominated diphenyl ethers (PBDEs), PCBs and OCPs were purchased from Chiron AS (Trondheim, Norway). Hexabromocyclododecane (HBCD) and all PFASs, except for perfluorohexane sulfonic acid (PFHxS) purchased from Santa Cruz Biotechnology Inc. (Dallas, USA), were obtained from Sigma-Aldrich (St. Louis, USA). Compounds were dissolved in acetone, cyclohexane or chloroform and added to corn oil (Jasmin, fully refined, Yonca Gida San A.S., Manisa, Turkey). The solvent was evaporated under N<sub>2</sub>-flow and the mixture was incorporated into AIN-93G mouse feed (TestDiets, St. Louis, USA). The control diet contained corn oil from which the solvent had been evaporated. An additional AIN-93G reference diet was also made using corn oil instead of soybean oil. Mixture composition and concentrations of individual compounds are presented in Table 1.

## Animals, experimental design, and sample collection

Timed-pregnant CD-1 mice (F0) were obtained from Charles River Laboratories (Wilmington, USA) and gave birth 4–5 d after arrival to the F1 generation. At 3 weeks of age, F1 females were randomly assigned to a dose: Control (n = 28), Low (n = 27), or High (n = 20). Exposure continued throughout mating (at 10 weeks of age with CD-1 males bred in-house), gestation and lactation. Offspring (F2) produced by the F1 dams were given the AIN-93G reference diet from 3 weeks of age and only exposed to the mixture of POPs *in utero*, through lactation and by nibbling on their mothers' feed prior to weaning. The study design is illustrated in S1 Fig.

All mice were housed in closed Type III individually ventilated cages (IVC) (Allentown Inc, USA) except during mating when animals were moved to open Makrolon Type III cages (Techniplast, Buguggiate, Italy). All cages contained standard aspen bedding, red polycarbonate houses and cellulose nesting material (Scanbur BK, Karlslunde, Denmark). Water and feed were available *ad libitum*. Cages, bedding, nesting material and water bottles were changed weekly. The animal room was on a 12:12 light-dark cycle, with a room temperature of 21 ± 2 °C and 45 ± 5% relative humidity.

Dams (F1) were euthanized at gestation d 17 (pregnant; n = 12, 16 and 8 for the Control, Low and High doses, respectively) or at 21 d post-partum (post-pregnant; n = 14, 10 and 11 for the Control, Low and High doses, respectively) with a total exposure time of 9 or 13 weeks, respectively. Mating was unsuccessful for 3 dams (2 in Control and 1 in High). One dam (Low dose) died prior to mating.

Female offspring (F2) were sacrificed at 3 (weaning), 6 (pubertal) and 9 (adult) weeks of age (n = 12, 14 and 14 for all doses, respectively). Male offspring (F2) were sacrificed at 9 or 30 weeks of age (n = 15 for all doses) to further investigate the hepatic alternations observed in females. Due to a low number of males produced by dams exposed to the High dose, all males in this dose were sacrificed at 9 weeks of age. Thus, 30-week-old male offspring were only sampled from the Control and Low doses.

Mice were sacrificed under anesthesia (isoflurane gas obtained from Baxter, San Juan, Puerto Rico) by cardiac puncture and cervical dislocation. The 4<sup>th</sup> MG (left), the ovaries and a section of the liver were fixed in 10% neutral buffered formalin for histopathological examinations. The remaining liver was frozen on dry ice and stored at -80 °C for analysis of chemicals

Table 1. POP concentrations in feed.

| Compounds                                    | Nominal concentrations Low | Measured concentrations Low | Nominal concentrations High | Measured concentrations High |
|--|----------------------------|-----------------------------|-----------------------------|------------------------------|
| <b>Polychlorinated biphenyls (PCBs)</b>      |                            |                             |                             |                              |
| PCB-28                                       | 5.8                        | 3.1                         | 117                         | 46                           |
| PCB-52                                       | 13.8                       | 15.0                        | 275                         | 182                          |
| PCB-101                                      | 23.3                       | 25.4                        | 467                         | 377                          |
| PCB-118                                      | 40.4                       | 37.2                        | 808                         | 612                          |
| PCB-138                                      | 57.5                       | 53.8                        | 1150                        | 957                          |
| PCB-153                                      | 57.5                       | 61.4                        | 1150                        | 981                          |
| PCB-180                                      | 15.4                       | 17.4                        | 308                         | 263                          |
| $\Sigma$ 7PCBs                               | 213.7                      | 213.3                       | 4275                        | 3418                         |
| <b>Organochlorine pesticides (OCPs)</b>      |                            |                             |                             |                              |
| HCB  | 50.0                       | 37.4                        | 1000                        | 588                          |
| $\alpha$ -chlordane                          | 37.5                       | 45.0                        | 750                         | 723                          |
| Oxychlordane                                 | 12.5                       | 9.8                         | 250                         | 297                          |
| <i>Trans</i> -nonachlor                      | 12.5                       | 14.9                        | 250                         | 264                          |
| $\alpha$ -HCH                                | 21.7                       | 21.2                        | 433                         | 421                          |
| $\beta$ -HCH                                 | 17.5                       | 22.3                        | 350                         | 398                          |
| $\gamma$ -HCH (Lindane)                      | 23.8                       | 31.4                        | 475                         | 435                          |
| $\Sigma$ 7OCPs                               | 175.5                      | 182.0                       | 3508                        | 3126                         |
| <i>p,p'</i> -DDE                             | 119.6                      | 136.0                       | 2392                        | 2390                         |
| Dieldrin                                     | 75.0                       | 70.4                        | 1500                        | 1470                         |
| <b>Brominated flame retardants (BFRs)</b>    |                            |                             |                             |                              |
| BDE-47                                       | 40.4                       | 39.7                        | 808                         | 642                          |
| BDE-99                                       | 7.9                        | 8.6                         | 158                         | 126                          |
| BDE-100                                      | 6.3                        | 5.6                         | 125                         | 91                           |
| BDE-153                                      | 1.3                        | 1.5                         | 25                          | 22                           |
| BDE-154                                      | 2.5                        | 2.8                         | 50                          | 38                           |
| $\Sigma$ BDE-47-154                          | 58.4                       | 58.2                        | 1166                        | 919                          |
| BDE-209                                      | 62.5                       | 64.8                        | 1250                        | 1141                         |
| HBCD   | 12.5                       | 9.9                         | 250                         | 203                          |
| <b>Perfluoroalkylated substances (PFASs)</b> |                            |                             |                             |                              |
| PFHxS  | 4.9                        | 1.7                         | 98                          | 42                           |
| PFOS   | 10.8                       | 3.2                         | 217                         | 74                           |
| PFOA   | 18.3                       | 6.0                         | 367                         | 121                          |
| PFNA   | 5.8                        | 2.1                         | 117                         | 42                           |
| PFDA   | 7.9                        | 3.1                         | 158                         | 57                           |
| PFUnDA                                       | 4.0                        | 1.6                         | 80                          | 28                           |
| $\Sigma$ PFASs                               | 51.7                       | 17.7                        | 1037                        | 364                          |

Nominal and measured concentrations (ng/g feed) of the PCBs, OCPs, BFRs and PFASs in AIN-93G mouse feed at the Low and High doses (5000x and 100 000x human estimated daily intake, respectively). Adapted from Table 1 in [39].

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and cytochrome P450 activity. The 4<sup>th</sup> MG (right) was peeled away from the inner skin surface and spread out on a microscope slide for whole mount analysis.

### Analysis of mammary glands

**Whole mount preparation, development scoring and Sholl analysis.** Whole mounts were used to assess alterations in MG development of female offspring (F2). The 4<sup>th</sup> MG from the right side was placed with the skin side down on a charged microscope slide and fixed in

Carnoy's solution (6:3:1 of ethanol:chloroform:acetic acid) at 4°C overnight. Whole mounts were rinsed in 70% ethanol, gradually transitioned to water and stained overnight in carmine alum stain (2 g/L carmine and 5 g/L aluminum potassium sulfate). After staining, the whole mounts were dehydrated gradually to 100% ethanol and de-fatted in xylene overnight until visibly clear. Multiple pictures were taken of each whole mount at 10x magnification using a Zeiss Axio Imager light microscope and camera (M2, Oberkochen, Germany), and a mosaic [40] was created using ImageJ [41].

Qualitative development scoring was conducted on a scale from 1 to 4 (1 = poor development, 4 = best development). The following criteria were evaluated: lateral and longitudinal epithelial growth into the surrounding fat pad; branching degree; alveolar budding; lobule formation; and the presence or absence of terminal end buds (TEBs). Scores were assigned by two assessors and repeated 3x for each whole mount to give a median score from each assessor. The final score was obtained by averaging the two median scores. The assessors were initially not blinded to Control as to establish a baseline of MG development. Subsequently, the glands were evaluated blinded to dose within each age class. TEBs were defined as ends with a diameter of  $\geq 100 \mu\text{m}$  and counted using ImageJ.

MG branching density was quantitatively assessed using ImageJ and the modified Sholl analysis [42]. In brief, the color channels were separated, and noise were removed from the images using various methods supplied by ImageJ. The images were skeletonized, made binary and dilated 1x to fill in gaps created by skeletonization. Longitudinal distance of the gland (termed mammary epithelial length) was measured between the base of the epithelial tree and the tip of the most distal branch on the skeletonized images. The mammary epithelial area (MEA) and the lymph node area (LNA) were defined by the periphery of the total skeletonized gland and the area covered by the lymph node, respectively. The total number of intersections (N) within the MEA was determined by Sholl analysis. Branching density was calculated by  $N/(\text{MEA}-\text{LNA})$ . A detailed description of the method is given by Stanko and Fenton [43].

Two whole mount images (6 weeks Control) were ruined during processing. In addition, three glands were partly torn during whole mount preparation (1 from 3 weeks High, 1 from 9 weeks High and 1 from 9 weeks Control). Thus, a total of 5 whole mounts were excluded from the Sholl analysis.

**Image analysis of glandular tissue.** The 4<sup>th</sup> MG from the left side of female offspring (F2) were fixated in formalin for at least 24 h. Glands were transferred to 70% ethanol prior to paraffin embedding and sectioned horizontally using a microtome (5  $\mu\text{m}$ ). Gland orientation for sectioning was ensured identical to whole mount orientation. Sections were attached onto glass slides and stained with haematoxylin and eosin (HE). Sections were examined blindly by a board-certified pathologist.

Digital photomicrographs of the glands were taken at 100x magnification using Zeiss Axio Imager light microscope, Axiocam 506 color camera and Zen microscope software (Carl Zeiss Microscopy GmbH, Germany). Photomicrographs were stitched and reconstructed digital images of the whole glands were created. Image-Pro Plus (Media Cybernetics Inc., MD, USA) was used to measure area fractions occupied by glandular tissue.

## Analysis of ovaries

Both ovaries from female offspring (F2) were fixated in 10% neutral buffered formalin (for 24 h), transferred to 70% ethanol and embedded in paraffin. Sections (8  $\mu\text{m}$ ) were made using a microtome, every 10<sup>th</sup> section was mounted onto glass slides and stained with HE. Histological examination was conducted using a light microscope on every 2<sup>nd</sup> glass slide throughout one randomly chosen ovary. Between 9 and 12 females were randomly selected for evaluation from

each dose and sampling time. One observer, blinded to treatment, counted the numbers of healthy follicles, including primordial follicles, primary follicles, preantral follicles and antral follicles, as categorized by Flaws and colleagues [44]. Primordial follicles were defined as having a single layer of squamous granulosa cells surrounding an oocyte, while primary follicles consisted of an oocyte surrounded by one layer of cuboidal granulosa cells. Preantral follicles were defined as being surrounded by at least two layers of cuboidal granulosa cells and theca cells, and antral follicles consisted of a fluid-filled antral space adjacent to the oocyte and surrounded by many layers of theca cells and cuboidal granulosa cells. The preantral and antral follicles were only registered if the oocyte showed nuclear material. Primordial and primary follicles were counted despite showing a nucleus.

