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Systematic Transcriptome-wide Meta-Analysis across Endocrine Disrupting Chemicals Reveals Shared and Unique Liver Pathways, Gene Networks, and Disease Associations

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Abstract

Cardiometabolic disorders (CMD) are a growing public health problem across the world. Among the known cardiometabolic risk factors are compounds that induce endocrine and metabolic dysfunctions, such as endocrine disrupting chemicals (EDCs). To date, how EDCs influence molecular programs and cardiometabolic risks has yet to be fully elucidated, especially considering the complexity contributed by species-, chemical-, and dose-specific effects. Moreover, different experimental and analytical methodologies employed by different studies pose challenges when comparing findings across studies. To explore the molecular mechanisms of EDCs in a systematic manner, we established a data-driven computational approach to meta-analyze 30 human, mouse, and rat liver transcriptomic datasets for 4 EDCs, namely bisphenol A (BPA), bis(2-ethylhexyl) phthalate (DEHP), tributyltin (TBT), and perfluorooctanoic acid (PFOA). Our computational pipeline uniformly re-analyzed pre-processed quality-controlled microarray data and raw RNAseq data, derived differentially expressed genes (DEGs) and biological pathways, modeled gene regulatory networks and regulators, and determined CMD associations based on gene overlap analysis. Our approach revealed that DEHP and PFOA shared stable transcriptomic signatures that are enriched for genes associated with CMDs, suggesting similar mechanisms of action such as perturbations of peroxisome proliferator-activated receptor gamma (PPARγ) signaling and liver gene network regulators *VNN1* and *ACOT2*. In contrast, TBT exhibited highly divergent gene signatures, pathways, network regulators, and disease associations

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CRediT authorship contribution statement

Zacary Zamora: Conceptualization, Data curation, Formal Analysis, Methodology, Visualization, Writing – original draft. **Susanna Wang:** Writing – review & editing. **Yen-Wei Chen:** Methodology. **Graciel Diamante:** Supervision, Visualization, Writing – review & editing. **Xia Yang:** Conceptualization, Supervision, Writing - review & editing.

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.

from the other EDCs. In addition, we found that the rat, mouse, and human BPA studies showed highly variable transcriptomic patterns, providing molecular support for the variability in BPA responses. Our work offers insights into the commonality and differences in the molecular mechanisms of various EDCs and establishes a streamlined data-driven workflow to compare molecular mechanisms of environmental substances to elucidate the underlying connections between chemical exposure and disease risks.

Keywords

Cardiometabolic disease; Liver; Transcriptome; Endocrine disrupting chemicals; Meta-analysis; Network analysis

1. Introduction

Cardiometabolic disorders (CMDs) such as metabolic syndrome (MetS), obesity, diabetes, hypertriglyceridemia, cardiovascular disease (CVD), and non-alcoholic fatty liver disease (NAFLD) contribute to a fast-growing health epidemic worldwide and inflict high rates of mortality and morbidity (Hurt et al., 2012; Raghavan et al., 2019; Roth et al., 2020). Both genetic and environmental factors, as well as the interactions between the two, can contribute to CMD predisposition and progression. Among the known cardiometabolic risk factors are exposure to endocrine disrupting chemicals (EDCs) (Janesick et al., 2011). EDCs are a class of mostly man-made exogenous chemicals that are used in industrial products, and many have been observed in human tissue samples such as liver and blood (Papalou et al., 2019). EDCs have been shown to alter pathways important in CMDs such as lipid and glucose metabolism pathways, PPAR signaling, and steroid hormone biosynthesis (Grün and Blumberg, 2007; Casals-Casas et al., 2008; Schug et al., 2011; Alves-Bezerra and Cohen, 2017; Heindel et al., 2017). However, the risk that environmental factors such as EDCs pose on CMD development, and the underlying mechanisms are still not fully understood.

Among the EDCs identified to date, bisphenol A (BPA), bis(2-ethylhexyl) phthalate (DEHP), tributyltin (TBT), and perfluorooctanoic acid (PFOA) are of particular interest due to their prevalence and capacity to confer CMD. BPA is an estrogenic chemical that is used in synthetic polymer goods and as the lining of various food containers (Schecter et al., 2010). Notably, BPA has been linked to various cardiometabolic risk factors such as hypertension, dyslipidemia, and abdominal obesity (Plourde et al., 2002; Benmohammed et al., 2011; Li et al., 2015). DEHP is an anti-androgenic chemical that is used in the production of flexible plastics, found in building materials, toys, food containers, and present in certain medical devices (Jarfelt et al., 2005; Shea, 2003). Similar to bisphenols, human DEHP exposure has been linked to increased body weight, increased plasma triglyceride levels, and elevated blood pressure (Mohammad et al., 2018). Marine biocides such as tributyltin, are used in paints that are applied to ships and fishing nets because of their disinfecting and anti-fouling properties (Ximenes et al., 2017). Despite the limited number of epidemiological studies of human exposure to organotins like TBT, they are classified as EDCs because TBT and its metabolites have been linked to increased adipogenesis and lipid accumulation in mammals and have been found in the blood and liver

of humans (Heindel and Blumberg, 2019; Jia et al., 2016; Ronconi et al., 2018). Another EDC is PFOA, a chemical used in commercial household products, such as cookware, food packaging, cosmetics, and other products that resist heat, oil, stains, and grease (Begley et al., 2005). PFOA does not readily break down in the environment, allowing it to easily bioaccumulate (Steenland et al., 2010). Moreover, PFOA exposure in mouse and human studies has been linked to increased weight, waist circumference, cholesterol levels, and prevalence of diabetes (Halldorsson et al., 2012; Fei et al., 2007; Hines et al., 2009; He et al., 2018). All these compounds have been observed in human samples (Calafat et al., 2004; Koch et al., 2006; Antizar-Ladislao, 2008; Calafat et al., 2007). However, there is a lack of systematic molecular comparison across EDCs and the similarities and differences in the mechanisms that contribute to CMD risks are still not fully understood. Therefore, it is important to discern how these chemicals alter important biological pathways and gene networks that may lead to CMD development and progression.

Due to the risks that chemicals such as EDCs pose to human health, several research programs such as Toxicology in the 21st Century (Tox21) and Toxicity Forecaster (Toxcast) have been launched to predict and characterize chemical effects in an efficient and high throughput manner (Attene-Ramos et al., 2013; Dix et al., 2007). The goals of these initiatives influenced the increase of numerous high-throughput molecular studies using transcriptome profiling. Many of these datasets have been deposited into the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO), an open access international genomics repository that archives various high-throughput data, including microarray, RNAseq and other omics datatypes (Barrett et al., 2013). While these studies are a valuable resource, the variability in species, dosage, treatment windows, platforms/technologies used to generate, analyze, and interpret the data, leaves a fragmented picture of the biological effects of these EDCs and makes comparisons across studies and chemicals difficult. Harmonizing and meta-analyzing these datasets are a major challenge but it is crucial to conduct a systematic investigation of the biological effects within and across EDCs to ascertain consistent/different pathways and mechanisms of these toxicants. To this end, we developed an analytical workflow to uniformly consolidate and process different types of high throughput sequencing data to characterize and compare transcriptomic profiles and molecular networks across EDCs. To do this, we 1) streamlined and standardized microarray and RNA sequencing data acquisition and processing, 2) calculated gene expression changes from individual studies varying in EDC type, species, exposure route, sex, and dosage, 3) clustered the studies based on similarity in gene expression changes and identified consensus differentially expressed genes (DEGs) for each cluster (a group of studies sharing similar gene expression changing patterns) for each EDC, 4) conducted weighted key driver analysis to identify potential regulators and gene networks induced by EDC exposure, and 5) linked the genes and pathways to CMDs based on overlaps between EDC signatures and disease candidate genes. Specifically, we meta-analyzed 30 liver transcriptomics studies for BPA, DEHP, PFOA, and TBT deposited in GEO, to understand how these EDCs perturb the transcriptome and affect cardiometabolic risks. We focused on liver as it is a critical tissue for lipid, glucose, and cholesterol metabolism (Maradonna and Carnevali, 2018), xenobiotic biotransformation and clearance (Guomao et al., 2016), and CMDs (Monami et al., 2003; Duvnjak et al., 2007). This study

