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Identification of phthalate mixture exposure targets in the human and mouse ovary *in vitro*

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ABSTRACT

Chemical health risk assessment is based on single chemicals, but humans and wildlife are exposed to extensive mixtures of industrial substances and pharmaceuticals. Such exposures are life-long and correlate with multiple morbidities, including infertility. How combinatorial effects of chemicals should be handled in hazard characterization and risk assessment are open questions. Further, test systems are missing for several relevant health outcomes including reproductive health and fertility in women. Here, our aim was to screen multiple ovarian cell models for phthalate induced effects to identify biomarkers of exposure. We used an epidemiological cohort study to define different phthalate mixtures for *in vitro* testing. The mixtures were then tested in five cell models representing ovarian granulosa or stromal cells, namely COV434, KGN, primary human granulosa cells, primary mouse granulosa cells, and primary human ovarian stromal cells. Exposures at epidemiologically relevant levels did not markedly elicit cytotoxicity or affect steroidogenesis in short 24-hour exposure. However, significant effects on gene expression were identified by RNA-sequencing. Altogether, the exposures changed the expression

Abbreviations: BMI, body mass index; CCDC163, coiled-coil domain-containing protein 163; DCAKD, dephospho-CoA kinase domain containing; DEG, differentially expressed genes; DEHP, di(2-ethylhexyl) phthalate; DMEM/F12, Dulbecco's modified Eagle's/Ham's F-12 medium; DMSO, dimethyl sulfoxide; EDC, endocrine disrupting chemicals; ELISA, enzyme-linked immunosorbent assay; EPIDEM, epidemiological mixture; EQUI, equimolar mixture; FBS, fetal bovine serum; FDR, false discovery rate; FSH, follicle stimulating hormone; GO, gene ontology; Hs-GC, human primary granulosa cells; IFI6, interferon alpha inducible protein 6; IVF, *in vitro* fertilization; LC-MS-MS, liquid chromatography with tandem mass spectrometry; LOD, limit of detection; MBP, monobutyl phthalate; MBzP, monobenzyl phthalate; MCPP, mono(3-carboxypropyl) phthalate; MECPP, mono(5-carboxy-2ethylpentyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono(ethylhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxyhexyl) phthalate; MEP, monoethyl phthalate; MiBP, monoisobutyl phthalate; Mm-GC, mouse primary granulosa cells; MWHS, the midlife women's health study; PFAS, phosphoribosylformylglycinamidine synthase; POI, primary ovarian insufficiency; RT, room temperature; SPRY4-AS1, SPRY4 Antisense RNA 1; UMAP, Uniform Manifold Approximation Projection; WQS, weighted quantile sums; WQS-m, WQS-based mixture.

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of 124 genes on the average (9–479 genes per exposure) in human cell models, without obvious concentration or mixture-dependent effects on gene numbers. The mixtures stimulated distinct changes in different cell models. Despite differences, our analyses suggest commonalities in responses towards phthalates, which forms a starting point for follow-up studies on identification and validation of candidate biomarkers that could be developed to novel assays for regulatory testing or even into clinical tests.

1. Introduction

Endocrine disrupting chemicals (EDCs) are exogenous substances that interfere with the endocrine system leading to adverse health outcomes [1]. They present a challenge to chemical health risk assessment due to properties that mimic hormonal modes of action instead of conventional toxicity. For example, just like hormones, EDCs can work at low exposure levels, they do not necessarily follow traditional concentration-response relationships, and the exposure-associated adverse outcomes are not always immediate [2]. In addition, combinatorial effects of EDCs take place, which complicates risk assessment further because everyone is exposed to extensive mixtures of chemicals instead of single compounds.

Epidemiological studies have shown that women exposed to EDCs are in higher risk of precocious puberty, primary ovarian insufficiency (POI), endometriosis, miscarriage, longer time-to-pregnancy, lower probability to succeed in infertility treatments, and pregnancy complications such as preterm birth [3,4]. However, these findings are based on correlating EDC concentrations with selected outcomes and do not provide causal proof or information on mechanisms of action. In addition, epidemiological studies traditionally have not accounted for mixture effects. Therefore, the biggest challenges are represented by the need to establish causal proof supporting the associations, and the combination effects of EDCs. Humans are exposed to complex mixtures of chemicals and the net effects cannot be reliably predicted based on the individual chemicals present in the mixture [5]. Yet, risk assessment is carried out one chemical at a time. Safety factors have been proposed to account for the combination effects in analogy to the assessment factors used to account for cross-species and inter-individual differences in risk assessment [6]. This approach has not yet been implemented, and it is not clear how big safety factors would be needed to account for all combination effects.

To evaluate the risks of EDC mixture exposures, we have recently suggested a novel approach that is based on combining epidemiological analyses with experimental studies [7]. The approach requires well-characterized cohorts with information on EDC exposures and adverse outcomes and well-established experimental models for that outcome. The lack of validated and human-relevant model systems is a limitation in reproductive toxicity studies, particularly concerning females. Current regulatory guideline assays are carried out using young fertile rats, and focus on the reproductive organ weights, estrus cyclicity, puberty onset, histology, and pup counts as signs of endocrine disruption targeting female reproductive system [8], but these endpoints are not sensitive to EDC exposures [9] and their relevance to humans can be questioned. There is an urgent need to identify sensitive endpoints and to develop test systems for identification and prioritization of chemicals that disrupt fertility in females. Ideally, these test systems should not be based on animals as the European Parliament has expressed an ambition to phase out the use of animals in research [10].

Phthalic acid diesters, also known as phthalates, form a large group of synthetic chemicals with known EDC effects. Phthalates are non-persistent, and they undergo extensive metabolism in biological systems [11]. The phthalate metabolites have been shown to possess higher bioactivity than their parent compounds. In addition to human urine samples, mixtures of phthalate metabolites can be detected in saliva, breast milk, serum and follicular fluid [12–15]. Animal studies have demonstrated that phthalates inhibit both testicular [16] and ovarian [17–19] steroidogenesis. In addition, epidemiologically defined phthalate mixtures have been found to decrease mouse antral follicle growth and induce oocyte fragmentation *in vitro,* with associated disruption of steroidogenic enzyme expression [20], as well as to exhibit multi- and transgenerational effects on female reproduction [21,22]. In humans, urinary monoisobutyl phthalate (MiBP) correlated negatively to serum estradiol and positively to follicle stimulating hormone (FSH) levels in women suffering from POI compared with a fertile control group, suggesting that MiBP affects the ovarian-hypothalamic feedback mechanism [23]. In addition, di(2-ethylhexyl) phthalate (DEHP) exposure has been associated with an increased risk of decreased ovarian reserve and disruption of gonadotropin stimulated follicle growth in women [13,24]. Despite several studies suggesting phthalates and their mixtures adversely affect ovaries and fertility in women, the exact cell-types and processes influenced by exposure have not been defined. The discovery of such mechanisms will undoubtedly help in the development of novel assays for screening and identification of EDCs that disrupt female fertility.

Here, our goal was to study the impact of endocrine-disruptive, environmentally relevant phthalate mixtures on the key female reproductive organ, the ovary, using *in vitro* cell culture models. The phthalate mixtures were determined based on urinary metabolite concentrations of phthalates reported in the midlife women's health cohort study (MWHS) [25,26] and prepared for experimental studies aiming to identify phthalate target genes in primary human and mouse granulosa cells, immortalized granulosa cell tumor cell lines, and ovarian stromal cell cultures (Fig. 1).

2. Methods

2.1. Definition, preparation, and quality control of the phthalate mixtures

2.1.1. Chemicals

Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (USA). Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) (98% purity), racemic mono(ethylhexyl) phthalate (MEHP) (98% purity), mono (2-ethyl-5-oxyhexyl) phthalate (MEOHP) (97% purity), racemic mono (5-carboxy-2ethylpentyl) phthalate (MECPP) (96% purity), MiBP (98% purity), monobenzyl phthalate (MBzP) (98% purity), mono(3-carboxypropyl) phthalate (MCPP) (97% purity), monoethyl phthalate (MEP) (98% purity), and monobutyl phthalate (MBP) (98% purity) were purchased from Toronto Research Chemicals (Canada).