## Analysis of liver

**Chemical analysis.** The analysis of PCBs, OCPs, BFRs and PFASs was conducted on liver of dams (F1) and female offspring (F2) at the Laboratory of Environmental Toxicology, NMBU, Oslo, Norway. Samples of the left lobe were pooled according to their respective dose (Control, Low and High) and sampling time (pregnant, post-pregnant, and 3, 6 and 9 weeks of age). The method for PCB, OCP and BFR quantification is based on Brevik [45] and Polder and colleagues [46], and is accredited (except for BDE-206, -207, and -208) by the Norwegian Accreditation for chemical analysis in biota (requirements of the NS-EN ISO/IEC 17025, TEST 137). Quantification of PFASs is not accredited, but is validated according to the same procedures and quality control measures, and is described by Grønnestad and colleagues [47]. Details on method modification for the present study are elaborated in [S1 File](#). Method quality control measures were approved as they were within accreditation requirements (see [S1 File](#)). Compounds detected at concentrations outside the accepted range of recovery (70–130%) were corrected for recovery (herein *p,p'*-dichlorodiphenyldichloroethylene (DDE), BDE-206 and HBCD). Concentrations below limit of detection (LOD) were replaced with the LOD to be included in the  $\Sigma$ PCBs,  $\Sigma$ OCPs,  $\Sigma$ PBDEs and  $\Sigma$ PFASs.

**Histopathological examination.** Cross sections of the left lobe from dams (F1), and female and male offspring (F2) were embedded in paraffin after fixation. Sections were made (3  $\mu$ m) using a microtome, transferred to microscope glass slides, and stained with HE. Examination was conducted by a board-certified pathologist under light microscope blinded by dose. Classification was based on the liver nomenclature guidelines recommended by the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) [48]. Pre-neoplastic and neoplastic lesions were noted as present (1) or absent (0). Non-neoplastic lesions were graded on a severity scale from 0 to 4 (0 = no, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe change) and included bile duct hyperplasia, fatty change (diffuse), extramedullary hematopoiesis, inflammation (chronic active), hepatocyte fatty change (diffuse), hepatocyte centrilobular hypertrophy, Ito cell hypertrophy, and oval cell hyperplasia.

**Liver microsomal preparation and cytochrome P450 activity analysis.** Liver samples (right, caudate, and quadrate lobes) from female offspring (F2) were shipped (on dry ice) to the Department of Molecular Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden, for cytochrome P450 (CYP) activity analysis. Approximately 1 g tissue were used to prepare hepatic microsomes by a calcium aggregation method previously described [49]. Protein concentrations in the microsomes were measured with a commercially available kit (Bio-Rad Laboratories Inc., Hercules, USA) according to the manufacturer's instructions and using bovine serum albumin as standard. Microsomes were diluted to a protein concentration of 4 mg/mL and stored at -80°C until use.

The activities of CYP1A and CYP2B10 were determined as a rate of 7-ethoxyresorufin (EROD, CYP1A1), 7-methoxyresorufin (MROD, CYP1A) and 7-pentoxyresorufin (PROD,

CYP2B10) O-dealkylation [50]. CYP3A11 activity were established as a rate of and benzyloxyresorufin (BROD) O-dealkylation [51]. CYP2E1 and CYP2A5 activities were determined as a rate of p-nitrophenol (PNPH) and coumarin-7- hydroxylation (CoH), respectively [52]. These isoforms were selected because of their well-known role in xenobiotic metabolism. Experimental conditions for activity assays are reported in [S1 File](#). Briefly, incubation mixtures were comprised of microsomal protein (0.2 mg for all enzymes except for PNPH with 0.5 mg), phosphate buffer (50 mM, pH 7.4) and the appropriate substrate (1  $\mu$ M for EROD, 2  $\mu$ M for MROD and BROD, 10  $\mu$ M for PROD, and 200  $\mu$ M for PNPH and CoH). Reactions were initiated by addition of 0.5 mM NADPH. The solutions were incubated in a water bath at 37°C for 5 (EROD), 7 (MROD and BROD), 15 (CoH), 20 (PROD), or 30 (PNPH) min. Reactions were terminated by adding 0.5 mL ice-cold methanol (40% TCA for PNPH) and the mixtures were centrifuged (7500 g at 4°C for 5 min). The amount of formed resorufin in the supernatants (for CYP1A, CYP2B10 and CYP3A11) was measured using HPLC with fluorescence detector (560 and 586 nm for excitation and emission wavelengths, respectively). The amount of formed p-nitrochatecol (for CYP2E1) was measured using HPLC with UV detector (345 nm). The amount of formed hydroxycoumarin (for CYP2A5) was measured using HPLC with fluorescence detector (338 and 458 nm for excitation and emission wavelengths, respectively). Formation of all metabolites were linear with microsomal protein concentrations and incubation times. All enzymatic activities were expressed as pmol of reaction product per mg protein and min.

### Statistical analysis

Box and bar plots were created using R Studio version 3.6.1 [53] and the packages ‘ggplot2’ [54] and ‘ggpubr’ [55]. Statistical analyses were conducted in JMP Pro 13® (SAS, Cary, USA) and a p-value  $\leq 0.05$  was considered statistically significant.

The measurements of weights, the number of TEBs (3 and 6 weeks only) and the branching density were normally distributed and with satisfactory variance homogeneity. Thus, these variables were analyzed using multivariate linear regression by standard least square personality, generating least square mean values that were further analyzed using ANOVA. Body, liver and ovaries + uterus weights were analyzed within each sampling time and with dose and weight at weaning (dam body weight), number of offspring per litter (offspring body weight) or body weight (liver and ovaries + uterus weight) as explanatory variables. The number of TEBs (3 and 6 weeks only) and branching density were analyzed within each sampling time with dose and body weight as explanatory variables. P-values were generated by performing Dunnett’s test on least square mean values.

Contingency table analysis using Chi-square likelihood ratio was conducted on the ratio between males and females, and the severity scores of hepatic histopathology within each sampling time and using the Steel-Dwass method to compare doses. The number of live offspring and ovarian follicles were analyzed for differences between doses within each sampling time by ANOVA followed by Dunnett’s or Tukey HSD test, respectively. The MG development scoring, the number of TEBs (9 weeks), N, MEA, mammary epithelial length and glandular area, as well as the hepatic enzyme activities, were not normally distributed and analyzed for differences between doses within each sampling time by non-parametric comparison using the Steel or Steel-Dwass methods.

## Results

### Hepatic internal dosimetry

The concentrations of PCBs, OCPs, BFRs and PFASs were measured in one pooled sample of liver tissue from each dose in dams (F1) and female offspring (F2). Results are presented in

Table 2. POP concentrations in pooled liver.

|                           | $\Sigma$ 6PCBs <sup>a</sup> | $\Sigma$ 7OCPs <sup>b</sup> | <i>p,p'</i> -DDE | $\Sigma$ BDE-28-183 <sup>c</sup> | $\Sigma$ BDE-206-209 | HBCD | $\Sigma$ PFASs <sup>d</sup> |
|---------------------------|-----------------------------|-----------------------------|------------------|----------------------------------|----------------------|------|-----------------------------|
| <b>Pregnant dams</b>      |                             |                             |                  |                                  |                      |      |                             |
| Control                   | 87.1                        | 9.4                         | <LOD             | 0.7                              | 0.4                  | <LOD | 2.8                         |
| Low                       | 45691.6                     | 2185.9                      | 907.0            | 373.2                            | 2260.7               | <LOD | 645.3                       |
| High                      | 87012.6                     | 32585.7                     | 13883.9          | 6923.4                           | 35303.5              | <LOD | 16183.3                     |
| <b>Post-pregnant dams</b> |                             |                             |                  |                                  |                      |      |                             |
| Control                   | 21.4                        | 4.2                         | <LOD             | 0.7                              | 7.9                  | <LOD | 6.5                         |
| Low                       | 7787.9                      | 802.6                       | 922.2            | 345.5                            | 4534.1               | <LOD | 1248.5                      |
| High                      | 52743.1                     | 15343.4                     | 14189.0          | 8197.3                           | 35411.8              | 40.2 | 22744.6                     |
| <b>3 weeks offspring</b>  |                             |                             |                  |                                  |                      |      |                             |
| Control                   | 48.8                        | 12.3                        | <LOD             | 0.7                              | 10.2                 | <LOD | 32.2                        |
| Low                       | 19629.9                     | 1779.2                      | 1200.9           | 515.5                            | 883.1                | <LOD | 403.7                       |
| High                      | 89968.8                     | 38043.8                     | 24412.2          | 11397.7                          | 16274.1              | <LOD | 8535.0                      |
| <b>6 weeks offspring</b>  |                             |                             |                  |                                  |                      |      |                             |
| Control                   | 65.3                        | 35.0                        | <LOD             | 0.7                              | 0.4                  | <LOD | 5.5                         |
| Low                       | 10144.7                     | 502.5                       | 153.6            | 79.2                             | 30.6                 | <LOD | 170.9                       |
| High                      | 50364.8                     | 11392.5                     | 1287.5           | 1102.7                           | 293.9                | <LOD | 3490.1                      |
| <b>9 weeks offspring</b>  |                             |                             |                  |                                  |                      |      |                             |
| Control                   | 81.0                        | 38.1                        | <LOD             | 0.7                              | 4.1                  | <LOD | 6.3                         |
| Low                       | 5318.4                      | 357.0                       | 10.2             | 38.5                             | 22.7                 | 27.6 | 142.3                       |
| High                      | 30278.5                     | 5068.8                      | 283.7            | 397.5                            | 165.6                | <LOD | 2521.8                      |

Concentrations of  $\Sigma$ 6PCBs,  $\Sigma$ 7OCPs, *p,p'*-DDE, PBDEs, HBCD and  $\Sigma$ PFASs in pooled liver samples from pregnant (gestation d 17) and post-pregnant (21 d post-partum) dams and female offspring (3, 6 and 9 weeks of age). Dams were dietary exposed to the mixture of POPs at Control, Low or High doses (0x, 5000x or 100 000x human estimated daily intake, respectively). Female offspring were exposed *in utero* and through lactation (ending at 3 wk). Values are presented as ng/g lipid weight for PCBs, OCPs, *p,p'*-DDE, PBDEs and HBCD, and ng/g wet weight for PFASs. Limit of detection (LOD), concentrations of individual chemicals and lipid (%) are presented in S1 File, and S1 and S2 Tables.

<sup>a</sup> $\Sigma$ 6PCBs: PCB-52, PCB-101, PCB118, PCB-138, PCB-153 and PCB-180.

<sup>b</sup> $\Sigma$ 7OCPs: HCB,  $\alpha$ -chlordane, oxychlordane, *trans*-nonachlor,  $\alpha$ -HCH,  $\beta$ -HCH and  $\gamma$ -HCH.

<sup>c</sup> $\Sigma$ BDE-28-183: BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154 and BDE-183.

<sup>d</sup> $\Sigma$ PFASs: PFHxS, PFOS, PFOA, PFNA, PFDA and PFUnDA.

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Table 2 as  $\Sigma$ 6PCBs,  $\Sigma$ 7OCPs, *p,p'*-DDE,  $\Sigma$ BDE-28-183,  $\Sigma$ BDE-206-209, HBCD and  $\Sigma$ PFASs. Individual concentrations are presented in S1 (ng/g lipid) and S2 Tables (ng/g wet weight). LOD, recovery (%) and fat (%) are presented in S1 File and S2 Table.

Multiple POPs were detected in dams and offspring exposed to the Control dose. However, all compounds had lower concentrations compared to Low (6-5652x). Furthermore, both dams and offspring had higher concentrations of all compounds in High compared Low (2-25x). PCB-52 was not detected in any samples. BDE-183 and HBCD were only detected in one (post-pregnant dam, High dose) and two (post-pregnant dam, High dose, and 9 weeks offspring, Low dose) samples, respectively. Debromination of BDE-209 was seen in both dams and offspring.