will lay the groundwork for the future development of a data-driven toxicogenomic database to better understand the toxicity and disease associations of other EDCs and environmental substances.

2. Materials and Methods

2.1 Dataset Identification and Curation

GEO was queried for various chemical names for the four EDCs of interest to capture all potential transcriptomic profiles. This search encompassed both *in vitro* and *in vivo* datasets to ensure full data coverage and notably, some GSE accession numbers contained broadscale profile surveys with multiple EDCs studied. BPA terms queried included: “Bisphenol A”, “4,4’-propane-2,2-diyldiphenol”, “BPA”. DEHP terms queried included: “Bis(2)ethylhexyl phthalate”, “Di-sec octyl phthalate”, “DEHP”. TBT queried terms included: “bis(tributyltin)oxide”, “TBTO”, “Tributyltin”, “TBT”. PFOA queried terms included: “perfluorooctanoic acid”, “perfluorooctanoate”, “PFOA”. The resulting datasets were curated to meet the following criteria for inclusion (Fig. 1A): 1. Publicly available in GEO; 2. Transcriptome data including both RNASeq and microarray (single channel) data types; 3. Datasets derived from humans, mice, and rats; 4. Data derived from liver tissue (including various *in vitro* hepatocyte cell lines); 5. Appropriate sample size ($n \geq 3$/group); 6. Not a transgenerational or a prenatal longitudinal exposure study; 7. Not a duplicate of another study (subseries/superseries). Lastly, all GEO transcriptomic profiles accessible at the end of July 2021 were considered.

2.2 Downloading and Processing Transcriptome Profiles

Microarray datasets were directly downloaded from GEO via the R (v 4.1.2) package GEOquery (v 2.62.2) (Davis and Meltzer, 2007; R Core Team, 2019). The meta-data containing the descriptive information of the overall experiments and individual samples was obtained, and cell lines/tissues were reannotated using the Brenda Tissue Ontology to consolidate liver tissue terms (Gremse et al., 2011). Microarray data submitted to GEO have previously been pre-processed and quality controlled, therefore, the resulting expression matrices were only checked for normalization and processed using log2 transformation if not normalized for further downstream analysis.

The raw RNAseq datasets were downloaded from the Sequence Read Archive (SRA), quality checked, and processed in the Anaconda environment (Leinonen et al., 2011; Anaconda Software Distribution, 2020). To summarize, we used the parallel-fastq-dump wrapper to retrieve FASTQ files from SRA (Valieris, 2020). We then conducted quality control on the FASTQ files using Trim Galore (v 0.6.6) and Cutadapt (v 2.1.0) (Krueger, 2020; Martin, 2011; Andrews, 2010). In brief, the Trim Galore wrapper was used to trim 3’ end of reads with low quality base-calls, then Cutadapt removed adaptor sequences (12-13bp based on adaptor used). After trimming, short read sequences (<20bp) were filtered from the data. The binary package Salmon (v 1.3.0) was then used to map the sequencing reads to the appropriate reference genomes (human genome build GRCh38.p13, mouse genome build GRCm39, and rat genome build Rnor_6.0) and to quantify the mapped reads (Patro et al., 2017). The R package Tximport (v 1.22.0) was then used to import and summarize Salmon

quantification results (Soneson et al., 2015). Lastly, we filtered out transcripts with 1< mean counts across replicates.

2.3 Differential Gene Expression Analysis and Clustering of Transcriptome Signatures

Differential gene expression analysis was performed using gold-standard tools appropriate for the different data types (Schurch et al., 2015): Linear Models for Microarray Data (LIMMA; v 3.50.3) was used for microarrays and DESeq2 (v 1.34.0) was used for RNAseq data to determine change in expression of genes, or "gene signatures" (Ritchie et al., 2015; Love et al., 2014). For studies with multiple doses, each dose was treated as a separate dataset to derive dose-specific gene signatures. To assess the similarity of global gene expression changes across studies, the log fold change (logFC) values of all genes between treatment group and control group were correlated across datasets using the Spearman rank option in the "cor" function in R. Genes were treated according to complete observations method, where genes with missing values (i.e. "NA") were removed when computing correlation coefficients, leaving thousands to over 10,000 remaining transcripts depending on the data platforms between pairs of studies. Higher positive correlation coefficients indicated better agreement and higher negative correlation coefficients indicated opposite changes between pairs of datasets. The ComplexHeatmap package (v 2.10.0) was then used to cluster and plot the resulting Spearman correlation coefficients across datasets to visualize the similarity in gene signature patterns of different studies using the hierarchical clustering algorithm (Gu et al., 2016). For each EDC, we identified clusters of signatures with highly similar transcriptome responses (i.e., positive correlation coefficients). We then validated and determined the number of clusters using the Dunn Index from the package clValid (v 0.7) (Brock et al., 2008). The Dunn Index is calculated as the lowest intercluster distance divided by the highest intracluster distance, thus the cluster number with the higher Dunn Index was used unless Spearman correlation was negative.

For each cluster of studies sharing similarities in gene signatures, we then conducted meta-analysis across the studies in the given cluster to derive consensus DEGs using the Robust Rank Aggregation package (v 1.2.1) (Kolde et al., 2012), which allows for identification of DEGs perturbed by an EDC across studies that vary in species, dosage, and sample size but share similar gene expression changes. Rank aggregation was performed separately for both up-regulated and down-regulated genes and was used to identify consensus DEGs across studies in each cluster at a Bonferroni-adjusted p-value of <0.01. To compare DEGs between study clusters, the package GeneOverlap (v 1.3.0) was then used to determine if gene overlaps between cluster DEGs were significant using Fisher's exact test (Shen, 2014), with the corresponding fold enrichment calculated using the following formula:

$$O/(A \times B)/N$$

Where O is the number of overlapping genes, A is the number of genes in group 1, B is the number of genes in group 2, and N is the total number of unique genes expressed across datasets (16,900).