2.1.2. Definition of the phthalate mixtures

Data on the nine urinary phthalates present in a cohort of almost 800 women enrolled in the MWHS [26,27] formed the basis for the mixtures studied. Urinary phthalates were measured by liquid chromatography with tandem mass spectrometry (LC-MS-MS) from four separate spot urine samples collected over four consecutive weeks and pooled for each woman as previously described [27]. The measured levels were specifically gravity-adjusted and presented as ng/mL. Because the endocrine mode of action of many phthalates is disruption of steroidogenesis, and estradiol is one of the main products of steroidogenesis in women, we used serum estradiol levels as an outcome variable to define an endocrine disrupting phthalate mixture. Serum samples were collected at four visits over four consecutive weeks and used for steroid hormone measurements as described [25]. The levels were averaged to obtain mean hormone concentrations for each woman during one menstrual cycle. For statistical modelling, phthalate levels were divided into

quartiles and estradiol levels log-transformed (Supplementary table 1). Two different statistical approaches were used: linear modelling for the individual chemicals, and weighted quantile sums (WQS) regression encompassing all measured phthalates. In the WQS analysis, both positive and negative associations were tested. Chemicals of concern for the WQS based mixture (WQS-m) were identified as those that had a threshold of $>11.1\%$ (100% divided by 9 chemicals). Individual chemicals were also tested using linear models [28,29]. In all models, covariates used were menopausal status, race, body mass index (BMI), and smoking. Statistical analyses were done using gWQS package in R [30]. Based on the analysis, three different phthalate mixtures were identified: i) the epidemiological mixture (EPIDEM); ii) the equimolar mixture (EQUI), and iii) the WQS-m consisting of those phthalates identified as chemicals of concern in the WQS model. The WQS-m has also been described in [31].

2.1.3. Mixture preparation

Individual phthalates were diluted in DMSO to make the final mixture stock solutions. EPIDEM and EQUI mixtures contained MEHHP, MEHP, MEOHP, MECPP, MiBP, MBzP, MCPP, MEP and MBP, and WQS-m contained MEHHP, MiBP, MBzP and MEP. MEHHP was used as a single phthalate control for comparison to mixtures. Mixtures were diluted to a total volume of 700 μL and then distributed into 10 μL aliquots and stored at −20 °C. Aliquots were shipped to collaborating laboratories overnight on dry ice. These stocks underwent a maximum of three freeze-thaw cycles and were used in exposure studies by adding to media at 0.75 μL/mL to obtain 100x working concentrations. These concentrations were then further diluted with media to obtain the 1x working concentrations. The mixtures were used in cell culture models at two different concentrations (1x and 100x), value referring to the geometrical means in urine of the women in the cohort. Treatment media concentration of DMSO did not exceed 0.075%, which has been used in previous cultures without detrimental effects [32].

2.1.4. Phthalate measurements

Since phthalate metabolites differ in their solubility and can be differentially metabolized in cell culture systems, concentrations of individual phthalates were measured in the conditioned media of the primary cell culture systems following 24 h exposure by LC-MS-MS (Supplementary table 2) using a published method [27,33]. Conditioned media from two replicates were analyzed for all treatment groups: DMSO vehicle control, EPIDEM, EQUI, WQS-m and MEHHP. Standards for MEHHP, MEHP, MEOHP, MECPP, MiBP, MBzP, MCPP, MEP, and MBP were used for LC-MS-MS (adapted from [34]).

2.2. Cell lines

The human granulosa-like tumor cell line KGN was purchased from the RIKEN cell bank (Japan) and the human ovarian granulosa tumor cell line COV434 was purchased from the European Collection of Cell Cultures (ECACC; #07071909). Cell line authenticity was confirmed by Eurofins Genomics Europe Applied Genomics GmbH using short tandem repeats. KGN and COV434 cells were cultured in Dulbecco's modified Eagle's /Ham's F-12 medium (DMEM/F12) (Life Technologies, USA). The basal media was supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 1% Glutamax (all sourced from Life Technologies). Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Primary ovarian stromal cell and granulosa cell culture

2.3.1. Study participants and ethical approval

Three patients undergoing assisted reproductive technology by *in vitro* fertilization (IVF) as well as three patients undergoing gender reassignment surgery at the Karolinska University Hospital Huddinge, Sweden were recruited to the study as granulosa cell (Hs-GC) and ovarian stromal cell donors, respectively. All participants received oral and written information on the project and gave their written informed consent. Follicular fluid was obtained during the standard oocyte pick-up process and once oocytes were collected for patient's treatment, the leftover fluid and cells were donated for research. Ovaries were removed from patients undergoing bilateral salpingo-oophorectomy and hysterectomy for gender reassignment, and ovarian tissue that would otherwise have been discarded was given to research. The tissue was collected in the operating theatre and transported in warm Dulbecco's phosphate-buffered saline supplemented with calcium, magnesium, glucose, and pyruvate (Thermo Fisher Scientific, USA) to the research laboratory for processing and culturing within 10 min. All patients were generally healthy and had no known existing morbidities (Supplementary table 3). This study was approved by the Swedish Ethical Review Authority (license number 2015–798/31, amendment 2016/1523–32).

2.3.2. Human granulosa cell isolation and culture

Hs-GC isolation was initiated by centrifuging the follicular fluid at 400x g for 5 min at room temperature (RT) after which the supernatant was removed and the cells resuspended in 5 mL of DMEM/F-12 base medium containing 20% knockout serum replacement (KSR) (Thermo Fisher Scientific, USA), 1% penicillin/streptomycin (Thermo Fisher Scientific), and Primocin (100 μg/mL) (InvivoGen, USA). The cell suspension was transferred onto an equal volume of 50% (v/v) Percoll (Merck KGaA, Germany) and centrifuged at 500x g for 20 min at RT. After centrifugation, the cells were collected from the interphase between base medium and Percoll into a new 15-mL centrifuge tube containing 5 mL base medium, and centrifuged at 300x g for 5 min. After removing supernatant, the cells were washed once with the base medium, and centrifuged at 300x g for 5 min. Subsequently, supernatant was removed, and the cell pellet was resuspended in dissociation solution containing 2 mL of hyaluronidase (500 U/mL) (Sigma-Aldrich) and 2 μL DNase I (10 U/mL) (Roche, Germany) and incubated in a shaking water bath at 37 °C for 10 min. Enzymatic reaction was stopped by adding 4 mL of base medium. Subsequently the cell suspension was pipetted through a 100 μm cell strainer and centrifuged at 300x g for

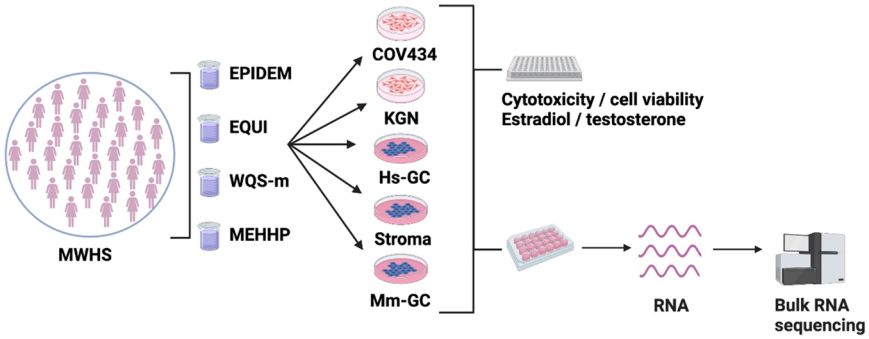

Fig. 1. Schematic representation of the study. The midlife women's health cohort study (MWHS) acted as the starting point for the epidemiological- (EPIDEM); equimolar- (EQUI), and the weighted quantile sum-based mixtures (WQS-m). Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) was used as a single phthalate control. Mixtures were tested on five different cell models, namely granulosa cell cancer cell lines COV434 and KGN, human granulosa cells (Hs-GC), human ovarian stromal cells, and mouse granulosa cells (Mm-GC). Assays studied the effects of phthalate mixtures on cytotoxicity, cell viability, steroid hormone levels as well as gene expression. Figure created with BioRender.com.

5 min. After removing supernatant, the cells were washed once with the base medium and strainer and centrifuged at 300x g for 5 min, after which the supernatant was discarded. The collected Hs-GC were cultured in hESC-Qualified Corning® Matrigel® (Merck KGaA) pre-coated wells in base medium supplemented with 8 ng/mL fibroblast growth factor 2 (R&D Systems, USA) and 1 U/mL FSH (Fostimon; IBSA, Italy) at 2.5×10^4 cells per cm^2.

2.3.3. Human ovarian stromal cell isolation and culture

Freshly collected tissue was processed using sterile scalpels and a spatula in a laminar flow hood to small cubes and cryopreserved as previously described [35]. For thawing for experiments, cryo-tubes were slightly opened and equilibrated at RT for 30 s. Closed tubes were then placed into a 37 °C water bath for 1.5 min, and the pieces were transferred through three different thawing solutions of decreasing sucrose and increasing human serum albumin concentration for 2, 3, and 5 min.