$\Sigma$ 6PCBs and  $\Sigma$ 7OCPs were slightly higher (1.6-6x) in pregnant dams compared to post-pregnant dams. On the other hand,  $\Sigma$ BDE-28-183,  $\Sigma$ BDE-206-209 and  $\Sigma$ PFASs were similar or 2x lower in pregnant dams compared to post-pregnant dams. All compounds (except PCB-52, BDE-183 and HBCD) were detected in 3-week-old offspring from the Low and High doses.  $\Sigma$ 6PCBs,  $\Sigma$ 7OCPs and  $\Sigma$ BDE-28-183 were 1.3–2.5x higher, while  $\Sigma$ BDE-206-209 and  $\Sigma$ PFASs were 2.2–5.1x lower, in 3-week-old offspring compared to post-pregnant dams. After end of

perinatal exposure, all compounds (except BDE-153 and HBCD) had decreasing concentrations with increasing age of offspring.

### Biometrical measurements

The body and liver weights of dams (F1) and offspring (F2) are presented in [S3 Table](#). The POPs did not affect the body weight of dams (F1), female offspring (F2) or 30-week-old male offspring (F2). On the other hand, male offspring perinatally exposed to the Low dose had significantly higher body weights compared to controls at 9 weeks ( $p = 0.02$ ).

The High dose caused a significant increase in liver weights of pregnant and post-pregnant dams ( $p < 0.01$  and  $p = 0.05$ , respectively). In addition, the High dose increased the liver weight of 6-week-old female offspring ( $p = 0.02$ ). No changes in liver weights were seen in 3, 9 and 30-week old offspring.

The number of live offspring and the ratio between male and female offspring were not significantly different from Control ([S4 Table](#)). However, the Low dose led to the production of more males while the High dose produced more females ( $p = 0.02$ ). The collective uterus and ovary weights from 6 and 9-week-old female offspring were not significantly different from Control ([S3 Table](#)).

### Mammary gland histology

The effect of perinatal exposure to the mixture of POPs on MG development was investigated in female offspring (F2). Complete results are presented in [S5 Table](#).

The development score was not affected by exposure. However, a trend towards less developed mammary glands was evident in the High dose at 3 weeks ( $p = 0.08$ ). This trend was supported by a significantly decreased number of TEBs (High dose), compared to Control, at 3 weeks ( $p = 0.04$ ). In addition, less TEBs were seen at 9 weeks in the Low dose, but this decrease was not significant from Control ( $p = 0.07$ ). The number of TEBs was not affected by exposure at 6 weeks.

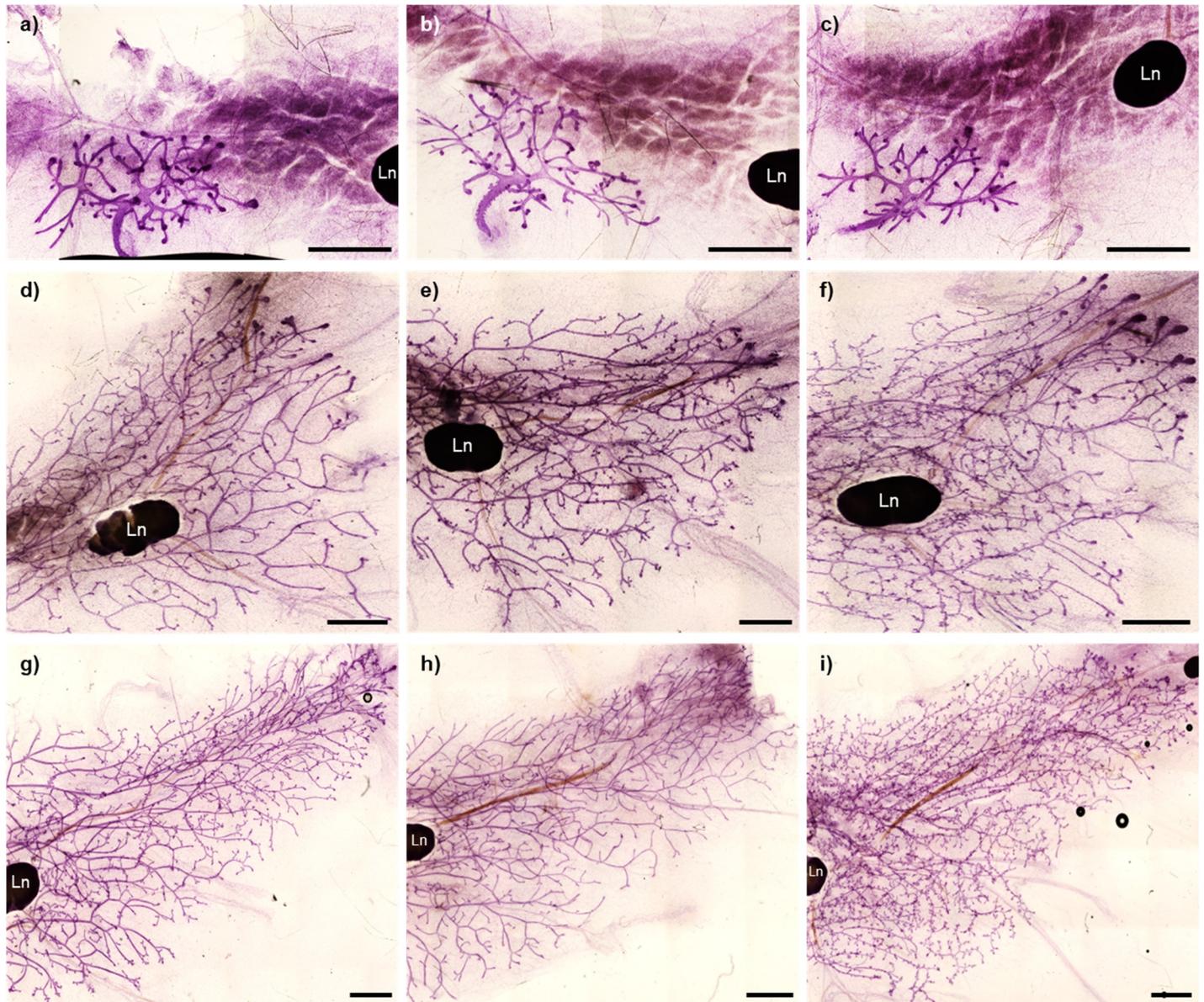
A visual difference in MG morphology between exposed and control mice was apparent, especially, for the High dose. At 6 weeks, the High dose had a significantly higher branching density compared to Control. An increased branching density was also apparent at 9 weeks, but not significantly different from Control ( $p = 0.1$ ). The total number of epithelial intersections was not affected by perinatal POP exposure. On the other hand, the mammary epithelial area was reduced by the Low dose, compared to Control, at 6 and 9 weeks ( $p = 0.05$  and  $p = 0.01$ , respectively). The epithelial length was also reduced by the Low dose at 9 weeks ( $p = 0.02$ ). MG whole mounts representative of each dose and age are shown in [Fig 1](#).

Histological examination of MGs did not reveal any significant effects of perinatal POP exposure. However, a trend towards more glandular tissue occupying the fat pad was seen by the Low dose at 6 weeks ( $p = 0.08$ ).

### Ovarian histology

Alterations in ovarian follicles at different stages in folliculogenesis were investigated in female offspring (F2) after perinatal exposure to the mixture of POPs. Results are presented in [Fig 2](#).

The number of primordial follicles was not affected by exposure. The Low dose significantly reduced the number of primary follicles at 3 weeks, compared to Control ( $p < 0.01$ ), but not at 6 and 9 weeks. The number of preantral follicles did not differ in exposed mice compared to controls. However, the Low dose caused the formation of significantly less preantral follicles than the High dose at 6 ( $p = 0.01$ ) and 9 weeks ( $p < 0.01$ ), with the Control showing an



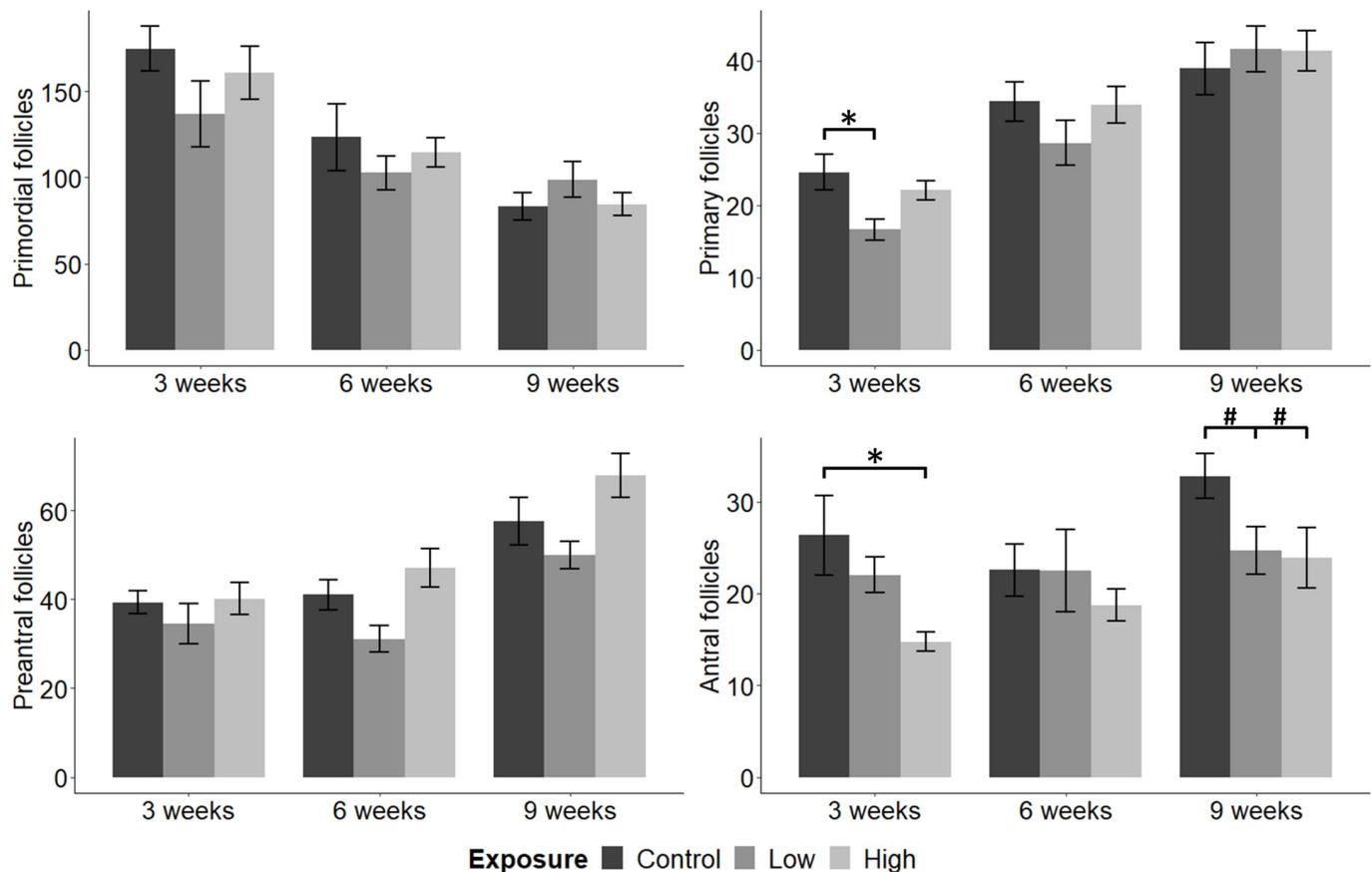
**Fig 1. Mammary gland whole mounts.** Whole mounts from 3 (a, b and c), 6 (d, e and f) and 9-week-old (g, h and i) female offspring maternally exposed to a mixture of POPs at Control, Low or High doses (0x, 5000x or 100 000x human estimated daily intake, respectively). Pictures illustrate the morphology representative of each dose; Control (a, d, and g), Low (b, e, and h) or High (c, f, and i). Each whole mount is composed of multiple images taken at 10-fold magnification and put together as a mosaic using ImageJ software. Scale bar = 2000  $\mu$ m. Ln = Lymph node.