2.4 Pathway and Disease Gene Enrichment Analysis

To better understand the biological processes represented by the cluster specific DEGs, pathway enrichment analysis was performed. Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG) was used to functionally annotate the cluster DEGs (Kanehisa and Goto, 2000). We matched orthologous gene symbols of mice and rats to humans from the HGNC consortium to ensure consistency in pathway enrichment across species (Tweedie et al., 2021). Similarly, after mapping RNAseq dataset transcripts to their respective reference genomes, mouse and rat gene symbols were converted to human symbols. Enrichment analysis was then performed on the significant up and down-regulated DEGs separately utilizing the "enrichR" package (Xie et al., 2021). Pathways were considered significant at FDR<0.05.

To assess the association of the DEGs of each signature cluster with various diseases, disease-associated genes from DisGeNET were evaluated for overlap with DEGs using enrichR and associations with an FDR<0.05 were considered significant (Pinero et al., 2015).

2.5 Weighted Key Driver Analysis (wKDA) of DEGs

To identify potential gene regulatory networks and key drivers (KDs) that regulate the DEGs from EDCs, we used Mergeomics and a previously constructed liver-specific Bayesian network to conduct wKDA (Shu et al., 2016; Ding et al., 2021). Up- and down-regulated DEG sets for each EDC cluster were separately used for wKDA analysis. Genes whose subnetworks were enriched for cluster specific up- or down-regulated DEGs with an FDR<0.05 were considered significant KDs. The top 5 KDs for each DEG set and their subnetworks were visualized using Cytoscape (Shannon et al., 2003).

3. Results

3.1 Curated liver transcriptome datasets were primarily from adult, male, rodents

A total of 30 transcriptome liver datasets passed our selection criteria for inclusion (Table 1**)**, including 11 for BPA, 9 for DEHP, 4 for TBT, and 6 for PFOA. Using the 30 studies, we derived 45 gene signature responses across the EDCs. The majority of these studies were conducted using adult rodent males, with only nine signatures from females which were limited to *in vitro* study designs. Overall, species- and sex-coverage for the EDCs examined was uneven.

3.2 Clustering based on similarities across chemical gene signatures reveals lower study heterogeneity for DEHP and PFOA but higher variability for BPA and TBT studies

To determine the similarities and differences in liver gene responses to EDC exposures across studies, Spearman correlations of the log fold change (logFC) of liver genes between treatment and control groups of each study were calculated against the logFC values from each of the other studies to assess similarity in gene expression changing patterns between studies. Hierarchical clustering was then applied to the correlation coefficients to define studies with similar gene expression changes upon EDC exposures. Clusters were confirmed using the Dunn index (Supplementary Table 1). Notably, many PFOA studies involving

different species, doses, and exposure routes showed positive correlation between the signatures, suggesting consistency in the gene expression changes (Fig. 2). It is important to note that the PFOA studies (PFOA_13044 and PFOA_9786) that showed the highest correlation were derived from a single research group. However, we note that additional PFOA studies (e.g. PFOA_14712 and PFOA_119441) also showed positive correlation with the other PFOA studies. Similarly, a majority of DEHP studies also showed consistent gene expression responses to DEHP. Interestingly, PFOA and DEHP studies are intermingled in the largest cluster in Figure 2, suggesting similarities between the two EDCs in their gene response signatures. In contrast, for BPA and TBT, only a few studies showed clear clustering patterns, meaning that gene expression changes across studies were not strongly correlated (Fig. 2). The few BPA or TBT studies that showed better concordance were mostly among datasets generated from dose-response curves within the same study. These results indicate that the downstream effects of PFOA and DEHP are more stable and consistent across studies despite differences in study designs, whereas the gene expression changes induced by BPA and TBT are less consistent between studies and more specific to individual study designs. Our cross-EDC comparisons also revealed high similarities between PFOA and DEHP in terms of liver gene expression responses which were distinct from both BPA and TBT.

Next, we focused on each EDC to evaluate the influence of various study parameters such as species, exposure route, and dosage and to define study clusters that share similar transcriptomic responses to EDCs (Figure 3). Of note, the GSE19662 dataset for three chemicals (BPA, DEHP, PFOA) correlated with each other but not with their respective EDCs (Figure 2) despite the robust correlations observed across other DEHP and PFOA signatures, suggesting GSE19662 is an outlier. Therefore, we excluded datasets in GSE19662 from downstream analysis. Hierarchical clustering of BPA studies based on gene signature similarity revealed two major clusters. The first cluster named “BPA Human” was composed of two human female *in vitro* studies of various BPA doses (Table 2), while the second cluster named “BPA Rat” was composed of four rat studies of different doses, all of which were adult males exposed to BPA via oral gavage (Fig. 3A). Six BPA studies from different species, exposure route, and dosages did not show strong correlations with other signatures. These results again suggest highly variable liver responses to BPA. For DEHP, apart from the lone human study (GSE28878) and a single rodent study, all other studies clustered into a single group and was termed “DEHP All” (Fig. 3B). The TBT studies yielded a single “TBT Human” cluster, which was composed of a single female *in vitro* study at multiple doses (GSE86259), while the other TBT studies did not correlate well with each other (Fig. 3C). The PFOA studies clustered into a single group termed “PFOA All”, except an *in vitro* rat study (Fig. 3D). Altogether the transcriptome signatures yielded 5 clusters (Table 2), with BPA and TBT clusters segregated by study design, whereas DEHP and PFOA clusters were inclusive of studies from varying study designs. We focus on these identified clusters in the following analysis since they represent the reproducible signatures for the 4 EDCs.

3.3 Differentially expressed gene (DEG) and clustering analyses suggest higher similarities between PFOA and DEHP signatures and species-specific EDC effects

Next, we derived a consensus list of DEGs for the 5 clusters identified using a rank-based aggregation method which mitigates the issues of heterogeneous study designs and hence, differences in statistical power across studies in each cluster. We identified 2,170 DEGs for the BPA Human cluster; 1,353 DEGs for the BPA Rat cluster; 1,521 DEGs for the DEHP All cluster; 1,826 DEGs for the TBT Human cluster; 2,284 DEGs for the PFOA All cluster at Bonferroni-adjusted $p<0.01$ (Fig. 4A; full list of DEGs in Supplementary Table 2). Many of the DEGs were unique to each cluster, indicating different liver molecular signatures between EDCs especially when considering the directionality of the DEGs (up-regulated DEGs shown in Fig. 4B and down-regulated DEGs shown in Fig. 4C). To assess the significance of DEG overlaps between clusters, we used Fisher's exact test in the GeneOverlap package. The most significant DEG overlaps were between the DEHP All and PFOA All clusters for both the up- ($p = 4\times10^{-236}$; fold enrichment = 7.1; Fig. 4D) and down-regulated DEGs ($p = 4\times10^{-144}$; fold enrichment = 5.6, Fig. 4E). Despite having the smallest number of DEGs among all clusters, the BPA Rat cluster yielded significant overlapping DEGs in both directions with the DEHP and PFOA All clusters, whereas the BPA Human cluster showed significant overlap with the DEHP All and PFOA All clusters among the down-regulated DEGs only. Between the two BPA clusters (BPA Rat vs BPA Human), significant overlap was found only for the up-regulated DEGs ($p = 4\times10^{-4}$ and fold enrichment = 1.5). Significant gene overlaps were found among rodent clusters (BPA Rat, DEHP All, and PFOA All) and human clusters (BPA Human and TBT Human) (Fig. 4E). Taken together, the pattern of cluster DEG overlaps suggests higher similarities between PFOA and DEHP signatures and species-specific EDC effects.