Thawed samples were then cut using scalpels into pieces of ~0.3 mm^3 and enzymatically digested in DMEM/F12 (Thermo Fisher Scientific) containing 5% FBS, 1 mg/mL collagenase IA (Merck KGaA), 50 μg/mL Liberase™ (Roche) and 1000 U DNase I (Roche) in a shaking 37 °C water bath for up to 50 min. Digestion was stopped with medium containing 10% FBS and cell suspension was centrifuged for 7 min on 300g. Cells were resuspended in Dulbecco's Phosphate Buffered Saline, 2% FBS and passed through a 40 μm cell strainer (VWR, USA), counted and used for subsequent experiments. The cells were cultured in low-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (all sourced from Thermo Fisher Scientific).

2.4. Mouse granulosa cell culture

2.4.1. Animals

CD-1 female mice were obtained from Charles River Laboratories (Wilmington, MA). The mice were housed at the College of Veterinary Medicine Animal Facility at the University of Illinois Urbana-Champaign. Animals were subjected to 12 h light-dark cycles and a temperature range of 22 ± 1 °C was maintained. Food (Harlan Teklad 2918) and water were provided for *ad libitum* consumption. The Institutional Animal Use and Care Committee at the University of Illinois Urbana-Champaign approved all animal procedures including animal care, euthanasia, and tissue collection.

2.4.2. Mouse granulosa cell isolation and culture

Cycling CD-1 female mice aged 32–42 days were euthanized with carbon dioxide, and the ovaries from 5 to 10 mice were aseptically removed and placed in supplemented α-minimal essential media (α-MEM, Life Technologies, Grand Island, NY) pre-warmed to 37 °C. Mouse primary granulosa cells (Mm-GC) were isolated from antral follicles with watchmaker's forceps. Cells were passed through a 23-gauge needle 3–4 times and then through a 40 μm cell strainer. Following isolation, cells were seeded in a 100 mm culture dish and expanded for 3 days. Cells were then lifted and seeded in a 96 well plate. Cell density at plating was 1×10^4 cells/well for 96-well plates. Media were supplemented with the following (12.6% total, by volume): 1 × ITS (10 μg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL sodium selenite, Sigma-Aldrich, USA), 1 × antibiotic antimycotic (100 U/mL penicillin, 100 μg/mL streptomycin, 25 ng/mL amphotericin B (Thermo Fisher Scientific), 10% FBS, and 5 IU/mL recombinant FSH (Dr. A.F. Parlow).

2.5. Cell viability and cytotoxicity assays

The cells were seeded onto 96-well plates (COV434: 2×10^4 cells per cm^2; KGN and ovarian stromal cells: 1.5×10^4 cells per cm^2; Hs-GC: 2.5×10^4 cells per cm^2; Mm-GC: 3×10^4 cells per cm^2) and exposed 24 h after seeding (Mm-GC), or on day three (human cell models) to the compounds in cell culture media for 24 h (cytotoxicity assay) or 7 days (cell viability assay), after which assays were conducted. For the 7-day exposure, the media were changed on days one and four after plating.

Cell viability was determined by CellTiter-Glo® luminescent cell viability assay (Promega, USA) according to the manufacturer's standard protocol. Briefly, 100 μL of CellTiter-Glo® reagent was added in each well and the content was mixed for two minutes on an orbital shaker to induce cell lysis, after which the plate was incubated for 10 min at RT and the luminescence was read with SpectraMax i3X Elisa Plate Reader (Molecular Devices, USA).

Cytotoxicity was studied with the CytoTox-Glo™ cytotoxicity assay (Promega) following the manufacturer's protocol. After 24 h exposure, 50 μL of CytoTox-Glo reagent was added to each well. The plate was briefly shaken in an orbital shaker and incubated for 15 min at RT, after which luminescence was read with SpectraMax i3X. Subsequently, 50 μL of lysis reagent was added, mixed, and incubated for 15 min at RT. The luminescence was read after the incubation. Luminescence measured after lysis was adjusted to reflect the live cell contribution by subtracting the initial dead cell signal. The experiments were conducted in three replicates with two wells per condition.

2.6. Steroid measurements in culture medium samples

After 24 h phthalate exposure, media were collected from cells in each well and subjected to enzyme-linked immunosorbent assays (ELISAs) for measurement of the levels of estradiol and testosterone, which are both essential for normal female reproductive function. The levels of these hormones were measured using ELISA kits purchased from DRG International, INC (USA) according to manufacturer's instructions. The detection ranges were 0.083–16 ng/mL for testosterone, and 10.6–2000 pg/mL for estradiol. Samples were run in triplicate. Samples were diluted as needed to match the dynamic range of each ELISA kit, and absorbance was read with BioTek Synergy LX multi-mode plate reader according to the protocol provided with each ELISA kit. LOD was determined as the lowest concentration in the range of detection as stated in ELISA manufacture's protocol.

2.7. RNA sequencing and data analysis

After 24 h phthalate exposure (and media collection for steroid analysis), the cells were lysed using RLT buffer with β-mercaptoethanol. Subsequently, the total RNA was extracted from cell samples with the RNeasy® Plus Micro and Mini kits (Qiagen, Germany), according to the manufacturer's instructions. The concentration and quality of purified RNA were assessed with a fluorometric assay using the RNA High Sensitivity Assay kit (Qubit™, Thermo Fisher Scientific) and 2100 Bioanalyzer with the RNA 6000 Pico kit (Agilent Technologies, USA), respectively. Ten ng of each sample with the RNA integrity number ≥ 8 was used for RNA sequencing library preparation with the Qiagen UPX 3' Transcriptome kit (Qiagen). Samples were pooled according to the cell type after the addition of sample-specific index and unique molecular identifiers (UMIs) during cDNA synthesis. Pooled libraries were sequenced on the Illumina NextSeq 500 instrument (Illumina, USA) using the High Output Kit v2.5 (Illumina) with paired-end configuration of 100 + 27 bp according to the recommendations of the sequencing library kit provider.

The fastq files were demultiplexed using umi_tools (v1.0.0). First, we used the umi_tools whitelist function to whitelist the indexes. Then the umi_tools extract function was used to extract and mark the fastq sequence names with the UMI and indexes which were then used to demultiplex the data. STAR 2.7.9a [36] was used to index the human (hg38/GRCh38) and mouse (mm10/GRCm38) reference genome and align the resulting fastq files. The aligned bam files were deduplicated with the umi_tools dedup function based on their UMI sequence. The deduplicated mapped reads were then counted in annotated exons using featureCounts v1.5.1 [37]. The gene annotations, Homo_sapiens. GRCh38.101.gtf and Mus_musculus.GRCm38.86.gtf, and reference genomes were obtained from Ensembl. The count table from featureCounts

was imported into R/Bioconductor and converted to a DGEList and normalized using Trimmed Mean of *M*-values (TMM) normalization with the edgeR package [38]. Differential gene expression analysis was performed using voom/limma. The gene counts were transformed with voomWithQualityWeights [39] and subsequent differential gene expression analysis was performed in limma [40]. Genes with a false discovery rate (FDR) adjusted p-value < 0.1 were termed significantly regulated. GO analysis was performed using the camera algorithm [41] from the limma package [40] with genesets obtained from molecular signatures database (http://www.gsea-msigdb.org/gsea/msigdb/collections.jsp). Plotting was performed in R using the ggplot2 [42] package and the ComplexHeatmap [43] package for heatmaps. UMAP analysis was carried out with the uwot R package.

Baseline gene expression of steroidogenesis genes, nuclear receptor genes and other relevant genes that had more than 10 counts and expressed in more than 10 samples were considered as expressed in most of the samples and thus plotted from normalized data using ggplot2 package [41].

2.8. Statistical analysis

Statistical analyses were performed in GraphPad Prism (9.4.1). Outliers were identified by Grubb's test and removed from data sets. Data were assessed for normal distribution by Shapiro-Wilk analysis. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* comparisons to vehicle control. For the ELISAs, the mean values of each sample were used for statistical analysis. If the measurement was lower than the limit of detection, the value was substituted with the limit of detection (LOD) divided by the square root of 2. For treatment comparisons, statistical significance was determined by p-value ≤ 0.05.

3. Results

3.1. Phthalate mixtures

Three phthalate mixtures were defined based on data from the MWHS [26,27]: i) EPIDEM, ii) EQUI, and iii) WQS-m. The cohort characteristics are provided in Table 1. While the EPIDEM mixture and EQUI mixtures were based on all urinary phthalates in either same concentrations as the cohort geometrical mean or in equimolar concentration, the WQS-m consisted only of those phthalates that were identified as chemicals of concern using WQS regression.