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intermediate follicle number. The High dose reduced the number of antral follicles at 3 weeks ( $p < 0.01$ ), but not at 6 weeks, compared to Control. At 9 weeks, the number of antral follicles were lower than but not significantly different from Control (with  $p = 0.06$  and  $p = 0.07$  for High and Low, respectively).

### Hepatic histology

Histopathological examination was conducted on livers from dams (F1) and offspring (F2) dietary or perinatally exposed to the mixture of POPs. No pre-neoplastic or neoplastic lesions



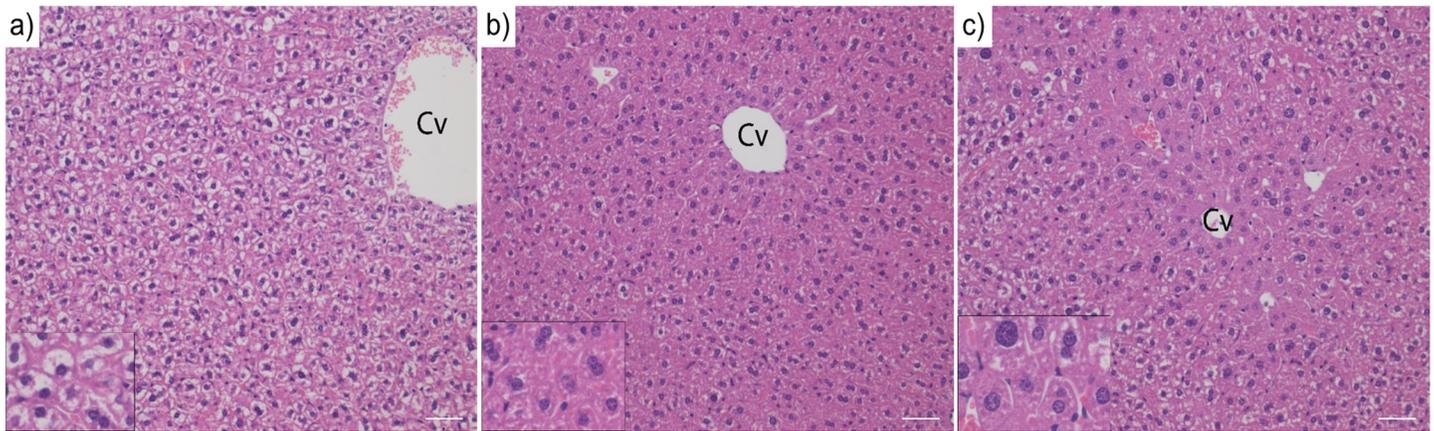
**Fig 2. Ovarian follicle numbers.** The number of primordial, primary, preantral and antral follicles in ovaries of 3, 6 and 9-week-old female offspring maternally exposed to a mixture of POPs at Control, Low or High doses (0x, 5000x or 100 000x human estimated daily intake, respectively). Histopathological examination was conducted on every 10th ovarian section. Results are presented as mean  $\pm$  standard error. 3 weeks: n = 9, 11 and 12 for Control, Low and High, respectively. 6 weeks: n = 11, 10, and 11 for Control, Low and High, respectively. 9 weeks: n = 10, 11 and 9 for Control, Low and High, respectively. \*p-values  $\leq$  0.05. #p-values  $\leq$  0.1.

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were noted. Focal fatty lesions were seen in 5 9-week-old female offspring perinatally exposed to the High dose but did not differ significantly from Control.

Dietary exposure to the Low and High doses increased the severity of hepatocellular hypertrophy (of the centrilobular region) in pregnant and post-pregnant dams compared to Control ( $p < 0.01$ ). Perinatal exposure to the High dose caused hypertrophy (with increased severity) in female offspring at 3 weeks ( $p = 0.02$ ). At 6 and 9 weeks, both the Low and High doses caused hepatocellular hypertrophy with increased severity from Control ( $p < 0.01$ ). Hepatocellular hypertrophy was also seen in 9-week-old male offspring with a higher severity than Control in both the Low and High doses ( $p < 0.01$ ). This hypertrophy persisted until 30 weeks in male offspring, which had a significantly higher severity compared to Control (Low dose only,  $p < 0.01$ ). Fig 3 illustrates the hepatocellular hypertrophy representative of each dose in female offspring at 9 weeks.

Extramedullary hematopoiesis was seen in female offspring at 3, 6 and 9 weeks with decreasing incidence and severity with increasing age and was not affected by perinatal exposure. Mean severity scores of the hepatocellular hypertrophy and hematopoiesis ( $\pm$  standard error) in dams and offspring are presented in S6 Table. No other non-neoplastic lesions were seen.



**Fig 3. Hepatic morphology.** Representative hepatic morphology of 9-week-old female offspring maternally exposed to a mixture of POPs at Control (a), Low (b) or High (c) doses (0x, 5000x or 100 000x human estimated daily intake, respectively). Hepatocytes have increased size and frequently enlarged hyperchromatic nuclei, which were graded on a scale from 0 to 4 (0 = no, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe change). The Low (b) and High (c) doses exhibited mild and severe hypertrophy, respectively, compared to the normal morphology of the Control (a). Magnification at 200-fold (inset at 400-fold). Scale bar = 50 µm. Cv = Central vein.

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### Hepatic cytochrome P450 activity

The activities of hepatic CYP enzymes were measured in female offspring (F2) perinatally exposed to the mixture of POPs (Fig 4).

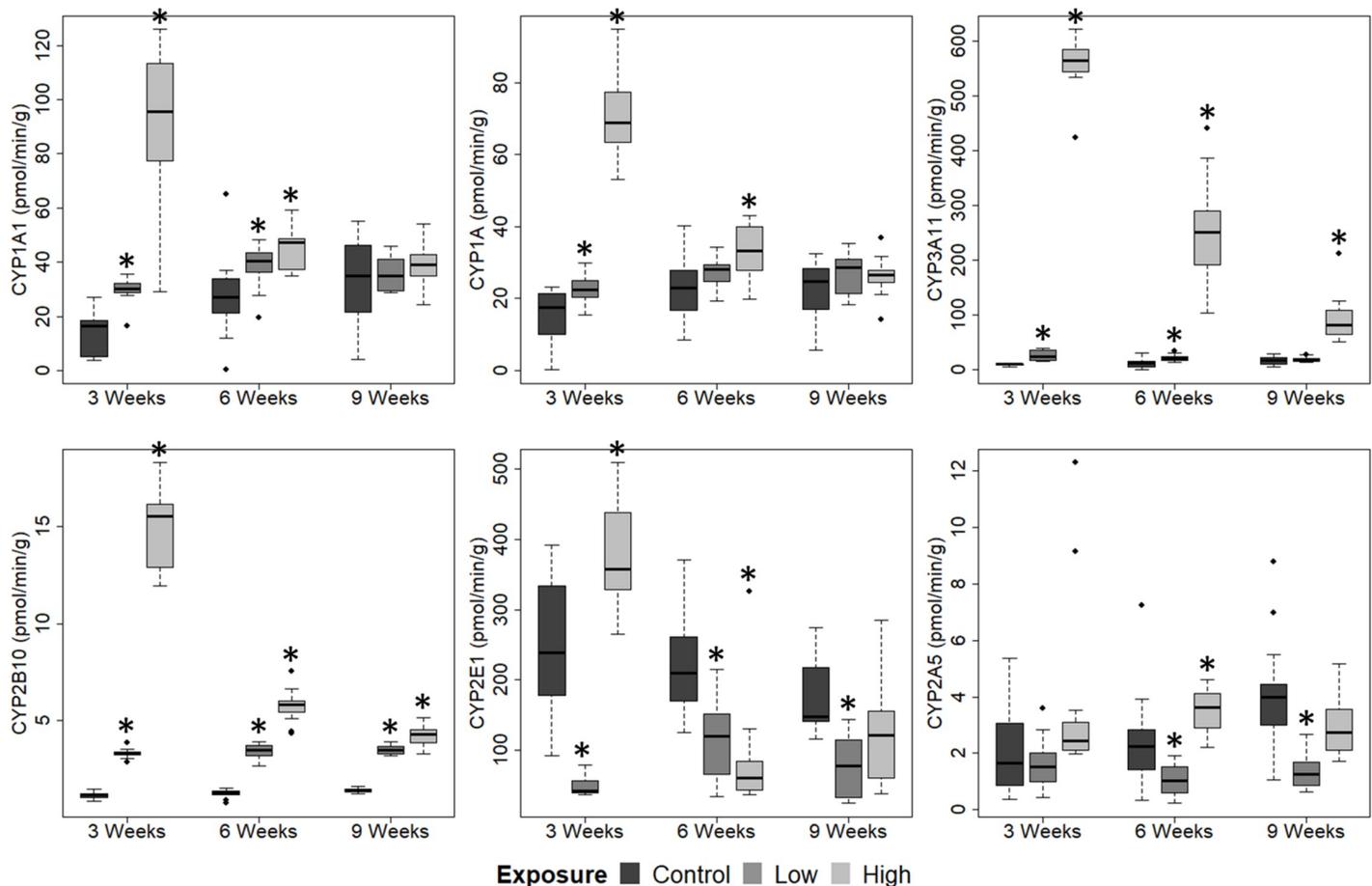
Perinatal exposure significantly induced the activity of all CYPs (except CYP2A5) at 3 weeks ( $p < 0.01$  for all, except for CYP1A for the Low dose with  $p = 0.05$ ), with the High dose inducing the largest change from Control for CYP1A1, CYP1A, CYP3A11 and CYP2B10. For CYP2E1, the activity was decreased by the Low dose, and increased by the High dose, compared to Control ( $p < 0.01$ ).

At 6 weeks, the activities of all CYPs were affected by the High dose ( $p \leq 0.01$ , except for CYP2A5 with  $p = 0.02$ ). Furthermore, the Low dose caused higher activities of CYP1A1 ( $p = 0.02$ ), CYP3A11 ( $p < 0.01$ ) and CYP2B10 ( $p < 0.01$ ) compared to Control. The activity of CYP2E1 was reduced by both the Low and High doses ( $p < 0.01$ ). The Low dose also reduced the activity of CYP2A5 ( $p < 0.01$ ), while the High dose increased the CYP2A5 activity compared to Control ( $p = 0.02$ ).

At 9 weeks, the CYP1A1 and CYP1A activities were no longer affected by perinatal exposure. However, the High dose still increased the activity of CYP3A11 and CYP2B10 compared to Control ( $p < 0.01$ ). In addition, the Low dose elevated the activity of CYP2B10 ( $p < 0.01$ ). On the other hand, the Low dose reduced the activities of CYP2E1 and CYP2A5 compared to Control ( $p < 0.01$ ).

### Discussion

The present study demonstrated that *in utero* and lactational exposure to the mixture of POPs altered MG development and ovarian follicle maturation and caused persistent hepatocellular hypertrophy and enzyme induction in CD-1 mice. In dams, the POPs (apart from PCB-52 and HBCD) were readily taken up from the diet and distributed to the hepatic tissue. PCBs and OCPs seemed to be more efficiently transferred to offspring during late gestation and lactation compared to BFRs and PFASs. However, all compounds (except PCB-52, BDE-183 and HBCD) were detected in weaning offspring, thus, confirming maternal transfer of the mixture. After the end of lactational exposure, the POPs decreased in concentration over time suggesting dilution due to weight gain, metabolism and excretion.



**Fig 4. Hepatic enzymatic activity.** The activities of cytochrome P450 (CYP) 1A1, 1A, 3A11, 2B10, 2E1 and 2A5 in female offspring maternally exposed to a mixture of POPs at Control, Low or High doses (0x, 5000x or 100 000x human estimated daily intake, respectively). Samples were taken at 3 (n = 12), 6 (n = 14) and 9 (n = 14) weeks of age. Activities were measured as a rate of 7-ethoxyresorufin (CYP1A1), 7-methoxyresorufin (CYP1A), benzyloxyresorufin (CYP3A11), 7-pentoxoresorufin (CYP2B10) dealkylation, and p-nitrophenol (CYP2E1) and coumarin-7 (CYP2A5) hydroxylation. Boxplots consist of 25<sup>th</sup>, 50<sup>th</sup> (median) and 75<sup>th</sup> percentiles, whiskers (extending to 1.5 interquartile range) and outliers (•). Significant difference from Control ( $p \leq 0.05$ ) is marked by \*.