3.4 DEGs of EDC clusters were enriched for diverse pathways

To better understand the biological functions of the DEGs, we carried out pathway enrichment analysis of cluster DEGs (Table 3; full list of enriched KEGG pathways in Supplementary Table 3). For both BPA Rat and Human clusters, cell cycle and DNA replication were down-regulated, while steroid biosynthesis was up-regulated (Table 3). Both BPA clusters also yielded pathways that are downstream of ER signaling, such as AMPK (up-regulated) and PI3K (down-regulated) pathways (Supplementary Table 3). Additionally, the Rat BPA cluster was enriched in pathways involved in immune response such as adipocytokine signaling (up-regulated) and cytokine-cytokine receptor interactions (down-regulated). For the DEHP cluster, the top up-regulated pathways included PPAR signaling, fatty acid metabolism/degradation, lipid metabolism, while down-regulated pathways included pentose and glucuronate interconversions and chemical carcinogenesis (Table 3). The PFOA All cluster up-regulated DEGs were enriched for lipid metabolism, PPAR signaling, and fatty acid metabolism/degradation (Table 3). The top up-regulated pathways in the TBT Human cluster included the AGE-RAGE signaling pathway in diabetes, small cell lung cancer, and proteoglycans in cancer; down-regulated pathways included DNA replication, and P53 signaling (Table 3). Of note, the TBT Human cluster also showed enrichment for pathways of other diseases such as lupus, measles, and alcoholism (Table 3).

Comparison across the four EDCs revealed a small number of broad overlapping pathways, including the general “metabolic pathways” term, terpenoid backbone biosynthesis, and P53 signaling (Fig. 5A; Table 3). However, there was high agreement in the enriched pathways identified for PFOA and DEHP clusters in both the up- and down-regulated DEGs (Fig. 5B-C). Specifically, complement/coagulation cascade and steroid hormone biosynthesis were down-regulated (Fig. 5C), while PPAR signaling, peroxisome, and fatty acid metabolism/degradation were up-regulated (Fig. 5B). We also observed other PPAR-related genes such as PPARγ coactivators *PPARGC1A* and *PPARGC1B* as DEGs (Supplementary Table 2). Taken together, although all the chemicals had limited overlap, our analysis suggests some general mechanisms are shared between the analyzed EDCs.

There were also numerous enriched pathways that were unique to each EDC, especially among the down-regulated pathways. For BPA, unique pathways included FoxO signaling and HIF-1 signaling, pathways critical in cellular processes and glucose metabolism (Fig. 5C). Interestingly, DEHP down-regulated the type II diabetes mellitus KEGG pathway, and up-regulated glutathione metabolism, a protective pathway that has roles in antioxidant defense and nutrient metabolism (Supplementary Table 3). Unique pathways for PFOA included up-regulation of genes involved in non-alcoholic fatty liver disease and oxidative phosphorylation (Supplementary Table 3). Among the top pathways unique to the TBT Human cluster are cancer-related pathways (Table 3; Supplementary Table 3). These unique pathways support disparate effects across EDCs.

3.5 Network modeling identifies overlapping key drivers (KDs) for DEHP and PFOA clusters and their subnetworks

In order to identify potential regulatory genes or KDs that drive EDC-induced transcriptomic alterations, significant DEGs within each cluster were subject to weighted key driver analysis (wKDA) using a Bayesian gene regulatory network previously constructed from tens of human and mouse liver datasets (Shu et al., 2016). Tens to hundreds of KDs at FDR<0.05 were identified for the DEGs of individual EDC clusters (top 5 KDs listed in Table 3 and illustrated in Fig. 6A-B; full KD list in Supplementary Table 4): BPA Human – 30 KDs (13 down and 17 up), BPA rat – 134 KDs (48 down and 86 up), DEHP All – 311 KDs (88 down and 223 up), PFOA All – 509 KDs (283 down and 226 up), and TBT Human – 23 (17 down and 6 up).

Although there were no KDs shared across all 5 EDC clusters, there was significant overlap between the DEHP and PFOA clusters (210 overlapping KDs) which is in agreement with the similarities observed at the DEG and pathway levels. Among KDs shared between DEHP and PFOA All clusters were genes involved in blood clotting/coagulation such as *SERPINF2* and *VTN* (Fig 6B), the fatty acid oxidation gene *ACOT2*, lipid metabolism genes *ALDH3A2* and *VNN1,* and a peroxisome biogenesis and PPAR signaling gene *PEX11A* (Fig 6A). All these genes and pathways are highly relevant to CMDs. The top unique KDs for the DEHP All cluster included *AKR1D1, C9, EHHADH*, while the top unique KDs for PFOA All cluster included *CFI, PLG,* and *ECI1.* Although there were EDC-specific top KDs between the two clusters, some KDs have largely overlapping biological roles; *C9* (DEHP KD), *CFI and PLG* (PFOA KDs) play roles in the immune

system response; *EHHADH and ECI1* are involved in lipid metabolism and PPAR signaling (Fig. 6A-B). These results further suggest that these two EDCs affect related pathways and potentially similar mode of action due to the high number of overlapping KDs.

Between BPA and the other EDCs, KDs were mostly shared with PFOA (55), however the majority belonged exclusively to BPA (79). To compare the effects of BPA on human versus rats, we examined the KDs and their associated functions from the 2 clusters. Top KDs for the BPA Human cluster included cell cycle/mitosis genes *MCM6* and *CDCA8;* glucose and cell metabolism genes *DCXR, IGFBP1,* and *GPSN2*; cholesterol and sex steroid biosynthesis genes *FDFT1, HMGCR, HSD17B7* and *IDI1*; and *ALG5,* which is involved in glucosyltransferase activity (Fig. 6A-B). The top KDs identified for the BPA Rat cluster included cell cycle/proliferation genes *CCNA2, MKI67, RACGAP1, CDCA8, CDC20,* and *BTG2* (Fig. 6A-B) as well as *CEBPD*, which has been shown to have a role in immune response and inflammation. Our analysis revealed direction-specific overlaps in the top KDs for the BPA clusters, where glucose and cholesterol metabolism genes such as *ACAT2, FDFT1,* and *IGFBP1* (Fig. 6A-B) were shared up-regulated KDs. However, no KDs were shared for the down-regulated DEGs. The highly significant enrichment of KDs in the cell cycle pathway of the BPA Rat cluster further suggest BPA plays a role in proliferation and has the potential to cause cancer in rodents such as liver carcinoma and neo-plasms (Gao et al., 2015). Moreover, the functions of KDs influenced by BPA in the human cluster suggests a variety of biological pathways altered, not just cell-cycle/proliferation. The limited overlap in KDs between BPA Human and Rat clusters highlight species differences in the genes responsive to BPA, coupled with the low agreement among mouse studies and between species, suggests BPA-exposure effects may be difficult to translate from rodents to humans.