WQS is a statistical model for highly correlated data such as those encountered in environmental exposures. This dimensionality reduction mixture effect strategy assesses associations of the mixture with health outcomes and determines contributions of each component in the mixture to the overall effect. The WQS model was built on the subset of women where data on outcome and covariates were in place (n = 654) (Table 1). The phthalate mixture was found to be positively associated to serum estradiol (p = 0.05) using the WQS regression (Supplementary table 1). There was a 0.1 unit increase in log-transformed estradiol for every quartile increase of the WQS index (Supplementary table 1). Chemicals of concern (and their weights) were MEHHP (22.3%), MiBP (21.5%), MEP (21.1%) and MBzP (16.0%).

To compare the WQS findings with individual chemical analyses, linear models were used. Significant positive associations were found for MEHHP and MiBP. Women exposed in the third quartile of MEHHP (B = 0.13; SE = 0.0015; p-value = 0.048) and fourth quartile of MiBP (B = 0.14; SE = 0.004; p-value = 0.044) had higher estradiol levels compared to women exposed to their respective first quartiles (Supplementary table 1). Borderline positive association was also found for the fourth quartile of MEHP (B = 0.13; SE= −0.0082; p-value = 0.066) (Supplementary table 1). The EPIDEM and WQS-m mixtures were prepared for experimental studies based on the geometrical mean values from the full cohort (n = 782); the final mixture compositions are presented in Table 2.

The phthalate concentrations measured by LC-MS-MS in conditioned media from three different culture systems (Hs-GC, Mm-GC and stromal cells) after 24 h culture indicated that actual concentrations closely reflected the target nominal concentrations, mirroring the concentrations found in women's urine from the MWHS cohort (Supplementary table 2). In addition, phthalate concentrations were similar between culture systems completed in different laboratories.

3.2. Impact of phthalate mixtures on cell viability and cytotoxicity

Our purpose was to focus on endocrine disruptive effects of phthalates in the absence of overt cytotoxicity or reduced viability. Therefore, both acute cytotoxicity in short exposure (24 h) and impacts on cell viability over long exposure (7 days) were investigated at a baseline concentration (1x) as well as at a hundred-fold concentration (100x). Neither of the concentrations of mixtures or MEHHP induced cytotoxicity in the studied models (Fig. 2). The mixtures did not have prominent effects on cell proliferation either as only 1x EQUI mixture significantly

Table 1
MWHS cohort characteristics.

Characteristic	WQS cohort N = 654	Full cohort N = 782
Estradiol, median (min-max) (ng/mL)	56.2 (5.6–355.1)	56.6 (5.6–378.0)
Missing, n (%)	0	1 (0)
BMI, median (min-max)	26.5 (15.6–67.7)	26.5 (15.6–67.7)
Missing, n (%)	0	7 (1)
Menopausal status, n (%)		
Premenopausal	384 (59)	463 (59)
Perimenopausal	270 (41)	305 (39)
Postmenopausal	0	1 (0)
Missing	0	13 (2)
Race, n (%)		
White	447 (68)	504 (65)
Black	207 (32)	238 (30)
Other	0	30 (4)
Missing	0	10 (1)
Smoking status, n (%)		
Never smoker	358 (55)	422 (54)
Former smoker	229 (35)	271 (35)
Current smoker	67 (10)	80 (10)
Missing	0	9 (1)

Table 2
Composition of the three phthalate mixtures at the 1x dilution that corresponds to the geometrical mean concentrations in urine of the MWHS women.

Metabolite (parent phthalate)	EPIDEM ng/mL (nM) %	EQUI ng/mL (nM) %	WQS-m ng/mL (nM) %	Single phthalate ng/mL (nM) %
MEHHP (DEHP)	45.98 (156.4) 8.1	62.7 (213.3) 11	45.98 (156.4) 9.9	45.98 (156.4) 100
MEHP (DEHP)	6.90 (24.8) 1.3	59.3 (213.3) 11	-	-
MEOHP (DEHP)	17.53 (60.0) 3.1	62.3 (213.3) 11	-	-
MECPP (DEHP)	37.69 (122.4) 6.4	65.7 (213.3) 11	-	-
MiBP (DiBP)	22.67 (102.1) 5.3	47.4 (213.3) 11	22.67 (102.1) 6.5	-
MBzP (BBzP)	12.90 (50.4) 2.6	54.6 (213.3) 11	12.90 (50.4) 3.2	-
MCPP (DOP/DBP)	3.74 (14.8) 0.8	53.8 (213.3) 11	-	-
MEP (DEP)	244.6 (1260.6) 65.6	41.4 (213.3) 11	244.6 (1260.6) 80.3	-
MBP (DBP)	28.65 (129.0) 6.7	47.4 (213.3) 11	-	-
SUM	**420.6 (1919.9)**	**495.2 (1919.7)**	**326.1 (1569.5)**	**45.98 (156.4)**

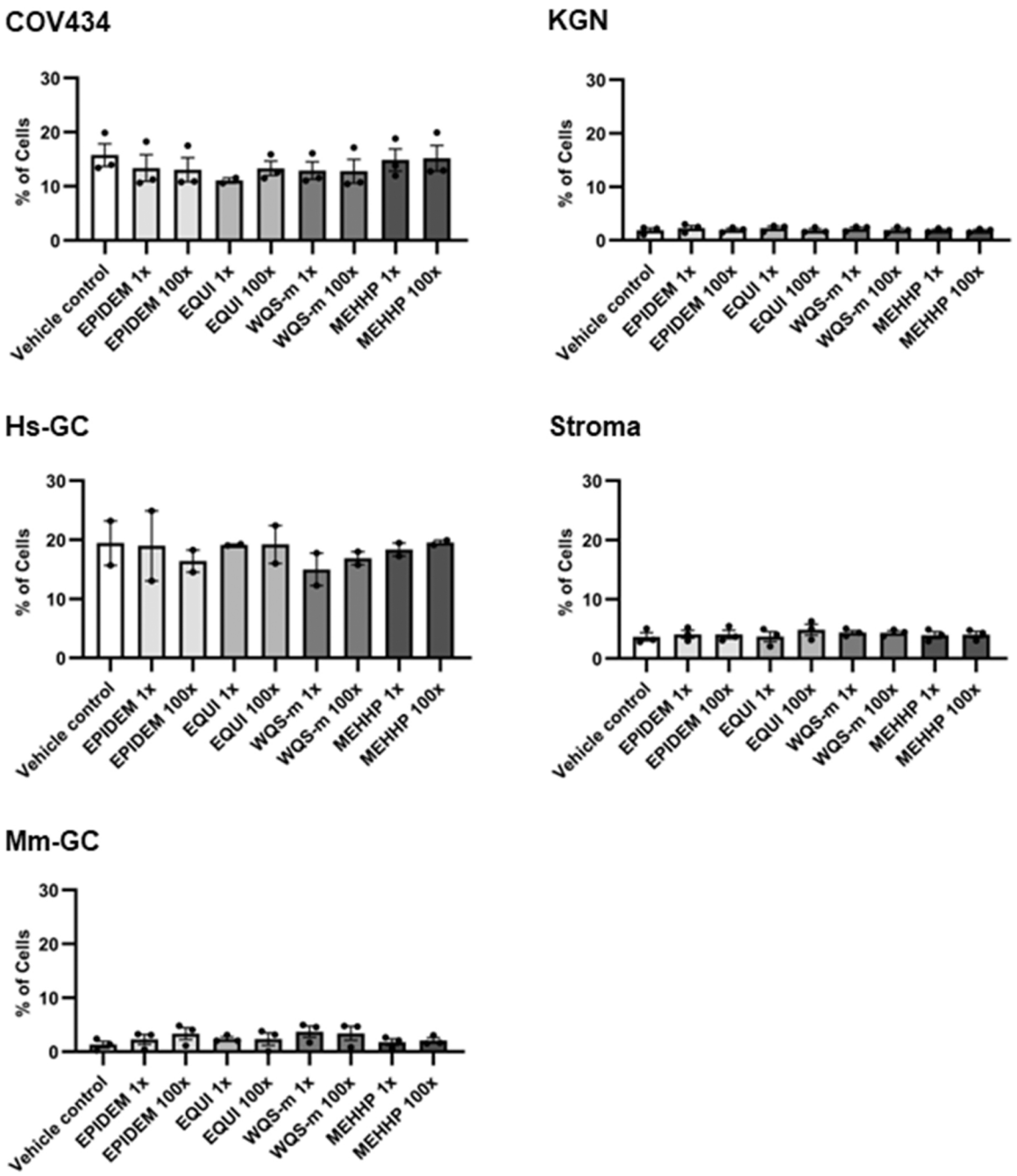

Fig. 2. Acute cytotoxicity of phthalate exposures. The percentage of dead cells after 24-hour exposure to phthalates was measured by CytoTox-Glo assay. None of the studied phthalates at 1x or 100x concentrations induced significant cytotoxicity in COV434, KGN, Hs-GC, human ovarian stromal cells, or Mm-GC compared to vehicle control. Data presented as mean ± SEM and the individual measurements are depicted with dots (N = 2–3; *p < 0.05 *vs* vehicle control, ANOVA followed by Dunnett's test).

reduced the cell proliferation of human ovarian stromal cells compared to vehicle control (−8.3%) (Supplementary figure 1). No other statistically significant effects were observed between treatment groups. The absence of direct toxicity suggests that the phthalates and exposure levels were appropriate for identification of endocrine disruptive effects.