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Previously, Johanson and colleagues [8] showed that perinatal exposure to the Low dose resulted in 2-35x higher POP concentrations in 20-week-old A/J Min/+ mice (liver and adipose tissue) than the average blood levels reported in the Scandinavian population [39]. In the present study, Low dose female offspring at 9 weeks had comparable (from 2x lower to 5x higher) lipid-adjusted concentrations to those found previously [8]. This may suggest slight strain-dependent difference in metabolism and excretion of POPs and that the Low dose could be considered relevant to humans.

Results from the traditional qualitative scoring of MG development in female offspring showed a trend towards reduced development in 3-weeks-old mice perinatally exposed to the High dose. In addition, the number of TEBs were significantly lower in these mice. As previously described [12], MG development can be regarded as accelerated if the number of TEBs in the treated group is higher than that of the control at 3 weeks of age. Exposure to hexachlorobenzene (HCB) has been shown to increase the number of TEBs in C57BL/6 mice [56], which could increase the risk of breast cancer [57–59]. However, the opposite was observed in the present study and, thus, perinatal exposure to the High dose of POPs seemed to restrict MG development in 3-week-old female mice.

The number of TEBs is also coincident with the number of epithelial ducts in the MG. A gland with a high branching density will, thus, have a higher amount of TEBs [12]. Although the number of TEBs was not affected by perinatal exposure at 6 and 9 weeks, the High dose increased the branching density significantly in pubertal and non-significantly in adult mice. This could suggest a retention of TEBs causing more branching and possibly indicate a continuation of the restricted MG development observed in 3-week-old mice. Furthermore, perinatal exposure to the Low dose decreased the size and length of the epithelial expansion throughout the MG in pubertal and adult mice, which suggested that the more human relevant dose of POPs may have prematurely arrested MG development.

Restricted MG development has previously been shown following *in utero* exposure to PFOA at concentrations as low as 0.01 mg/kg in CD-1 and C57BL/6 mice [15, 16, 18, 19]. Interestingly, the delayed gland development found by Macon and colleagues [18] was evident at lower doses of PFOA than those required to increase the liver weight and persisted for a relatively long time after end of exposure. *In utero* and lactational exposure to DE-71 (a mixture of PBDEs) also delayed MG development in 3-week-old female Long-Evans rats [60]. Furthermore, *in utero* exposures to other EDCs, such as 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), Bisphenol A and Atrazine metabolites, have been shown to restrict gland development in rats and mice [61–63]. However, as previously emphasized [17], caution should be taken when drawing conclusions from studies using a single mouse strain as differences have been observed in the sensitivity of strains to PFOA-induced alterations in MG development [17, 19].

Overall, perinatal exposure to the mixture of POPs affected ovarian folliculogenesis in CD-1 mice. The Low dose reduced the number of primary follicles, while the High dose reduced the number of antral follicles in weaning offspring. In addition, there was a non-significant decrease in the number of antral follicles in adult offspring. This may suggest a delayed rate of follicle maturation. Several previous studies exposing rats to single POPs (including TCDD, PCB-126, BDE-99, BDE-47, PFOA and perfluorooctanesulfonic acid (PFOS)) during early development have also reported delays in ovarian follicle maturation [64–69]. Furthermore, evidence indicates that POPs can disrupt ovarian folliculogenesis by directly affecting the ovary, by modulating endocrine pathways or by altering circulating hormone levels [66, 68, 70]. After primordial follicle assembly, the initial recruitment is triggered by stimulation from intraovarian and/or unknown factors promoting follicle growth into primary, preantral and antral follicles. Most antral follicles undergo atresia. However, when the optimal stimulus is present, a few follicles continue into cyclic recruitment and reach the preovulatory stage. This process is controlled and stimulated by gonadotropins (e.g. luteinizing hormone and follicle-stimulating hormone) that have been synthesized and excreted by the anterior pituitary. Gonadotropin production is regulated by the gonadotropin releasing hormone (released from the hypothalamus), which further is controlled by ovarian steroid hormones that have been synthesized in response to gonadotropin stimulation. This is known as a negative feedback loop which tightly controls follicle maturation [71, 72]. Due to the endocrine disrupting properties of many POPs, it is likely that perinatal exposure may have disturbed the hormonal regulation of ovarian folliculogenesis [73]. However, alterations in hormone levels were not investigated in the present study.

The reduced number of antral follicles could also suggest an increased rate of follicle atresia. However, the number of atretic follicles were not quantified in the present study. An increased number of atretic follicles has previously been reported following perinatal exposure to a mixture of PCB-101 and -118 in CD-1 mice [74] and *in utero* exposure to PCB-126 in Sprague-Dawley rats [65]. Increased follicle atresia can result in an acceleration of ovarian reserve depletion and induce premature ovarian failure, thus, shortening the reproductive lifespan

[20, 75]. In humans, multiple OCPs, PCBs and BFRs have been associated with premature menopause [76–80]. Unfortunately, no measures were taken to promote estrous cycle homogeneity in mice. Consequently, the pubertal and adult offspring may have been terminated at different stages of estrous cycle and possibly affecting the ovarian follicle numbers.

Dietary exposure to the High dose increased the relative liver weight in dams, an increase possibly caused by hepatocellular hypertrophy. In addition, hypertrophy was seen in dams given the Low dose proving the sensitivity of this endpoint in CD-1 mice exposed to POPs. Multiple other studies have also showed hepatocellular hypertrophy in response to POP exposure [17, 34, 81–86]. Hypertrophy of liver cells is often seen together with hyperplasia and increased enzymatic activity. Hyperplasia was not detected in the present study. However, dose-dependent increases in hepatic CYP activities were seen in female offspring following perinatal exposure to the mixture of POPs. Interestingly, combined hepatic hypertrophy, hyperplasia and heightened enzymatic activities have previously been evaluated as predictors of liver cancer [26, 87, 88].

Increased CYP activities may lead to the formation of reactive oxygen species and cause hepatotoxicity [28, 29, 89]. In addition, the enhanced activities of CYP1A, CYP3A and CYP2B are commonly used as biomarkers for AhR, PXR and CAR activation, respectively [31, 90–93]. In the present study, CYP1A1 and CYP1A, CYP3A11 and CYP2B10 were dose-dependently induced in offspring, indirectly indicating AhR, PXR and CAR activation. Furthermore, the induction was less prominent with time corresponding to the temporal decline in hepatic POP concentrations. Interestingly, the activities of CYP2E1 and CYP2A5 were (at some points) lower in perinatally exposed mice compared to controls. This may have been caused by a reduced capacity for xenobiotic metabolism after POP exposure during early development. Physiological consequences of this should be further investigated, as low activity of these enzymes might predispose animals to harmful health effects under conditions where CYP2E1 and CYP2A5 are required for detoxification.

Previously, alterations in rodent hepatic CYP activities have been shown following perinatal exposure to POPs, such as PBDEs [33, 36, 94, 95],  $\gamma$ -hexachlorocyclohexane (HCH) [96] and mixtures [97–99]. Other studies have also observed hepatocellular hypertrophy caused by *in utero* and lactational exposure to mixtures of POPs [36, 84, 98, 99] or single chemicals [36, 100, 101]. Generally, resolution of hepatocellular effects occurs when the chemical has been metabolized and excreted from the system [26]. This is dependent on the nature of the chemical and highly persistent compounds (e.g. POPs) may, thus, cause effects long after end of exposure. The persistent nature of the Low dose has recently been demonstrated with higher (< 136x) levels of POPs in A/J Min/+ mice 17 weeks after end of perinatal exposure to the mixture of POPs [8]. Furthermore, the present study showed sustained hepatocellular hypertrophy with a significantly increased severity in 30-week-old male offspring perinatally exposed to the Low dose compared to controls.

Notably, perinatal exposure to the Low dose increased the body weight of male but not female offspring at 9 weeks. Previously, perinatal exposure to BDE-47 elevated the body weight of male but not female Sprague-Dawley rats [102] and stimulated the development of obesity in male ICR mice [103]. Various POPs have also been associated with increased weight gain and obesity in humans [104–107]. Thus, the increased body weight of male mice observed could possibly suggest an obesogenic effect of developmental exposures to POPs. No other effects were seen on body weight in both dams and offspring. This could be explained by POP concentrations generally being below the no observed adverse effect level (NOAEL) for individual chemicals [39], as well as the differences in xenobiotic metabolism between mice and humans [108, 109].

In summary, the present study illustrated that perinatal exposure to a human relevant mixture of POPs modulated female mammary gland development and ovarian folliculogenesis in

CD-1 mice. Furthermore, the mixture caused persistent hepatocellular hypertrophy, enzymatic induction and slight liver enlargement suggesting modest hepatotoxic effects. Further studies should include measurements of multiple hormones to create a better understanding of the effects observed on the development of the female reproductive organs. Additionally, studies should focus on how human relevant mixtures of POPs can ultimately affect female reproduction or negatively impact liver function.

## Supporting information

**S1 Fig. Study design of the experiment.** Timed-pregnant CD-1 dams (F0) gave birth to F1 dams at approximately 1 week after arrival. F1 dams were exposed to the mixture of POPs through the diet at Control, Low or High doses (0x, 5000x or 100 000x human estimated daily intake, respectively) from 3 weeks of age until termination during pregnancy (gestation d 17) or post-pregnancy (d 21 post-partum). F2 offspring were only exposed to the mixture *in utero*, through lactation and by nibbling on their mother's food prior to weaning, and were terminated at 3 (females), 6 (females), 9 (females and males) or 30 (males) weeks of age. (TIF)

**S1 File. Supporting information on materials and methods.**  
(DOCX)

**S1 Table. Lipid adjusted concentrations of POPs.** Individual PCBs, OCPs, BFRs and PFASs in pooled liver samples of dietary exposed pregnant (gestation d 17) and post-pregnant (21 d post-partum) dams and maternally exposed female offspring (3, 6 and 9 weeks of age). Mice were exposed to the mixture of POPs at Control, Low or High doses (0x, 5000x or 100 000x human estimated daily intake, respectively). Values are presented as ng/g lipid weight for PCBs, OCPs and BFRs, and ng/g wet weight for PFASs.  
(DOCX)

**S2 Table. Lipid percentage and wet weight concentrations of POPs.** PCBs, OCPs, BFRs and PFASs (ng/g wet weight) and lipid (%) in pooled liver samples of dietary exposed pregnant (gestation d 17) and post-pregnant (21 d post-partum) dams and maternally exposed female offspring (3, 6 and 9 weeks of age). Mice were exposed to the mixture of POPs at Control, Low or High doses (0x, 5000x or 100 000x human estimated daily intake, respectively).  
(DOCX)

**S3 Table. Biometrical measurements.** Body and liver weight of pregnant and post-pregnant dams, and female (3, 6 and 9 weeks of age) and male (9 and 30 weeks of age) offspring. Combined ovaries and uterus weight of female offspring (6 and 9 weeks of age) is also presented. Dams were dietary exposed to a mixture of POPs at Control, Low or High doses (0x, 5000x or 100 000x human estimated daily intake, respectively). Offspring were exposed in utero and through lactation (ending at 3 weeks). Pregnant dams were euthanized on gestation d 17 (n = 12, 16 and 8 for Control, Low and High, respectively). Post-pregnant dams were euthanized 21 d post-partum (n = 14, 10 and n = 11 for Control, Low and High, respectively). Females 3 weeks: n = 12 (all groups). Females 6 and 9 weeks: n = 14 (all groups, except for ovary + uterus weight from 6 weeks Control where n = 13). Males 9 and 30 weeks: n = 15 (all groups, except for liver weight from 9 weeks Low where n = 14). Results are presented as least square mean  $\pm$  standard error. Bold marks significant differences ( $p \leq 0.05$ ) from Control.  
(DOCX)

**S4 Table. Number of live offspring, males, and females.** The number of live offspring produced by dams during dietary exposure to a mixture of POPs at Control, Low or High doses

(0x, 5000x or 100 000x human estimated daily intake, respectively) were counted on gestation d 17. Live offspring of the post-pregnant dams were counted, and gender was determined, at 21 d post-partum. Results are presented as mean  $\pm$  standard error. The numbers of males/females are given as the total number within each dose.