The TBT cluster showed highly divergent KDs from the other EDC clusters, and the top KDs were mostly unrelated to metabolism. Only one gene, *LPIN1,* was involved with lipid metabolism (Fig. 6A). The other KDs had roles in cell organization and cell-to-extracellular matrix interactions, such as *NID1, PHLDB1, TAGLN2* (Fig. 6B). Furthermore, KDs involved in cell cycle and cell differentiation were the most overrepresented such as *HSPB1*, *TUBB6, MCM6,* and *C8orf4.* These KDs are largely associated with various cancers such as liver carcinoma, squamous cell carcinoma, etc. (Nagaraja et al., 2012; Jiang et al., 2020; Liu et al., 2018; Sunde et al., 2004). KDs with other roles included *JUN* and *RGS16,* which participate in hepatocyte growth signaling and GTPase activator activity, respectively (Fig. 6A-B; Behrens et al., 2002; Derrien and Druey, 2001).

Overall, our wKDA indicated highly similar liver KDs genes for PFOA and DEHP involved lipid, PPAR, coagulation, and immune pathways; relatively unique KDs for the BPA involved cholesterol and steroid pathways; several unique TBT KDs involved in cell cycle, growth, and cell-cell signaling. Additionally, BPA studies showed between-cluster differences in the KDs, and subnetworks potentially driven by species or sex-specific effects, with more metabolic KDs reflected in the network for the BPA Human cluster versus more cell cycle-related KDs in the BPA Rat cluster.

3.6 EDC cluster DEGs except DEGs of the TBT cluster are enriched for CMD-associated genes

To identify the genes that potentially contribute to EDC-induced CMDs, we assessed the overlap of the EDC cluster gene signatures with genes exhibiting genetic association with 6 CMDs (obesity, diabetes, CVD, MetS, hypertriglyceridemia, NAFLD) extracted from human genome-wide association studies (GWAS) curated by DisGeNET (Supplementary Table 5). Both up- and down-regulated DEGs for the DEHP and PFOA All clusters yielded associations with all 6 CMDs. For BPA, only the down-regulated DEGs for the BPA Human cluster and mainly the up-regulated DEGs for the BPA Rat cluster showed significant enrichment for CMD genes. Interestingly, the TBT Human cluster (both down- and up-regulated genes) and the BPA Human up-regulated genes did not exhibit any associations with the selected CMDs.

Obesity- and diabetes-associated genes were the most over-represented among the DEGs of all the EDC clusters, with several disease genes also identified as KDs in our network analysis (e.g., *PEX11A, VTN*; Fig. 7A-B). CVD-associated genes were enriched among the down-regulated DEGs of all EDC clusters (Fig. 7B) as well as the up-regulated DEGs of the BPA Rat cluster (Fig. 7A). Several of these CVD-related genes were also among the top KD networks for both BPA clusters, such as *FDFT1*, and *IGFBP1*. MetS-related genes were also enriched among the DEGs of the chemicals analyzed. Several of these MetS genes were also network KDs for PFOA (*ACOT2*, *PLG*, and *VTN*), DEHP (*VTN* and *ACOT2*), BPA Human (*DCXR*), and BPA Rat (*CEBPD*, *FASN*, *FDFT1*, *IGFBP1*, and *MKI67*). NAFLD genes were enriched among the down-regulated genes of the BPA Rat cluster as well as both down- and up-regulated DEGs of DEHP and PFOA all clusters. Hypertriglyceridemia genes were enriched among the up-regulated DEGs of DEHP and the down-regulated DEGs of PFOA. Previous studies have shown BPA, DEHP, and TBT to be highly associated with metabolic diseases (obesity, diabetes, MetS, etc.); however, we did not identify any genetic associations for CMD in the TBT Human gene set (Feninchel et al., 2013; James-Todd et al., 2016; Freitas-Lima et al., 2018). PFOA studies have yielded inconsistent associations with CMDs (Christensen et al., 2019), however here we show prominent CMD gene enrichment in the DEGs of the PFOA All cluster.

Previous studies have also identified all four chemicals to be carcinogenic due to their ability to enhance cell proliferation as EDCs (Soto and Sonnenschein, 2010; Kumar et al., 2020). Notably, several significant cancer-related associations were observed for the DEGs of each cluster. BPA Rat DEGs were enriched for lymphoma adenocarcinoma genes; BPA Human DEGs for breast and prostate carcinoma genes; DEHP All and PFOA DEGs for liver neoplasms and carcinoma genes; TBT Human DEGs for genes involved in neoplasms of lung and prostate (Supplementary Table 5; Fig. 7B). These results affirm the chemicals' capacities to promote cancer pathogenesis. In addition, our results provide molecular support for the association of the various EDCs with six different CMDs based on the significant overlap between genes perturbed by EDCs and CMD-associated genes.

4. Discussion

Several studies have examined EDCs and their relationships with CMDs, however some chemicals presented volatile responses and the molecular mechanisms were unclear. In our current study, we devised a new computational pipeline to uniformly meta-analyze 30 RNAseq and microarray datasets from four different EDCs ubiquitously found in humans. The datasets were from liver tissue across three mammalian species, with a variety of exposure conditions. Using DEG, pathway, and network analyses, we evaluated similarities and differences between EDCs and identified key drivers that potentially mediate EDC-induced molecular perturbations in pathways and networks that affect CMD risk and development (Table 3). DEHP, PFOA, and BPA all showed significant perturbations in metabolic pathways and CMD associations. In contrast, TBT-affected liver genes were the most dissimilar compared to the signatures of the other EDCs and did not show significant overlap with CMD-associated genes. Our meta-analysis was also able to recapitulate the known oncogenic biology and pathways induced by these chemicals, such as cancer/cell cycle-related pathways and cancer associations such as liver cancer/neoplasms (Supplementary Table 3 and 5). Overall, our analysis gave insights into shared and chemical-specific molecular actions of EDCs that provide a more comprehensive evaluation of the MOA of EDCs in the liver.

Our meta-analysis across chemicals (Fig. 2) results suggests that the comparability across studies differ between EDCs and it is easier to derive MOAs for DEHP and PFOA than for TBT and BPA. In the case of DEHP and PFOA, fewer studies departed from their own chemical categories. It is plausible that the MOAs of each of these chemicals are more robust and consistent even with different study designs and conditions. Studies from these two chemicals also showed between-chemical mixing clustering in our analysis (Fig. 2). This potentially indicates that these two chemicals share a more similar MOA. Indeed, our DEG, pathway and network analyses revealed shared MOA including PPAR signaling and fatty acid metabolism/degradation which agree with previous reports (Casals-Casas, et al., 2008; Rosen et al., 2008; Huang and Chen, 2017). We speculate that the few DEHP and PFOA studies that did not cluster with their own chemical categories are potentially due to technical issues. In contrast, the BPA and TBT studies showed high variability between studies, between species, and between doses, making it challenging to infer their MOA. Many factors can contribute to this observation, some biological and some technical. One biological reason is that activity and effects of BPA and TBT are more complex and dynamic (Marmugi et al., 2012; Rodrigues-Pereira et al., 2022). In regards to BPA, it has been noted that there is high variability between studies and its exact MOA is still controversial. Recently, the Clarity-BPA project and other studies assessing dose-responses curves showed that several tissue types, including liver, have non-monotonic dose responses (e.g. U and W shape dose-response curves) to BPA (Heindel et al., 2020; Lagarde et al., 2015). Taken together, the lack of consistency or clustering in our comparative analysis across BPA studies agreed with the well-documented variability in the literature, supporting that the MOA of BPA is more specific to individual exposure conditions and likely inconsistent between conditions. In the case of TBT, the small number of studies makes it challenging to propose the cause of the poor clustering across studies or draw conclusions about its MOA.