3.3. *Effects of phthalate mixtures on the steroidogenesis of ovarian cells*

As the WQS modelling outcome was serum estradiol levels in women, and estradiol in pre-menopausal women is mainly produced by granulosa cells in the ovaries, we determined if the ovarian cell models produced steroids and whether the levels were affected by the phthalate exposures. Because testosterone is a precursor of estradiol, and it could also have an impact on fertility on its own, testosterone levels were measured as well. In general, the concentrations found in spent culture media samples indicated that Hs-GC and Mm-GC had the highest estradiol and testosterone levels of the studied cell models in our experimental settings (estradiol concentration in vehicle treated media 4799 and 88 ng/mL, testosterone concentration in vehicle treated media 1.8 and 0.8 ng/mL in Hs-GC and Mm-GC respectively). Meanwhile, without induction, the granulosa tumor cell lines KGN and COV434 had very low baseline estradiol and testosterone levels, with the measured values being mainly under the level of detection (estradiol concentration in vehicle treated media 12 and 13 ng/mL, testosterone concentration in

vehicle treated media 0.7 and 0.8 ng/mL in COV434 and KGN respectively). The stromal cells did not have significant steroidogenic potential *in vitro* (estradiol concentration in vehicle treated media 2 ng/mL, testosterone concentration in vehicle treated media 0.7 ng/mL). Therefore, only results from the primary granulosa cell cultures are presented. In Mm-GC, the 100x EPIDEM mixture showed significantly higher estradiol levels compared to the vehicle control, whereas 100x MEHHP decreased the estradiol levels significantly (Fig. 3). No differences in testosterone levels of mouse cells were found in response to the mixtures. The mixtures did not impact testosterone or estradiol levels in Hs-GC (Fig. 3).

3.4. Transcriptomic effects of phthalates

To study the acute impacts of the phthalate mixtures and MEHHP on gene expression across the cell models, highly multiplex transcriptomic profiling was performed after short 24 h exposure to 1x and 100x concentrations. Uniform manifold approximation projection (UMAP) of the RNA-sequencing results with differentially expressed genes (DEGs) showed that the main transcriptomic differences in the human dataset were driven by differences between the cell models, with KGN and stromal cells being the most similar to each other, and COV434 and Hs-GC being the most different from each other (Fig. 4A). We also compared the human cell responses to Mm-GC (Fig. 4B). For this purpose, those DEGs with orthologs in mice were considered. The clustering indicates that the Mm-GC were the most similar to KGN and stromal cells.

Because the cell models were clearly different from each other, they were kept separate in downstream bioinformatic analyses. DEGs were identified in each cell model as those that significantly changed in expression compared to vehicle control at FDR< 0.1. In general, the results did not indicate combinatory or concentration dependent effects of phthalates on the numbers of genes regulated in any cell model. In all cell lines, 9–479 DEGs were found in the different exposure groups (Fig. 5). In KGN cells, the exposures resulted in the greatest number of upregulated DEGs (average 72 upregulated DEGs/exposure compared to 17, 67, and 37 upregulated DEGs/exposure in Hs-GC, COV434, and stromal cells respectively), whereas COV434 cells demonstrated the greatest number of downregulated DEGs (average 230 DEGs/exposure compared to 30, 30 and 12 downregulated DEGs/exposure in KGN, Hs-GC and stromal cells respectively). In Mm-GC, the analysis did not result in the discovery of DEGs at FDR< 0.1.

Next, we studied how similar the responses to the different phthalate exposures were within a cell model by looking at the fractions of shared DEGs. The heatmap illustrating the fractions of shared DEGs between exposures in Fig. 6 shows that the similarity in responses was statistically highly significant ($p < 0.0001$ for all comparisons) with Jaccard coefficients varying from 0.02 to 0.48. The lowest fractions of shared genes between phthalate exposures were found in human stromal cells (Jaccard 0.02–0.14), and the highest in COV434 cell line (Jaccard 0.21–0.48) (Fig. 6). The different concentration groups (1x *vs* 100x) in each exposure within a cell model showed comparable levels of similarity. The single phthalate control MEHHP behaved similarly, suggesting that the level of shared genes by concentration is not affected by combination effects of phthalates. The clustering of the heatmaps further shows that there was no clear grouping based on the phthalate exposures (Fig. 6).

In order to explore the presence of universally affected DEGs, we studied the overlaps in phthalate exposure effects between the human cell models by identifying all unique DEGs by cell line. This gave in total 1006 DEGs in COV434, 614 in KGN, 253 in Hs-GC, and 285 in human primary stromal cells. The overlap between these was five genes, which were coiled-coil domain-containing protein 163 (*CCDC163),*

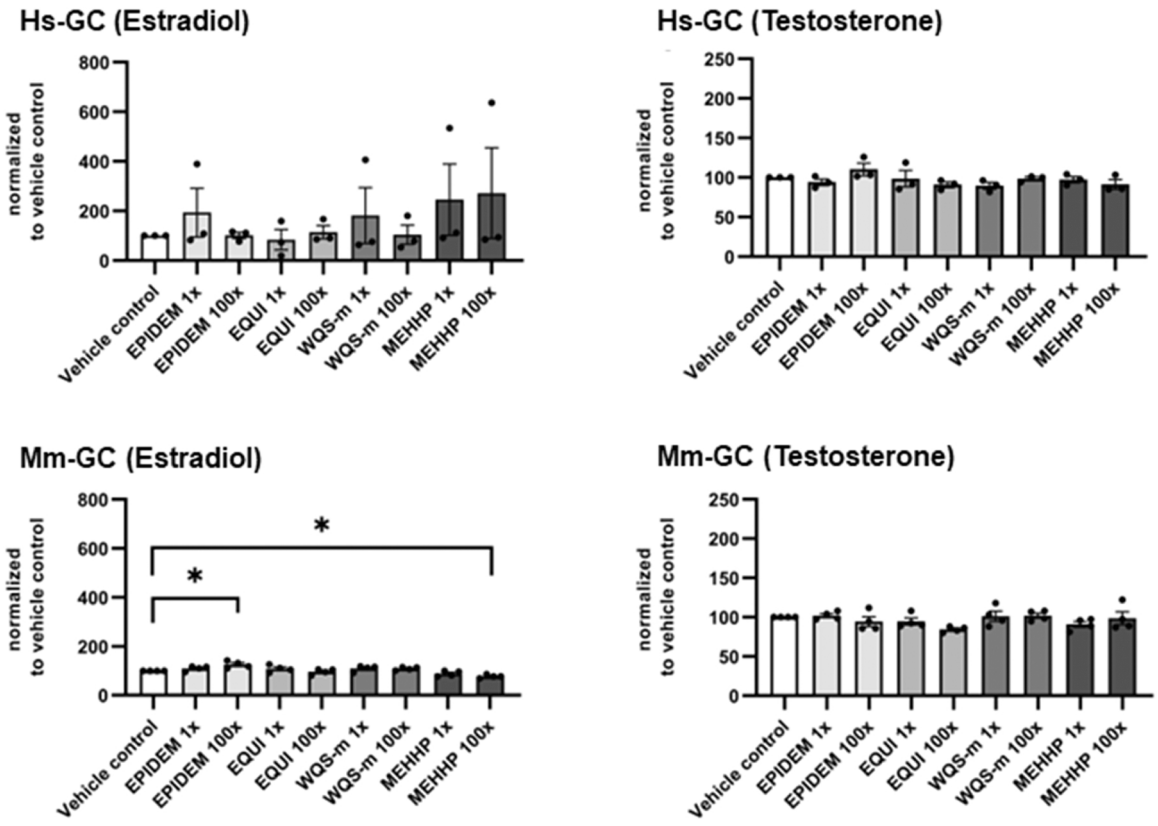

Fig. 3. Steroid production in granulosa cell culture. Estradiol and testosterone levels were measured by ELISA from the media of human and mouse primary granulosa cells exposed to phthalates for 24 h. The exposures did not significantly alter testosterone levels in the media, or the levels of steroids in human cell models. EPIDEM and MEHHP exposures significantly altered estradiol levels at 100x exposure in Mm-GC. Data presented as normalized to vehicle control ± SEM and the individual observations are depicted with dots (N = 3–4; *$p < 0.05$ *vs* vehicle control, ANOVA followed by Dunnett's test).