(DOCX)

**S5 Table. Complete results on mammary gland histology.** Mammary gland morphology in 3, 6 and 9-week-old female CD-1 mice maternally exposed to a mixture of POPs at Control, Low or High doses (0x, 5000x or 100 000x human estimated daily intake, respectively). Qualitative development scores were conducted on whole mounts of the 4<sup>th</sup> mammary gland from the right side using a scale from 1 to 4 (1 = poor development, 4 = best development). Terminal end buds (TEBs) were defined as ends with a diameter of  $\geq 100 \mu\text{m}$ . Branching density, sum of the number of intersections, mammary epithelial area and length were calculated using Sholl analysis in ImageJ software. Glandular area was calculated using ImageJ on one section of fixated 4<sup>th</sup> mammary gland from the left side. Results are presented as mean  $\pm$  standard error. At 3 weeks of age,  $n = 11$  for the High group and  $n = 12$  for the Control and Low groups except glandular area were  $n = 11$ . At 6 weeks,  $n = 12$  for the Control group and  $n = 14$  for the Low and High groups except glandular area were  $n = 12$ . At 9 weeks,  $n = 13$  for the Control and High groups, except Control group glandular area were  $n = 12$ . Furthermore,  $n = 14$  for the Low group except glandular area were  $n = 12$ . Numbers in bold mark significant difference from Control ( $p \leq 0.05$ ). P-values  $\leq 0.10$  are marked with \*.

(DOCX)

**S6 Table. Hepatic extramedullary hematopoiesis and centrilobular hypertrophy.** Severity of extramedullary hematopoiesis and centrilobular hypertrophy of hepatocytes in livers from dams (pregnant sampled at gestation d 17, and post-pregnant sampled at 21 d post-partum), and female (sampled at 3, 6 and 9 weeks of age) and male (sampled at 9 and 30 weeks of age) offspring. Dams were dietary exposed, and offspring were maternally exposed, to a mixture of POPs at Control, Low or High doses (0x, 5000x or 100 000x human estimated daily intake, respectively). Results are presented as mean  $\pm$  standard error. Severity was graded on a scale from 0 to 4 (0 = no, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe change).  $n = 12$ , 14 and 14 for female offspring in all groups at 3, 6 and 9 weeks, respectively.  $n = 15$  for male offspring in all groups at both sampling times.  $n = 12$ , 16 and 8, and  $n = 14$ , 10 and 11 for the Control, Low and High groups of pregnant and post-pregnant dams, respectively. Bold indicates significant ( $p \leq 0.05$ ) difference from Control.

(DOCX)

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## References

1. Secretariat of the Stockholm Convention [Internet]. What are POPs? 2019 [cited 02 April 2020]. Available from: <http://chm.pops.int/TheConvention/ThePOPs/tabid/673/Default.aspx>.
2. Secretariat of the Stockholm Convention. Ridding the world of POPs: a guide to the stockholm convention on persistent organic pollutants. Geneva, Switzerland: United Nations Environment Programme; 2010.
3. Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, Hauser R, Prins GS, Soto AM, et al. Endocrine-disrupting chemicals: an endocrine society scientific statement. *Endocr Rev.* 2009; 30:293–342. <https://doi.org/10.1210/er.2009-0002> PMID: 19502515
4. Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, et al. EDC-2: the endocrine society's second scientific statement on endocrine-disrupting chemicals. *Endocr Rev.* 2015; 36:E1–e150. <https://doi.org/10.1210/er.2015-1010> PMID: 26544531
5. Barr DB, Bishop A, Needham LL. Concentrations of xenobiotic chemicals in the maternal-fetal unit. *Reprod Toxicol.* 2007; 23:260–6. <https://doi.org/10.1016/j.reprotox.2007.03.003> PMID: 17386996
6. Winkens K, Vestergren R, Berger U, Cousins IT. Early life exposure to per- and polyfluoroalkyl substances (PFASs): a critical review. *Emerg Contam.* 2017; 3:55–68. <https://doi.org/10.1016/j.emcon.2017.05.001>
7. Vizcaino E, Grimalt JO, Fernández-Somoano A, Tardon A. Transport of persistent organic pollutants across the human placenta. *Environ Int.* 2014; 65:107–15. <https://doi.org/10.1016/j.envint.2014.01.004> PMID: 24486968
8. Johanson SM, Swann JR, Umu OCO, Aleksandersen M, Muller MHB, Berntsen HF, et al. Maternal exposure to a human relevant mixture of persistent organic pollutants reduces colorectal carcinogenesis in A/J Min/+ mice. *Chemosphere.* 2020; 252:126484. <https://doi.org/10.1016/j.chemosphere.2020.126484> PMID: 32199166
9. Landrigan PJ, Goldman LR. Children's vulnerability to toxic chemicals: a challenge and opportunity to strengthen health and environmental policy. *Health Affairs.* 2011; 30:842–50. <https://doi.org/10.1377/hlthaff.2011.0151> PMID: 21543423
10. Faustman EM, Silbernagel SM, Fenske RA, Burbacher TM, Ponce RA. Mechanisms underlying children's susceptibility to environmental toxicants. *Environ Health Perspect.* 2000; 108:13–21. <https://doi.org/10.1289/ehp.00108s113> PMID: 10698720
11. Barr DB, Wang RY, Needham LL. Biologic monitoring of exposure to environmental chemicals throughout the life stages: requirements and issues for consideration for the National Children's Study. *Environ Health Perspect.* 2005; 113:1083–91. <https://doi.org/10.1289/ehp.7617> PMID: 16079083
12. Rudel RA, Fenton SE, Ackerman JM, Euling SY, Makris SL. Environmental exposures and mammary gland development: state of the science, public health implications, and research recommendations. *Environ Health Perspect.* 2011; 119:1053–61. <https://doi.org/10.1289/ehp.1002864> PMID: 21697028
13. Fenton SE. Endocrine-disrupting compounds and mammary gland development: early exposure and later life consequences. *Endocrinology.* 2006; 147:S18–24. <https://doi.org/10.1210/en.2005-1131> PMID: 16690811

14. White SS, Calafat AM, Kuklennyik Z, Villanueva L, Zehr RD, Helfant L, et al. Gestational PFOA exposure of mice is associated with altered mammary gland development in dams and female offspring. *Toxicol Sci.* 2007; 96:133–44. <https://doi.org/10.1093/toxsci/kfl177> PMID: 17132714
15. White SS, Kato K, Jia LT, Basden BJ, Calafat AM, Hines EP, et al. Effects of perfluorooctanoic acid on mouse mammary gland development and differentiation resulting from cross-foster and restricted gestational exposures. *Reprod Toxicol.* 2009; 27:289–98. <https://doi.org/10.1016/j.reprotox.2008.11.054> PMID: 19095057
16. White SS, Stanko JP, Kato K, Calafat AM, Hines EP, Fenton SE. Gestational and chronic low-dose PFOA exposures and mammary gland growth and differentiation in three generations of CD-1 mice. *Environ Health Perspect.* 2011; 119:1070–6. <https://doi.org/10.1289/ehp.1002741> PMID: 21501981
17. Yang C, Tan YS, Harkema JR, Haslam SZ. Differential effects of peripubertal exposure to perfluorooctanoic acid on mammary gland development in C57Bl/6 and Balb/c mouse strains. *Reprod Toxicol.* 2009; 27:299–306. <https://doi.org/10.1016/j.reprotox.2008.10.003> PMID: 19013232
18. Macon MB, Villanueva LR, Tatum-Gibbs K, Zehr RD, Strynar MJ, Stanko JP, et al. Prenatal perfluorooctanoic acid exposure in CD-1 mice: low-dose developmental effects and internal dosimetry. *Toxicol Sci.* 2011; 122:134–45. <https://doi.org/10.1093/toxsci/kfr076> PMID: 21482639
19. Tucker DK, Macon MB, Strynar MJ, Dagnino S, Andersen E, Fenton SE. The mammary gland is a sensitive pubertal target in CD-1 and C57Bl/6 mice following perinatal perfluorooctanoic acid (PFOA) exposure. *Reprod Toxicol.* 2015; 54:26–36. <https://doi.org/10.1016/j.reprotox.2014.12.002> PMID: 25499722
20. Vabre P, Gatimel N, Moreau J, Gayrard V, Picard-Hagen N, Parinaud J, et al. Environmental pollutants, a possible etiology for premature ovarian insufficiency: a narrative review of animal and human data. *Environ Health.* 2017; 16:37. <https://doi.org/10.1186/s12940-017-0242-4> PMID: 28388912
21. Patel S, Zhou C, Rattan S, Flaws JA. Effects of endocrine-disrupting chemicals on the ovary. *Biol Reprod.* 2015; 93:1–9. <https://doi.org/10.1095/biolreprod.115.130336> PMID: 26063868
22. Rachoń D. Endocrine disrupting chemicals (EDCs) and female cancer: informing the patients. *Rev Endocr Metab Disord.* 2015; 16:359–64. <https://doi.org/10.1007/s11154-016-9332-9> PMID: 26831296
23. Coughtrie MW, Burchell B, Leakey JE, Hume R. The inadequacy of perinatal glucuronidation: Immunoblot analysis of the developmental expression of individual UDP-glucuronosyltransferase isoenzymes in rat and human liver microsomes. *Mol Pharmacol.* 1988; 34:729–35. PMID: 3143908
24. Milsap RL, Jusko WJ. Pharmacokinetics in the infant. *Environ Health Perspect.* 1994; 102:107–10. <https://doi.org/10.1289/ehp.94102s11107> PMID: 7737034
25. Guengerich FP. Reactions and significance of cytochrome P-450 enzymes. *J Biol Chem.* 1991; 266:10019–22. PMID: 2037557
26. Maronpot RR, Yoshizawa K, Nyska A, Harada T, Flake G, Mueller G, et al. Hepatic enzyme induction: histopathology. *Toxicol Pathol.* 2010; 38:776–95. <https://doi.org/10.1177/0192623310373778> PMID: 20585142
27. Nebert DW, Russell DW. Clinical importance of the cytochromes P450. *The Lancet.* 2002; 360:1155–62. [https://doi.org/10.1016/S0140-6736\(02\)11203-7](https://doi.org/10.1016/S0140-6736(02)11203-7) PMID: 12387968
28. Bondy SC, Naderi S. Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. *Biochem Pharmacol.* 1994; 48:155–9. [https://doi.org/10.1016/0006-2952\(94\)90235-6](https://doi.org/10.1016/0006-2952(94)90235-6) PMID: 8043018
29. Zangar RC, Davydov DR, Verma S. Mechanisms that regulate production of reactive oxygen species by cytochrome P450. *Toxicol Appl Pharm.* 2004; 199:316–31. <https://doi.org/10.1016/j.taap.2004.01.018> PMID: 15364547
30. Tolson AH, Wang H. Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR. *Adv Drug Deliver Rev.* 2010; 62:1238–49. <https://doi.org/10.1016/j.addr.2010.08.006> PMID: 20727377
31. Waxman DJ. P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. *Arch Biochem Biophys.* 1999; 369:11–23. <https://doi.org/10.1006/abbi.1999.1351> PMID: 10462436
32. Sanders JM, Burka LT, Smith CS, Black W, James R, Cunningham ML. Differential expression of CYP1A, 2B, and 3A genes in the F344 rat following exposure to a polybrominated diphenyl ether mixture or individual components. *Toxicol Sci.* 2005; 88:127–33. <https://doi.org/10.1093/toxsci/kfi288> PMID: 16107549
33. Szabo DT, Richardson VM, Ross DG, Diliberto JJ, Kodavanti PRS, Birnbaum LS. Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase 1 gene expression involved in thyroid hormone metabolism in male rat pups. *Toxicol Sci.* 2008; 107:27–39. <https://doi.org/10.1093/toxsci/kfn230> PMID: 18978342