One potential biological factor underlying the poor consistency across TBT studies could be dose differences, as we note in Fig. 2, four *in vitro* signatures within the dose range of 0.3uM-10uM clustered together, whereas the other two remaining signatures that did not cluster well reflected a significantly lower dose of 0.02nM and a very high *in vivo* dose of 200mg/kg/d. Previously, studies using zebrafish as a model have suggested that sub-acute TBT exposures promote metabolic dysfunction only at higher doses, especially those which are considered cytotoxic (Heindel and Blumberg, 2019; Martinez et al., 2020). Our study would cautiously support this claim, as the lone high-dose study happened to cluster with the DEHP and PFOA studies (Fig. 2), which were particularly associated with metabolic alterations.

Our analytical pipeline provided further insights on the molecular pathways and potential regulators involved in the perturbations induced by each of the EDCs that contribute to disease risk. It has been shown that the effects of EDCs like BPA are dependent on model organism, sex, tissue, and age, and that the liver is the organ most susceptible to EDC exposure (Cimmino et al., 2020; Le Magueresse-Battistoni et. al., 2018; Shu et al., 2019; Diamante et al., 2021). BPA is metabolized and cleared by humans at a faster rate compared to rodents, while fecal excretion of BPA by rodents allows enterohepatic recirculation to result in prolonged BPA exposure (Völkel et al., 2002). There are also distinct sex-differences in BPA glucuronidation rates, with female rats having higher levels of clearance enzyme activity (Takeuchi et al., 2004). Our analysis was able to uniformly process data from different model organisms, doses, and sex, and therefore able to make comparisons and uncover potential species or sex specific effects of BPA. Our results showed high diversity of KEGG pathways (cancer, immune and metabolism pathways represented), disparate CMD associations, and divergent top KDs between Human and Rat BPA clusters which support a more volatile BPA response and may underlie the controversies regarding BPA safety in previous studies (Vogel, 2009). Additionally, previous studies indicate that BPA can bind to estrogen receptors and influence biological processes such as cell proliferation and apoptosis (Gao et al., 2015; Can et al., 2005). Our study did not yield the ER-signaling pathway as significantly enriched; however, previous findings have shown that non-classical ER-alpha signaling can be mediated by membrane localized ERs (Björnström and Sjöberg, 2005). This signaling rapidly activates ER-signaling-related pathways, such as those observed in both BPA Rat and Human clusters (e.g., PI3K-, AMPK-signaling; Table 3). Our CMD association analysis also revealed divergent responses between species: the human cluster yielded more CMD associations in the down-regulated gene set and the rat cluster yielded more associations with CMDs in the up-regulated gene set. In addition, we identified KDs from the BPA clusters (Fig. 3A) that were associated with CMDs: for the BPA Human cluster, KD *DCXR* was associated with only obesity while the BPA Rat cluster KDs *CEBP*, *FASN*, *FDFT1*, *IGFBP1*, and *MKI67* were associated with all the CMDs evaluated. Taken together our study suggests that BPA exposure is a greater CMD risk factor in rat male models, and that the nuances of species-specificity in BPA exposure studies should be considered when extrapolating to human health.

In addition to its endocrine disrupting effects, TBT has also been linked to obesity, NAFLD, tumor development and cancer progression (Casey et al., 2015; Katz et al., 2020). Our study supports the oncogenic capacity of TBT in human cell lines by uncovering several enriched

cancer-related pathways that were unique to the TBT-induced DEGs, including small cell lung cancer, and proteoglycans in cancer. TBT has been linked to altered adipogenesis, increased lipid accumulation (Heindel and Blumberg., 2019; Jia et al., 2016) and has been indicated as an agonist for classic lipogenic pathways such as PPARγ and RXR (retinoic x receptor) in adipose, liver, and pancreas (Le Maire et al., 2009; Bertuloso et al., 2015). Interestingly, the DEGs from the TBT-treated female human cell lines only revealed the AGE-RAGE signaling pathway and the KD *LPIN* as relevant to metabolism and did not show significant overlap with genes associated with CMDs. It is important to note the TBT Human cluster (Fig. 3C) is from a single study at multiple doses which could induce potential biases. Though studies with transcriptome responses to TBT remain limited, our analysis suggests TBT's previously reported adipogenic effects are likely not mediated through liver tissue and that TBT is a potent carcinogen.

The myriad of health effects of DEHP have been well documented (James-Todd et al., 2016; Jaimes et al., 2017; Rowdhwal and Chen 2018), and our analysis of DEHP-exposed liver confirmed diverse disease associations and pathways, such as chemical carcinogenesis, metabolic dysfunction, and immune system perturbations (Supplementary Table 3 and 5). Notably, numerous metabolic pathways including, fatty acid metabolism/degradation, steroid biosynthesis, and PPAR signaling were enriched in both up- or down-regulated DEGs, suggesting a complex and broad mis-regulation of metabolic sub-pathways. Furthermore, KDs identified for DEHP have roles in both immune response (*C9*, *SERPINF2*, *VTN*) and metabolism (*EHHADH*, *PEX11A*, *VNN1*, *ACOT2*). Disease association analysis further supported the capacity of DEHP to be a CMD-inducing chemical, as DEHP DEGs were significantly enriched for diabetes and MetS genes. More importantly, several of them were identified as network KDs such as *VNN1* and *ACOT2*. Despite the heterogeneity of dose and exposure route in the study design, all but one DEHP transcriptomic study showed profiles that converged on a consensus signature (Fig. 3B), indicating that liver responses to DEHP were robust and reproducible across multiple conditions.

Prior studies have claimed that PFOA exposure leads to numerous health problems, such as metabolic, reproductive, immunological, developmental effects and cancer in humans, mice, and rats (Barry et al., 2013; Vieira et al., 2013; Filgo et al., 2014; Fenton et al., 2021). Disease gene set enrichment analysis associations revealed prominent liver diseases (e.g., NAFLD, cirrhosis, liver carcinoma; Supplementary Table 5). Additionally, previous rodent and human studies have demonstrated the ability of PFOAs to alter liver metabolic health via pathways such as cholesterol metabolism, PPAR activation, and fatty acid oxidation (Fletcher et al., 2013; Li et al., 2017). Indeed, our pathway analysis showed up-regulation of many of these metabolism-related pathways. Furthermore, our wKDA yielded regulatory genes (*ACOT2*, *PEX11A*, ECI1, and *VNN1)* and metabolic pathways (fatty acid metabolism, PPAR signaling, starch and sucrose metabolism) regardless of species. PFOA DEGs were also enriched for CVD, diabetes, MetS, and obesity associated genes, and several were also KDs (*ACOT2*, *VNN1,* and *PLG*). We hypothesize that these genes are potential mediators of PFOA-induced CMD and could serve as potential targets for further experimental validation.