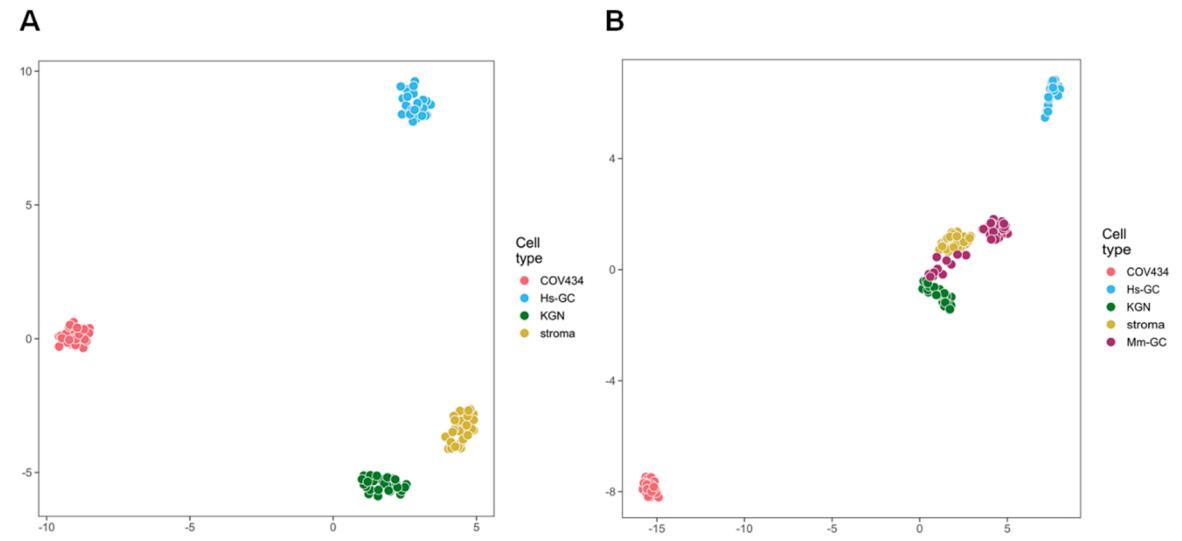

Fig. 4. Uniform Manifold Approximation Projection (UMAP) of the RNA-sequencing results. Cells were treated for 24 h with phthalate mixtures and transcriptomic responses were measured with RNA-sequencing. The overall results are visualized using the UMAP dimension reduction analysis based on the differentially expressed genes (DEGs) in human cell models (A) as well as in human and mouse cell models (B). For the human and mouse comparison, the mouse orthologues of the human DEGs were used and the mouse *vs* human batch effect was corrected. The clustering was driven by the cell models; KGN, stromal cells and Mm-GC cells were more similar to each other and different from Hs-GC and COV434.

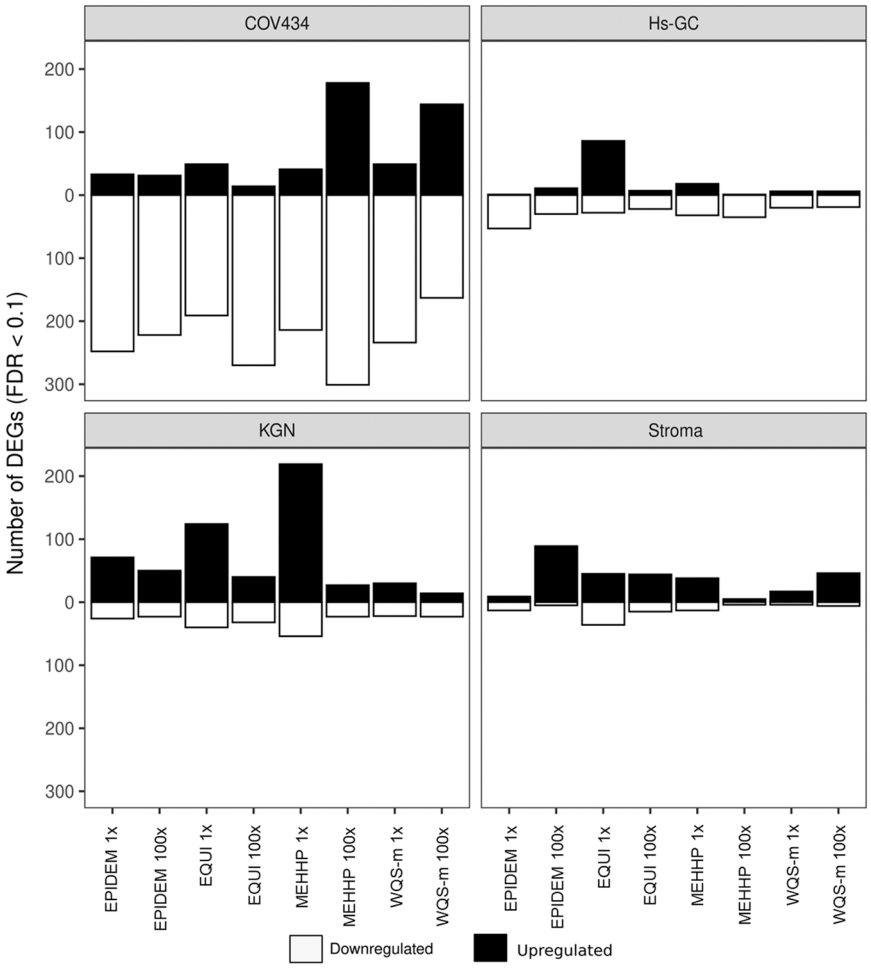

Fig. 5. Overview of numbers of differentially expressed genes (DEGs) in response to phthalate treatments in the different cell models. Differentially expressed genes (DEGs) were identified as those that differed in expression from the vehicle control group (FDR<0.1) after 24 h exposure to EPIDEM, EQUI, WQS-m and MEHHP at 1x and 100x concentrations. Numbers of up- and down-regulated genes by phthalate treatments are shown in the different cell models. There were no clear concentration-response trends in terms of numbers of genes regulated by the 1x and 100x treatments or by the complexity of the exposure. Overall, most DEGs were found in the COV434 cell line.