34. van der Ven LTM, van de Kuil T, Verhoef A, Leonards PEG, Slob W, Cantón RF, et al. A 28-day oral dose toxicity study enhanced to detect endocrine effects of a purified technical pentabromodiphenyl ether (pentaBDE) mixture in Wistar rats. *Toxicology*. 2008; 245:109–22. <https://doi.org/10.1016/j.tox.2007.12.016> PMID: 18243468
35. Pacyniak EK, Cheng X, Cunningham ML, Crofton K, Klaassen CD, Guo GL. The flame retardants, polybrominated diphenyl ethers, are pregnane X receptor activators. *Toxicol Sci*. 2007; 97:94–102. <https://doi.org/10.1093/toxsci/kfm025> PMID: 17324954
36. Dunnick JK, Shockley KR, Pandiri AR, Kissling GE, Gerrish KE, Ton TV, et al. PBDE-47 and PBDE mixture (DE-71) toxicities and liver transcriptomic changes at PND 22 after *in utero*/postnatal exposure in the rat. *Arch Toxicol*. 2018; 92:3415–33. <https://doi.org/10.1007/s00204-018-2292-y> PMID: 30206662
37. Bopp SK, Barouki R, Brack W, Dalla Costa S, Dorne J-LCM, Drakvik PE, et al. Current EU research activities on combined exposure to multiple chemicals. *Environ Int*. 2018; 120:544–62. <https://doi.org/10.1016/j.envint.2018.07.037> PMID: 30170309
38. Hernández AF, Parrón T, Tsatsakis AM, Requena M, Alarcón R, López-Guarnido O. Toxic effects of pesticide mixtures at a molecular level: their relevance to human health. *Toxicology*. 2013; 307:136–45. <https://doi.org/10.1016/j.tox.2012.06.009> PMID: 22728724
39. Berntsen HF, Berg V, Thomsen C, Ropstad E, Zimmer KE. The design of an environmentally relevant mixture of persistent organic pollutants for use in *in vivo* and *in vitro* studies. *J Toxicol Env Heal A*. 2017; 80:1002–16. <https://doi.org/10.1080/15287394.2017.1354439> PMID: 28854125
40. Preibisch S, Saalfeld S, Tomancak P. Globally optimal stitching of tiled 3D microscopic image acquisitions. *Bioinformatics*. 2009; 25:1463–5. <https://doi.org/10.1093/bioinformatics/btp184> PMID: 19346324
41. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012; 9:676–82. <https://doi.org/10.1038/nmeth.2019> PMID: 22743772
42. Stanko JP, Easterling MR, Fenton SE. Application of Sholl analysis to quantify changes in growth and development in rat mammary gland whole mounts. *Reprod Toxicol*. 2015; 54:129–35. <https://doi.org/10.1016/j.reprotox.2014.11.004> PMID: 25463529
43. Stanko JP, Fenton SE. Quantifying branching density in rat mammary gland whole-mounts using the Sholl analysis method. *J Vis Exp*. 2017; 125:55789. <https://doi.org/10.3791/55789> PMID: 28745626
44. Flaws JA, Langenberg P, Babus JK, Hirshfield AN, Sharara FI. Ovarian volume and antral follicle counts as indicators of menopausal status. *Menopause*. 2001; 8:175–80. <https://doi.org/10.1097/00042192-200105000-00006> PMID: 11355039
45. Brevik EM. Gas chromatographic method for the determination of organochlorine pesticides in human milk. *Bull Environ Contam Toxicol*. 1978; 78:281–6. <https://doi.org/10.1007/BF01685799> PMID: 647131
46. Polder A, Müller MB, Lyche JL, Mdegela RH, Nonga HE, Mabiki FP, et al. Levels and patterns of persistent organic pollutants (POPs) in tilapia (*Oreochromis sp.*) from four different lakes in Tanzania: geographical differences and implications for human health. *Sci Total Environ*. 2014; 488–489:252–60. <https://doi.org/10.1016/j.scitotenv.2014.04.085> PMID: 24836134
47. Grønnestad R, Villanger GD, Polder A, Kovacs KM, Lydersen C, Jenssen BM, et al. Maternal transfer of perfluoroalkyl substances in hooded seals. *Environ Toxicol Chem*. 2017; 36:763–70. <https://doi.org/10.1002/etc.3623> PMID: 27771942
48. Thoolen B, Maronpot RR, Harada T, Nyska A, Rousseaux C, Nolte T, et al. Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicol Pathol*. 2010; 38:5S–81S. <https://doi.org/10.1177/0192623310386499> PMID: 21191096
49. Rasmussen MK, Ekstrand B, Zamaratskaia G. Comparison of cytochrome P450 concentrations and metabolic activities in porcine hepatic microsomes prepared with two different methods. *Toxicol In Vitro*. 2011; 25:343–6. <https://doi.org/10.1016/j.tiv.2010.10.007> PMID: 20940039
50. Zamaratskaia G, Zlabek V, Ropstad E, Tajet H, Andresen O. Hepatic ethoxy-, methoxy- and pentoxyl-*O*-dealkylase activities in Landrace and Duroc pigs stimulated with HCG. *Reprod Domest Anim*. 2010; 45:e269–74. <https://doi.org/10.1111/j.1439-0531.2009.01553.x> PMID: 19930133
51. Pilipenko N, Ropstad E, Halsne R, Zamaratskaia G. Effect of naringenin, quercetin, and sesamin on xenobiotica-metabolizing CYP1A and CYP3A in mice offspring after maternal exposure to persistent organic pollutants. *BioMed Res Int*. 2017; 2017:8472312. <https://doi.org/10.1155/2017/8472312> PMID: 28567424
52. Zamaratskaia G, Zlabek V, Chen G, Madej A. Modulation of porcine cytochrome P450 enzyme activities by surgical castration and immunocastration. *Animal*. 2009; 3:1124–32. <https://doi.org/10.1017/S1751731109004510> PMID: 22444842

53. R Development Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing ed. Vienna, Austria: ISBN 3-900051-07-0, URL: <http://www.R-project.org>. 2008.
54. Wickham H. ggplot2: elegant graphics for data analysis: Springer-Verlag New York; 2016.
55. Kassambara A. ggpubr: 'ggplot2' based publication ready plots. 2020. URL: <https://rpkgs.datanovia.com/ggpubr/>.
56. Miret N, Rico-Leo E, Pontillo C, Zotta E, Fernandez-Salguero P, Randi A. A dioxin-like compound induces hyperplasia and branching morphogenesis in mouse mammary gland, through alterations in TGF-beta1 and aryl hydrocarbon receptor signaling. *Toxicol Appl Pharmacol*. 2017; 334:192–206. <https://doi.org/10.1016/j.taap.2017.09.012> PMID: 28923513
57. Miret NV, Pontillo CA, Zarate LV, Kleiman de Pisarev D, Cocca C, Randi AS. Impact of endocrine disruptor hexachlorobenzene on the mammary gland and breast cancer: the story thus far. *Environ Res*. 2019; 173:330–41. <https://doi.org/10.1016/j.envres.2019.03.054> PMID: 30951959
58. Macon MB, Fenton SE. Endocrine disruptors and the breast: early life effects and later life disease. *J Mammary Gland Biol Neoplasia*. 2013; 18:43–61. <https://doi.org/10.1007/s10911-013-9275-7> PMID: 23417729
59. Osborne G, Rudel R, Schwarzman M. Evaluating chemical effects on mammary gland development: a critical need in disease prevention. *Reprod Toxicol*. 2015; 54:148–55. <https://doi.org/10.1016/j.reprotox.2014.07.077> PMID: 25091782
60. Kodavanti PRS, Coburn CG, Moser VC, MacPhail RC, Fenton SE, Stoker TE, et al. Developmental exposure to a commercial PBDE mixture, DE-71: neurobehavioral, hormonal, and reproductive effects. *Toxicol Sci*. 2010; 116:297–312. <https://doi.org/10.1093/toxsci/kfq105> PMID: 20375078
61. Enoch RR, Stanko JP, Greiner SN, Youngblood GL, Rayner JL, Fenton SE. Mammary gland development as a sensitive end point after acute prenatal exposure to an atrazine metabolite mixture in female Long-Evans rats. *Environ Health Perspect*. 2007; 115:541–7. <https://doi.org/10.1289/ehp.9612> PMID: 17450222
62. Fenton SE, Hamm JT, Birnbaum LS, Youngblood GL. Persistent abnormalities in the rat mammary gland following gestational and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Toxicol Sci*. 2002; 67:63–74. <https://doi.org/10.1093/toxsci/67.1.63> PMID: 11961217
63. Markey CM, Luque EH, Munoz De Toro M, Sonnenschein C, Soto AM. *In utero* exposure to bisphenol A alters the development and tissue organization of the mouse mammary gland. *Biol Reprod*. 2001; 65:1215–23. <https://doi.org/10.1093/biolreprod/65.4.1215> PMID: 11566746
64. Heimler I, Trewin AL, Chaffin CL, Rawlins RG, Hutz RJ. Modulation of ovarian follicle maturation and effects on apoptotic cell death in Holtzman rats exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in utero and lactationally. *Reprod Toxicol*. 1998; 12:69–73. [https://doi.org/10.1016/s0890-6238\(97\)00101-9](https://doi.org/10.1016/s0890-6238(97)00101-9) PMID: 9431574
65. Muto T, Imano N, Nakaaki K, Takahashi H, Hano H, Wakui S, et al. Estrous cyclicity and ovarian follicles in female rats after prenatal exposure to 3,3',4,4',5-pentachlorobiphenyl. *Toxicol Lett*. 2003; 143:271–7. [https://doi.org/10.1016/s0378-4274\(03\)00175-9](https://doi.org/10.1016/s0378-4274(03)00175-9) PMID: 12849687
66. Lilienthal H, Hack A, Roth-Härer A, Grande Simone W, Talsness Chris E. Effects of developmental exposure to 2,2',4,4',5-pentabromodiphenyl ether (PBDE-99) on sex steroids, sexual development, and sexually dimorphic behavior in rats. *Environ Health Perspect*. 2006; 114:194–201. <https://doi.org/10.1289/ehp.8391> PMID: 16451854
67. Shirota M, Mukai M, Sakurada Y, Doyama A, Inoue K, Haishima A, et al. Effects of vertically transferred 3,3',4,4',5-pentachlorobiphenyl (PCB-126) on the reproductive development of female rats. *J Reprod Develop*. 2006; 52:751–61. <https://doi.org/10.1262/jrd.18062> PMID: 16983182
68. Talsness C E., Kuriyama S N., Sterner-Kock A, Schnitker P, Grande Simone W, Shakibaei M, et al. *In utero* and lactational exposures to low doses of polybrominated diphenyl ether-47 alter the reproductive system and thyroid gland of female rat offspring. *Environ Health Perspect*. 2008; 116:308–14. <https://doi.org/10.1289/ehp.10536> PMID: 18335096
69. Du G, Hu J, Huang Z, Yu M, Lu C, Wang X, et al. Neonatal and juvenile exposure to perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS): advance puberty onset and kisspeptin system disturbance in female rats. *Ecotox Environ Safe*. 2019; 167:412–21. <https://doi.org/10.1016/j.ecoenv.2018.10.025> PMID: 30368134
70. Baldrige MG, Stahl RL, Gerstenberger SL, Tripoli V, Hutz RJ. Modulation of ovarian follicle maturation in Long-Evans rats exposed to polychlorinated biphenyls (PCBs) *in-utero* and lactationally. *Reprod Toxicol*. 2003; 17:567–73. [https://doi.org/10.1016/s0890-6238\(03\)00095-9](https://doi.org/10.1016/s0890-6238(03)00095-9) PMID: 14555195
71. McGee EA, Hsueh AJW. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev*. 2000; 21:200–14. <https://doi.org/10.1210/edrv.21.2.0394> PMID: 10782364