All the EDCs in this current study have been reported to act as PPAR and RXR agonists (Huang and Chen, 2017). PPARα is highly expressed in the liver and regulates lipid and fatty acid metabolism, while also playing an important role in immune response (macrophage/monocytes); PPARγ is mainly expressed in adipose tissue (exhibits low expression in the liver to facilitate lipid uptake) and its activation improves insulin sensitivity and glucose homeostasis as well as regulate adipocyte differentiation (Tyagi et al., 2011). In our meta-analysis across 30 studies, only the DEGs of the DEHP All and PFOA All clusters (Fig. 3B& D) yielded PPAR signaling as a significant pathway and PPARγ coactivators as DEGs. It has been indicated that EDCs with stronger estrogenic potential were less likely to activate PPARγ (Riu et al., 2011; Faulds et al., 2012). Indeed, general PPAR signaling pathway was not enriched in the BPA clusters; however, key BPA DEGs that have roles associated with PPAR signaling were observed (PPARγ in the BPA Human cluster and RXR in both the Human and Rat clusters). Additionally, the clusters were enriched for the adipocytokine signaling pathway, where it has been reported that adipocytokines such as adiponectin, are stimulated by PPARγ signaling and can modulate PPARα signaling (Yamauchi et al., 2007; Fujita et al., 2008; Corrales et al., 2018). Thus, the complex interplay of the two main PPAR isoforms and their signaling cascades could be driving the differences in liver pathways and disease associations between the EDC clusters. Specifically, DEHP and PFOA may be greater disruptors of PPAR pathways through PPARγ coactivators (PPARGC1A/B; Oberkofler et al., 2003; Léveillé et al., 2020) compared to BPA and TBT in the liver. Interestingly, the KD *VNN1* shared between PFOA and DEHP is a PPARγ antagonist and may serve as a potential target for future studies of CMD therapies due to its role in hepatic gluconeogenesis and steatosis (Berruyer et al., 2006; Bartucci et al., 2019). Taken together, our data support the involvement of PPAR signaling in DEHP and PFOA effects in the liver, whereas the CMD-promoting effects of BPA and TBT exposures may be mediated through other mechanisms of action or through different tissue types.

Our systematic analysis was able to process diverse datasets and generate consensus DEGs utilizing a new approach to overcome study heterogeneity to robustly detect DEGs, regulatory genes (KDs), pathways, and networks involved in EDC-induced CMD. Additionally, our pipeline allowed us to identify and exclude datasets that are highly distinct from other studies and therefore likely outliers. Utilizing a large number of studies enhances the reproducibility of our findings and studying multiple chemicals across species also enables the discovery of similarities and differences across EDCs and species to facilitate comparative toxicology. In addition, the current study is a meta-analysis of transcriptomic data independent of phenotypic evaluation due to the missing phenotypic data in many of the studies. This highlights the need to coordinately submit phenotypic data and the associated publications along with omics datasets to GEO to enable phenotype-anchored molecular analysis. In our study, to overcome this we systematically conducted a functional and disease annotation of our gene signatures through pathway enrichment and disease-association analysis, and our results inferred the potential functional and phenotypic endpoints reflected by the gene signatures.

It is important to note several limitations of the current study. First, our meta-analysis is limited by the coverage of the existing datasets in GEO, which do not evenly cover both sexes, range of doses, exposure routes, and species across EDCs. Secondly, technical factors

such as different profiling platforms and differences in reference genome versions used for microarray probe annotation may confound our findings. Given that microarrays and their probe annotations accessible from GEO can be based on different genome versions, we acknowledge that differences in gene annotation may lead to missing transcripts that can be compared between datasets. Future efforts to re-annotate the different microarray platforms to a common gene annotation version will mitigate this concern. However, we note that without explicitly re-annotating probes across microarrays, the overlapping transcripts between datasets ranged from thousands to over ten thousand, which could still provide reasonable estimates for the overall correlative relationships and pathway/network level comparisons. We also observed that the BPA Human cluster was derived from two datasets from the same lab, and other BPA studies clustered poorly. The stratification of BPA studies may likely be the result of differences in technical factors as well as biological factors (species/exposure/sex differences), and our current study cannot tease these apart. However, the “DEHP All” and “PFOA All” clusters (Fig. 3B&D) incorporated varying technical and design factors, such as profiling platforms (microarray and RNAseq), species (mouse and rat), and exposure route (diet, drink, and oral gavage). Therefore, technical factors did not appear to be a major confounding factor in our findings at least for DEHP and PFOA, which may be extrapolated to BPA studies. However, caution is required to interpret the findings on BPA due to the difficulties in separating technical from biological factors. Thirdly, we focused only on studies whose gene signatures showed similarity and formed clusters with other studies in our meta-analysis of DEGs, and excluded those that did not cluster with others. These studies that were excluded could either be outliers with poor reproducibility, or capture unique EDC biology, which warrants further investigation. Fourth, although both exposure dose and window have a great impact on the magnitude of transcriptome alteration (Golestanzadeh et al., 2019), we did not analyze transgenerational or pre-natal longitudinal exposure studies. In addition, further experiments are needed to validate the key genes identified in our network analysis and their role in CMD risk and development. Lastly, we only examined the liver in the current study due to limited studies of other metabolic tissues (e.g., adipose, pancreas, skeletal muscle) in the EDC field, but we acknowledge the importance of other organs in CMD.

Conclusion

Our meta-analysis of 30 high throughput studies across four chemicals in three mammalian species offers unique insights into the functional changes associated with exposure to a select set of EDCs. We also analyzed key regulatory genes and pathways in the liver to identify those that potentially drive EDC-induced CMD risk. Despite inherent variations in study designs across the datasets, we found that DEHP and PFOA had significantly overlapping gene signatures. By contrast, BPA liver signatures were highly variable, and TBT had the most divergent profile compared to the other EDCs. The shared and chemical-specific genes, pathways, networks, and regulators identified from our study provide comprehensive insights into the molecular actions of EDCs in the liver and facilitate future mechanistic and translational studies. The bioinformatics workflow established by the current study will be used in a new toxicogenomics database, ToxiOmics, that we