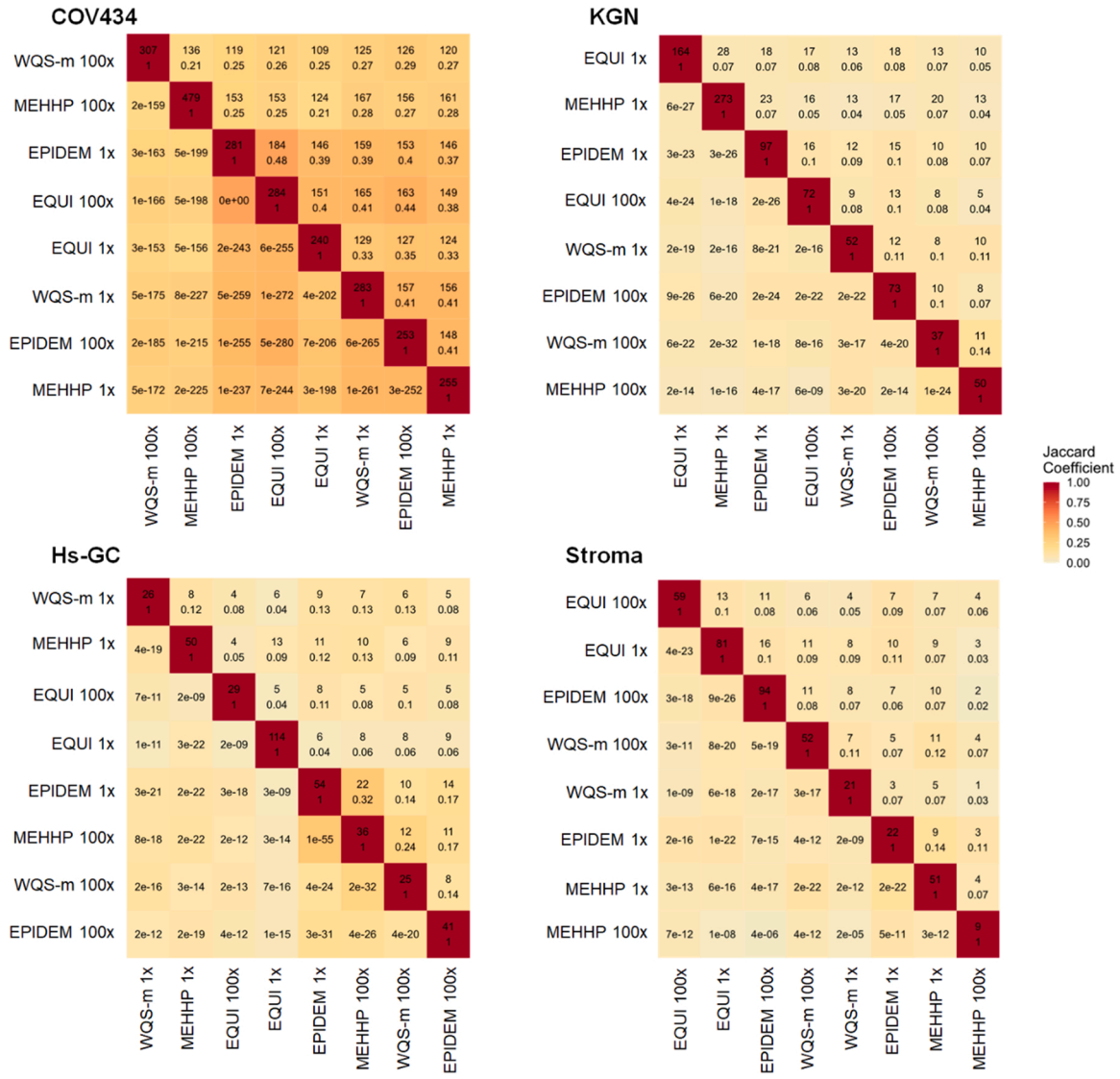

Fig. 6. Similarity in gene expression response to different phthalate mixtures. Jaccard coefficient was used to estimate the similarity in gene expression changes in response to different phthalate mixtures (EPIDEM, EQUI, WQS-m and MEHHP) in human cell models, namely COV434, KGN, Hs-GC and stroma. The plots display the number of shared DEGs between treatments and the Jaccard coefficient (top half) as well as the hypergeometric p-value (bottom half). The treatments are hierarchically clustered. The overlaps were statistically significant in every comparison. Highest Jaccard coefficients were found in the COV434 cells.

dephospho-CoA kinase domain containing *(DCAKD),* interferon alpha inducible protein 6 (*IFI6*), phosphoribosylformylglycinamidine synthase (*PFAS*), and SPRY4 antisense RNA 1 (*SPRY4-AS1*) (Fig. 7, Supplementary file 1).

To also include the mouse model, next we studied which cellular processes were affected by phthalate exposures in the different cell models. This was determined utilizing camera analysis and GOs as gene sets. This allows the inclusion of the mouse data into the analysis as the analysis is based on ranking all genes rather than focusing on DEGs. Using all expressed genes ranked by their test statistic, significantly enriched GOs were identified (Supplementary file 2). A great number of significantly enriched unique GOs were found in all cell models (total $n = 10{,}114$ at $p < 0.01$). Over half of the GOs were unique and enriched in only one model ($n = 5{,}339$) in line with the observation of cell model having the greatest impact on the transcriptomic results. Stromal cells had the greatest number of shared GOs ($n = 2{,}643$) with at least one other model, followed by Hs-GC ($n = 2{,}519$), KGN ($n = 2{,}485$), COV434 ($n = 2{,}210$) and Mm-GC ($n = 1{,}989$). To get an overview of the characteristics of the significant GOs, we prepared a heatmap based on ranking their p-values. For this analysis, only those GOs that were based on at least 5 genes present in our dataset were included, and the results were further visualized by UMAP. Both the heatmap and the UMAP show clear clustering by cell type, not by treatment (Supplementary figure 2). Hence, all phthalate exposures triggered similar GOs in a given cell model that are different from the top GOs in other cell models. The clustering of the heatmap suggests that KGN and stromal cells are closest to each other in terms of GO responses (Supplementary figure 2 A).

All analyses implicated clear differences between the cell models despite their ovarian origin. We hypothesized that it could depend on differential baseline expression of genes with central involvement in ovarian biology and phthalate responses, such as genes related to steroidogenesis (*e.g.*, *STAR*, *CYP11A1*, *CYP19A1),* granulosa cell function (*e.g.*, *FOXL2*) and nuclear receptors (*e.g.*, *ESR*, *GR*, *AR*). We therefore plotted the normalized expression levels of such genes that were expressed in at least 10 samples using RNA-sequencing data from untreated cells (Supplementary figure 3). Most of the classical steroidogenesis genes and nuclear receptors were not highly expressed in the samples. Several genes demonstrated similar trends between the human cell models, however, some characteristic differences in baseline expression of genes could be observed especially in the COV434 cell line

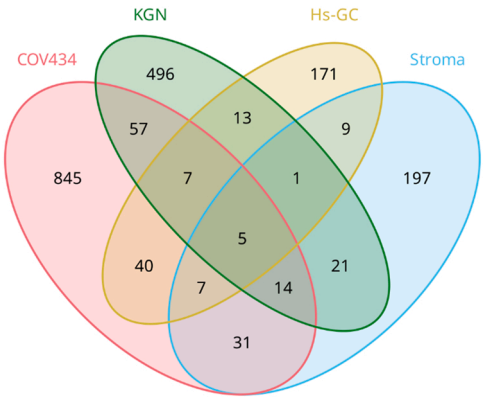

Fig. 7. Overlap in differentially expressed genes between the human cell models. Venn diagram illustrating the overlap in DEGs regulated by the phthalates in COV434, KGN, stroma and Hs-GC cell models. For the comparison, all identified DEGs per cell line were pooled and duplicates removed to study the overall similarity in responses to phthalates. The five universally shared DEGs were coiled-coil domain-containing protein 163 (*CCDC163),* dephospho-CoA kinase domain containing *(DCAKD),* interferon alpha inducible protein 6 (*IFI6*), phosphoribosylformylglycinamidine synthase (*PFAS*), and SPRY4 antisense RNA 1 (*SPRY4-AS1*).

and Hs-GC (Supplementary figure 3). For example, *StAR*, *HSD17B1* and *HAS15/RDH11* genes are highly expressed in Hs-GC, but expressions were relatively low in other human cells, whereas the COV434 cell line did not seem to express for example *AR* or *AHR*. Interestingly, only Mm-GC expressed *ESR1* while *ESR2* was not highly expressed in any models under this sequencing depth (data not shown). These observations indicate clear differences in expression levels of the traditional key targets of EDCs including phthalates; nuclear receptors and steroidogenic enzymes, in models that are commonly used to study ovarian biology *in vitro*.

Despite differences in responses, we set to identify commonalities across the exposures and cell models. We started by considering functional similarities between all identified significant GOs by studying their semantic similarity. When all GOs were displayed by UMAP, six main functional clusters were found (Fig. 8). Interestingly, these clusters were present in all five cell models. We annotated these functional GO clusters by identifying the most common words per cluster and found them to characterize functions related to: trans-membrane transport, metabolism, differentiation, chemotaxis, cell adhesion and kinase signaling (Fig. 8; Supplementary file 2). In addition, within the 10,114 significant GOs we found 66 that were shared between all five cell models (Supplementary table 4). Further inspection of these 66 GOs suggested that all mixtures affected genes involved in post-translational modifications, development and differentiation, circadian rhythms, and different metabolic processes. Of the five identified overlapping DEGs, two (*DCAKD* and *PFAS*) were associated with three of the 66 shared GOs. These GOs were coenzyme A biosynthetic process (*DCAKD*), ribonucleoside monophosphate biosynthetic process (*PFAS*), and cellular process (*DCAKD* and *PFAS*).

In summary, our transcriptomic analyses suggest that all phthalate exposures evoke gene expression changes in a wide variety of ovarian cell models. Similar numbers of genes were regulated in 1x and 100x exposures to mixtures and the single-phthalate treatment, suggesting that all exposures can have effects on the cells regardless of the mixture composition or the concentration at least in acute exposure settings. Interestingly, despite the differences between cell lines, and differing phthalate exposures (composition, concentration), some commonalities were found in the regulated genes and GOs.