72. Yamaji T, Dierschke DJ, Bhattacharya AN, Knobil E. The negative feedback control by estradiol and progesterone of LH secretion in the ovariectomized Rhesus Monkey. *Endocrinology*. 1972; 90:771–7. <https://doi.org/10.1210/endo-90-3-771> PMID: 4621570
73. Uzumcu M, Zachow R. Developmental exposure to environmental endocrine disruptors: consequences within the ovary and on female reproductive function. *Reprod Toxicol*. 2007; 23:337–52. <https://doi.org/10.1016/j.reprotox.2006.10.006> PMID: 17140764
74. Pocar P, Fiandanese N, Secchi C, Berrini A, Fischer B, Schmidt J-S, et al. Effects of polychlorinated biphenyls in CD-1 mice: reproductive toxicity and intergenerational transmission. *Toxicol Sci*. 2011; 126:213–26. <https://doi.org/10.1093/toxsci/kfr327> PMID: 22157103
75. Ge W, Li L, Dyce PW, De Felici M, Shen W. Establishment and depletion of the ovarian reserve: physiology and impact of environmental chemicals. *Cell Mol Life Sci*. 2019; 76:1729–46. <https://doi.org/10.1007/s00018-019-03028-1> PMID: 30810760
76. Cooper GS, Savitz DA, Millikan R, Kit TC. Organochlorine exposure and age at natural menopause. *Epidemiology*. 2002; 13:729–33. <https://doi.org/10.1097/00001648-200211000-00021> PMID: 12410018
77. Akkina JE, Reif JS, Keefe TJ, Bachand AM. Age at natural menopause and exposure to organochlorine pesticides in Hispanic women. *J Toxicol Env Heal A*. 2004; 67:1407–22. <https://doi.org/10.1080/15287390490483845> PMID: 15371229
78. Knox SS, Jackson T, Javins B, Frisbee SJ, Shankar A, Ducatman AM. Implications of early menopause in women exposed to perfluorocarbons. *J Clin Endocr Metab*. 2011; 96:1747–53. <https://doi.org/10.1210/jc.2010-2401> PMID: 21411548
79. Taylor KW, Hoffman K, Thayer KA, Daniels JL. Polyfluoroalkyl chemicals and menopause among women 20–65 years of age (NHANES). *Environ Health Perspect*. 2014; 122:145–50. <https://doi.org/10.1289/ehp.1306707> PMID: 24280566
80. Grindler NM, Allsworth JE, Macones GA, Kannan K, Roehl KA, Cooper AR. Persistent organic pollutants and early menopause in U.S. women. *PLoS One*. 2015; 10:e0116057-e. <https://doi.org/10.1371/journal.pone.0116057> PMID: 25629726
81. Roos R, Andersson PL, Halldin K, Håkansson H, Westerholm E, Hamers T, et al. Hepatic effects of a highly purified 2,2',3,4,4',5,5'-heptachlorobiphenyl (PCB 180) in male and female rats. *Toxicology*. 2011; 284:42–53. <https://doi.org/10.1016/j.tox.2011.03.013> PMID: 21458519
82. National Toxicology Programme. NTP technical report on the toxicology and carcinogenesis studies of 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) (CAS No. 35065-27-1) in female Harlan Sprague-Dawley rats (gavage studies). NTP TR 529, NIH Publication.: 2006 May. Report No.: 06–4465 Contract No.: 529.
83. Wade MG, Foster WG, Younglai EV, McMahon A, Leingartner K, Yagminas A, et al. Effects of subchronic exposure to a complex mixture of persistent contaminants in male rats: systemic, immune, and reproductive effects. *Toxicol Sci*. 2002; 67:131–43. <https://doi.org/10.1093/toxsci/67.1.131> PMID: 11961226
84. Bondy GS, Lefebvre DE, Aziz S, Cherry W, Coady L, MacLellan E, et al. Toxicologic and immunologic effects of perinatal exposure to the brominated diphenyl ether (BDE) mixture DE-71 in the Sprague-Dawley rat. *Environ Toxicol*. 2011; 28:215–28. <https://doi.org/10.1002/tox.20713> PMID: 21544923
85. Wolf DC, Moore T, Abbott BD, Rosen MB, Das KP, Zehr RD, et al. Comparative hepatic effects of perfluorooctanoic acid and WY 14,643 in PPAR-alpha knockout and wild-type mice. *Toxicol Pathol*. 2008; 36:632–9. <https://doi.org/10.1177/0192623308318216> PMID: 18467680
86. Butenhoff JL, Chang S-C, Olsen GW, Thomford PJ. Chronic dietary toxicity and carcinogenicity study with potassium perfluorooctanesulfonate in Sprague Dawley rats. *Toxicology*. 2012; 293:1–15. <https://doi.org/10.1016/j.tox.2012.01.003> PMID: 22266392
87. Allen DG, Pearse G, Haseman JK, Maronpot RR. Prediction of rodent carcinogenesis: an evaluation of prechronic liver lesions as forecasters of liver tumors in NTP carcinogenicity studies. *Toxicol Pathol*. 2004; 32:393–401. <https://doi.org/10.1080/01926230490440934> PMID: 15307212
88. Hall AP, Elcombe CR, Foster JR, Harada T, Kaufmann W, Knippel A, et al. Liver hypertrophy: a review of adaptive (adverse and non-adverse) changes—conclusions from the 3rd International ESTP Expert Workshop. *Toxicol Pathol*. 2012; 40:971–94. <https://doi.org/10.1177/0192623312448935> PMID: 22723046
89. Ioannides C, Lewis DF. Cytochromes P450 in the bioactivation of chemicals. *Curr Top Med Chem*. 2004; 4:1767–88. <https://doi.org/10.2174/1568026043387188> PMID: 15579107
90. Whitlock JJP. Genetic and molecular aspects of 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin action. *Annu Rev Pharmacol Toxicol*. 1990; 30:251–77. <https://doi.org/10.1146/annurev.pa.30.040190.001343> PMID: 2188570

91. Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, et al. Humanized xenobiotic response in mice expressing nuclear receptor SXR. *Nature*. 2000; 406:435–9. <https://doi.org/10.1038/35019116> PMID: 10935643
92. Maglich JM, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT, Kliewer SA. Nuclear pregnane X receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharm*. 2002; 62:638–46. <https://doi.org/10.1124/mol.62.3.638> PMID: 12181440
93. Yamada H, Ishii Y, Yamamoto M, Oguri K. Induction of the hepatic cytochrome P450 2B subfamily by xenobiotics: research history, evolutionary aspect, relation to tumorigenesis, and mechanism. *Curr Drug Metab*. 2006; 7:397–409. <https://doi.org/10.2174/138920006776873508> PMID: 16724929
94. Tseng L-H, Li M-H, Tsai S-S, Lee C-W, Pan M-H, Yao W-J, et al. Developmental exposure to decabromodiphenyl ether (PBDE 209): effects on thyroid hormone and hepatic enzyme activity in male mouse offspring. *Chemosphere*. 2008; 70:640–7. <https://doi.org/10.1016/j.chemosphere.2007.06.078> PMID: 17698168
95. Zhou T, Taylor MM, DeVito MJ, Crofton KM. Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol Sci*. 2002; 66:105–16. <https://doi.org/10.1093/toxsci/66.1.105> PMID: 11861977
96. Johri A, Dhawan A, Singh RL, Parmar D. Persistence in alterations in the ontogeny of cerebral and hepatic cytochrome P450s following prenatal exposure to low doses of lindane. *Toxicol Sci*. 2008; 101:331–40. <https://doi.org/10.1093/toxsci/kfm269> PMID: 17984293
97. Bonfanti P, Comelli F, Assi L, Casati L, Colciago A, Villa S, et al. Responsiveness of hepatic and cerebral cytochrome P450 in rat offspring prenatally and lactationally exposed to a reconstituted PCB mixture. *Environ Toxicol*. 2014; 29:856–66. <https://doi.org/10.1002/tox.21812> PMID: 22987612
98. Chu I, Bowers WJ, Caldwell D, Nakai J, Pulido O, Yagminas A, et al. Toxicological effects of gestational and lactational exposure to a mixture of persistent organochlorines in rats: systemic effects. *Toxicol Sci*. 2005; 88:645–55. <https://doi.org/10.1093/toxsci/kfi335> PMID: 16177236
99. Chu I, Bowers WJ, Caldwell D, Nakai J, Wade MG, Yagminas A, et al. Toxicological effects of *in utero* and lactational exposure of rats to a mixture of environmental contaminants detected in Canadian Arctic human populations. *J Toxicol Env Health A*. 2008; 71:93–108. <https://doi.org/10.1080/15287390701612811> PMID: 18080900
100. Filgo AJ, Quist EM, Hoenerhoff MJ, Brix AE, Kissling GE, Fenton SE. Perfluorooctanoic acid (PFOA)-induced liver lesions in two strains of mice following developmental exposures: PPAR $\alpha$  is not required. *Toxicol Pathol*. 2015; 43:558–68. <https://doi.org/10.1177/0192623314558463> PMID: 25398757
101. Quist EM, Filgo AJ, Cummings CA, Kissling GE, Hoenerhoff MJ, Fenton SE. Hepatic mitochondrial alteration in CD-1 mice associated with prenatal exposures to low doses of perfluorooctanoic acid (PFOA). *Toxicol Pathol*. 2015; 43:546–57. <https://doi.org/10.1177/0192623314551841> PMID: 25326589
102. Gao H, Li P, Liu L, Yang K, Xiao B, Zhou G, et al. Perigestational low-dose BDE-47 exposure alters maternal serum metabolome and results in sex-specific weight gain in adult offspring. *Chemosphere*. 2019; 233:174–82. <https://doi.org/10.1016/j.chemosphere.2019.05.277> PMID: 31173955
103. Wang D, Yan J, Teng M, Yan S, Zhou Z, Zhu W. *In utero* and lactational exposure to BDE-47 promotes obesity development in mouse offspring fed a high-fat diet: impaired lipid metabolism and intestinal dysbiosis. *Arch Toxicol*. 2018; 92:1847–60. <https://doi.org/10.1007/s00204-018-2177-0> PMID: 29523931
104. Valvi D, Mendez MA, Martinez D, Grimalt JO, Torrent M, Sunyer J, et al. Prenatal concentrations of polychlorinated biphenyls, DDE, and DDT and overweight in children: a prospective birth cohort study. *Environ Health Perspect*. 2012; 120:451–7. <https://doi.org/10.1289/ehp.1103862> PMID: 22027556
105. Karmaus W, Osuch JR, Eneli I, Mudd LM, Zhang J, Mikucki D, et al. Maternal levels of dichlorodiphenyl-dichloroethylene (DDE) may increase weight and body mass index in adult female offspring. *Occup Environ Med*. 2009; 66:143–9. <https://doi.org/10.1136/oem.2008.041921> PMID: 19060027
106. Lee DH, Lind L, Jacobs DR Jr., Salihovic S, van Bavel B, Lind PM. Associations of persistent organic pollutants with abdominal obesity in the elderly: the prospective investigation of the vasculature in Uppsala seniors (PIVUS) study. *Environ Int*. 2012; 40:170–8. <https://doi.org/10.1016/j.envint.2011.07.010> PMID: 21835469
107. Warner M, Rauch S, Coker ES, Harley K, Kogut K, Sjodin A, et al. Obesity in relation to serum persistent organic pollutant concentrations in CHAMACOS women. *Environ Epidemiol*. 2018; 2:e032. <https://doi.org/10.1097/EE9.000000000000032> PMID: 31106289
108. Walton K, Dorne JL, Renwick AG. Uncertainty factors for chemical risk assessment: interspecies differences in the *in vivo* pharmacokinetics and metabolism of human CYP1A2 substrates. *Food Chem Toxicol*. 2001; 39:667–80. [https://doi.org/10.1016/s0278-6915\(01\)00006-0](https://doi.org/10.1016/s0278-6915(01)00006-0) PMID: 11397514

109. Martignoni M, Groothuis GMM, de Kanter R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin Drug Metab Toxicol.* 2006; 2:875–94. <https://doi.org/10.1517/17425255.2.6.875> PMID: 17125407