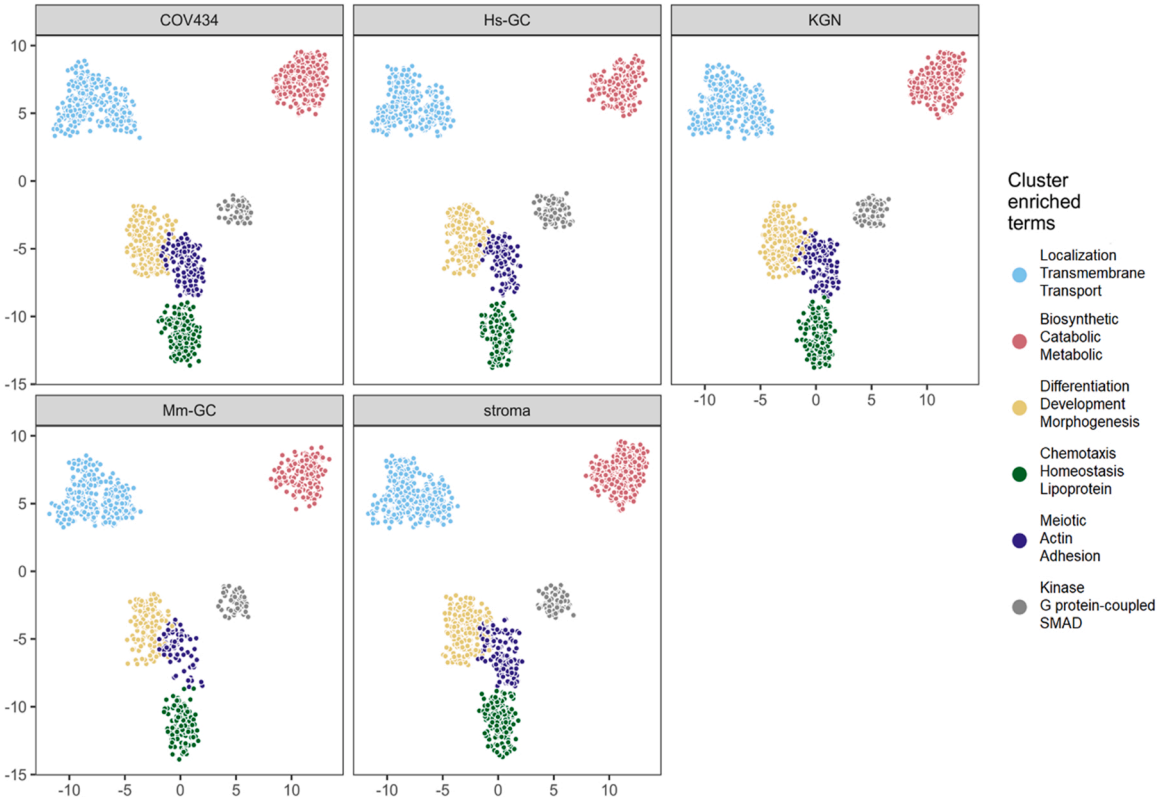

Fig. 8. UMAP of significantly enriched GO terms in human and mouse models based on their semantic similarity. Each dot represents one GO. In both human and mouse models, six main clusters were found. The most common words characterizing the clusters are given. Functional cluster members (1–6) are presented in the Supplementary file 2.

4. Discussion

Even though humans are exposed to mixtures of chemicals, chemical health risk assessment has traditionally been based on the evaluation of individual substances. An understanding of combined exposures and effects has shifted the focus and interest towards mixture risk assessment [5,44]. The most widely used method for mixture risk assessment is the dose-addition principle, which assumes that mixture effect is the sum of activities of its individual components [45]. Characterizing the composition of environmentally relevant mixtures for experimental purposes remains extremely challenging. One common approach to cumulative mixture risk assessment is to focus on grouping chemicals that are structurally related or have similar mechanism of action. In the current study, we focused on phthalates, and defined three phthalate mixtures from the MWHS cohort of almost 800 women for experimental *in vitro* studies. The urinary levels of phthalates in the MWHS cohort are in agreement with the Swedish SELMA study [46], the NHANES biomonitoring study [47], as well as several other exposure studies [48–51]. The measured concentrations of individual phthalates in the conditioned media in our experiments closely reflected the targeted concentrations. In general, phthalate metabolite levels are assumed to be higher in urine than in serum [48]. Due to their fast metabolism, serum levels are challenging to measure reliably, and they are therefore often estimated based on urinary levels, being 3–4 fold lower in serum [52]. Due to the lack of validated test system to study female fertility *in vitro* or to assess the differences across various species and cell types, the effects of the phthalate mixtures were studied in five different culture systems, namely granulosa cell cancer cell lines KGN and COV434; Hs-GC; Mm-GC and primary human ovarian stromal cells.

Phthalates have been demonstrated to dysregulate steroidogenesis both *in vivo* [53–55] and *in vitro* [53,54,56]. Majority of previous reports have shown decrease in estradiol levels [55–58] and few studies have also reported reduced testosterone levels [57,59]. However, for example in a test guideline assay for the *in vitro* H295R (adreno-carcinoma cell line) steroidogenesis, both DEHP and DINP increased estradiol production [60]. Here we observed significant changes in estradiol levels only in cultured Mm-GC, where, in line with most of the literature, MEHHP decreased estradiol levels. In line with MWHS study, EPIDEM increased the estradiol secretion, yet other human studies have shown either a negative- or no association between phthalate and steroid hormone levels [61–63]. It is noteworthy that the literature described above has focused on individual phthalates, not mixtures, and it is likely that in addition to the exposure, the results depend on interspecies and inter-individual differences. Furthermore, we only studied acute 24 h exposures, and longer exposure times may be needed to capture more effects on steroidogenesis in the cells. Steroid production was robust in the Hs-GC and Mm-GC in our studies, which is supported by their marked expression of steroidogenesis enzymes. In KGN or COV434, induction of steroidogenesis may be needed (for example, with forskolin). The transcriptomic results were in line with the observed modest impacts on steroidogenesis. No common signatures or genes related to core ovarian steroidogenesis were found to be affected by the exposures.

In the transcriptomic analysis, each cell line was considered separately due to the great differences identified among the cell models. While significant numbers of genes were regulated in response to the phthalates, overlaps in DEGs between the exposures in each cell model, and between the cell models, were rather small. Yet, if the results were random, no such overlaps would be expected at all, as indicated by the very low p-values. When focus was shifted from DEGs to more holistic analyses considering all genes, enrichment analyses revealed thousands of significantly enriched GOs. The GOs mostly described generic processes, which is in line with several previously reported transcriptomic alterations induced by phthalates, but also reflects the abundance of generic GOs over specific ones in the databases [64–67]. Another important issue highlighted by the transcriptomic analysis arises from the differences between the cell models. Granulosa cancer cell lines COV434 and KGN are very commonly used as ovarian cell models in *in vitro* toxicity testing. As commercial cell lines, they are readily available and easy to work with, yet our current results challenge the representativity of COV434 cells as an ovarian cell model. COV434 cells seem to cluster separately from other models both in terms of baseline levels of gene expression as well as in the responses.

The composition of phthalate mixtures did not play as crucial a role as expected, whereas the differences between the models used were more prominent than anticipated. This could depend on the short 24 h exposure that we chose to capture the acute effects of exposures. The observed responses could even represent a general acute cellular reaction to exogenous chemicals. This hypothesis should be further tested by exposure to wider selection of chemicals. Another possibility is that the responses were driven by MEHHP that was present in every tested mixture at similar levels. Further studies should test exposing the cells to mixtures that do not contain MEHHP.

We can, however, conclude that by utilizing several *in vitro* models, our study identified five overlapping DEGs and 66 GOs prevalent in all different cell models. Interestingly, the common GOs did not indicate traditional endocrine modes of action but rather mechanisms related to cellular metabolism, differentiation, cell adhesion and kinase signaling pathways. It has been long recognized that EDC research should expand beyond the so-called estrogen-androgen-thyroid-steroidogenesis (EATS) modalities. Our results will serve as a starting point to investigate possible 'phthalate signature effects', cellular responses to short chemical exposure that ideally could even be used to identify ovarian EDCs at large. Additional investigation is required to characterize and validate the candidate biomarker genes of phthalate exposure and to identify the most representative *in vitro* model(s) for studying female reproductive toxicity *in vitro*. Such models are urgently needed considering the substantial number of chemicals on the market and the lack of models that can predict reproductive disruption in women. For example, in the European Union around 100 000 chemicals are on the market and only 0.5% of them have been well-characterized for hazards and exposures [68]. Already in 2018, the European Commission flagged female reproduction as a main adverse outcome area where more research and test development is needed [69]. Future studies should identify a set of core markers that could be used as biomarkers of phthalate and EDC exposure and effect in ovaries.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The RNA-sequncing data have been deposited to GEO with number GSE223645.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2023.108393.

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