are developing to study a broad range of environmental toxicants and tissues to better understand their influence on complex human diseases such as CMDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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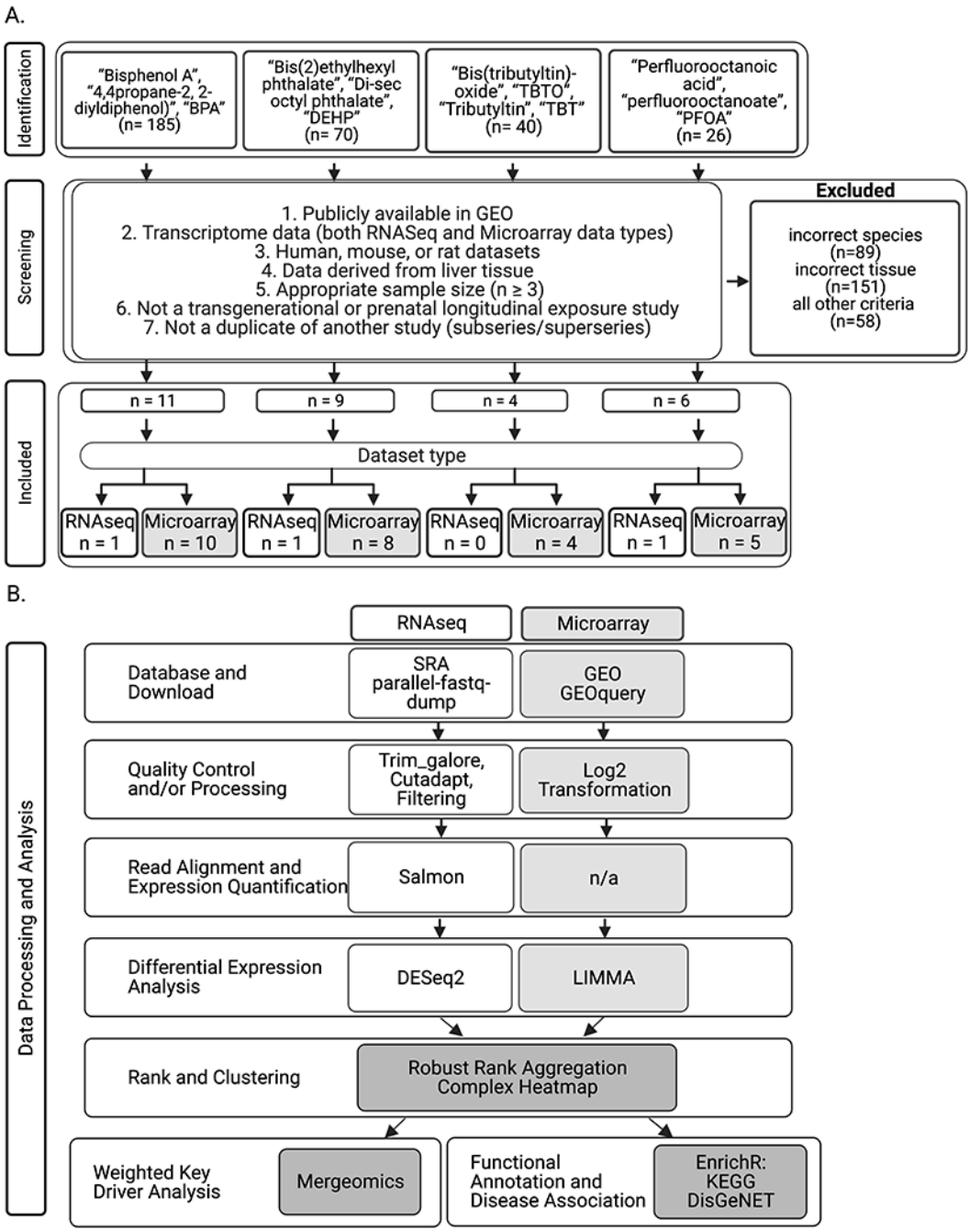

Fig 1. Overall data curation workflow and analysis pipeline.

(A) Criteria and selection workflow of GEO transcriptome datasets included in the study.

(B) Meta-analysis pipeline for both microarray and RNAseq data types.

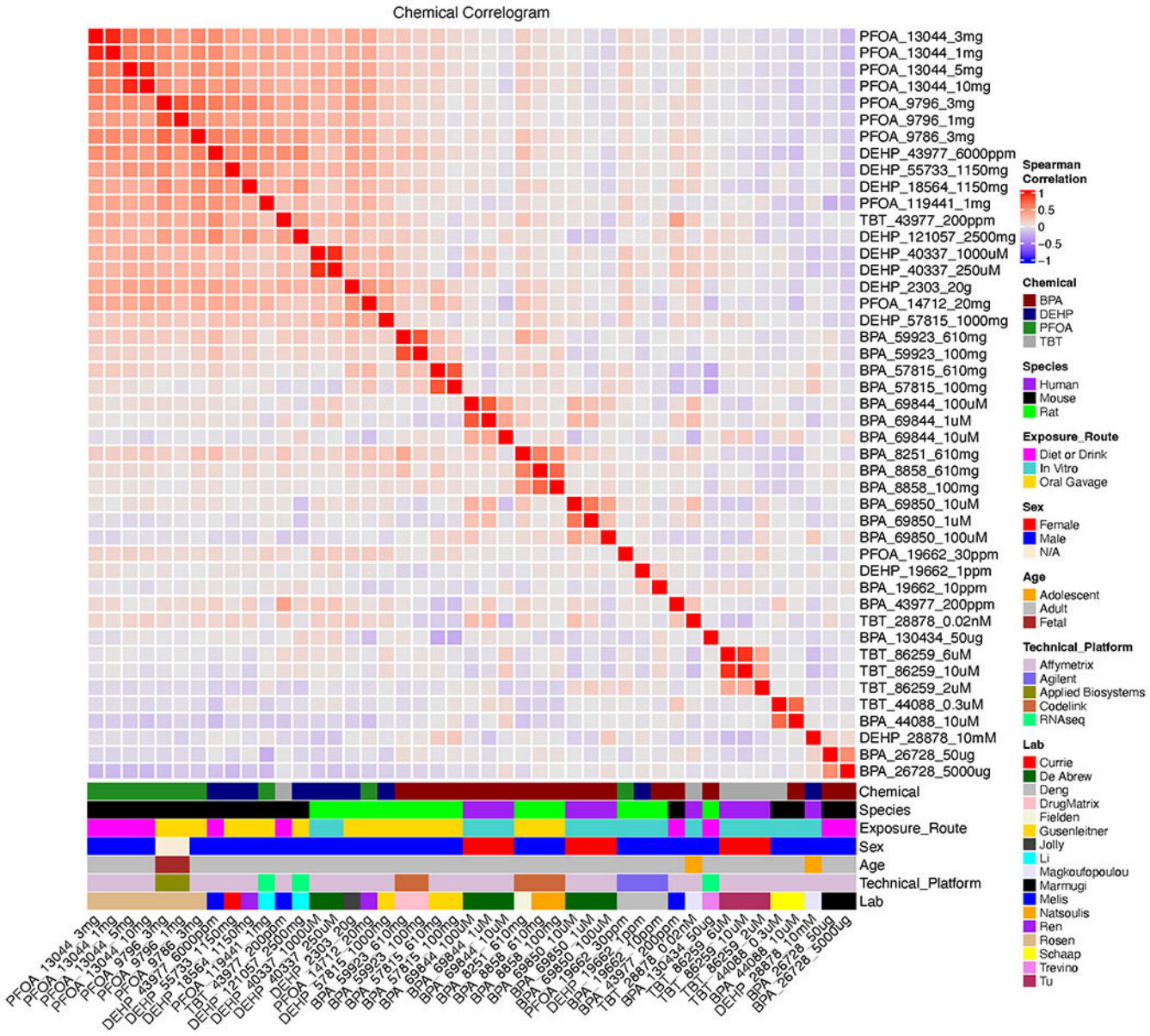

Fig. 2. Clustering analysis of EDC gene signatures across studies.

Correlogram of gene signatures of EDCs based on hierarchical clustering using Spearman's rank correlation of the log fold changes (logFC) of expressed genes between treatment and control groups in liver transcriptome across all EDC studies examined in the current study. Color of the heat map indicates Spearman's rank correlation coefficients, where red tones represent positive correlations and blue tones represent negative correlations. Below the cluster map, different factors such as chemical type, species, exposure route, sex, age, technical platform for transcriptome profiling, and lab/group (labeled by the last name of the first author) that carried out the studies are indicated by color bars.

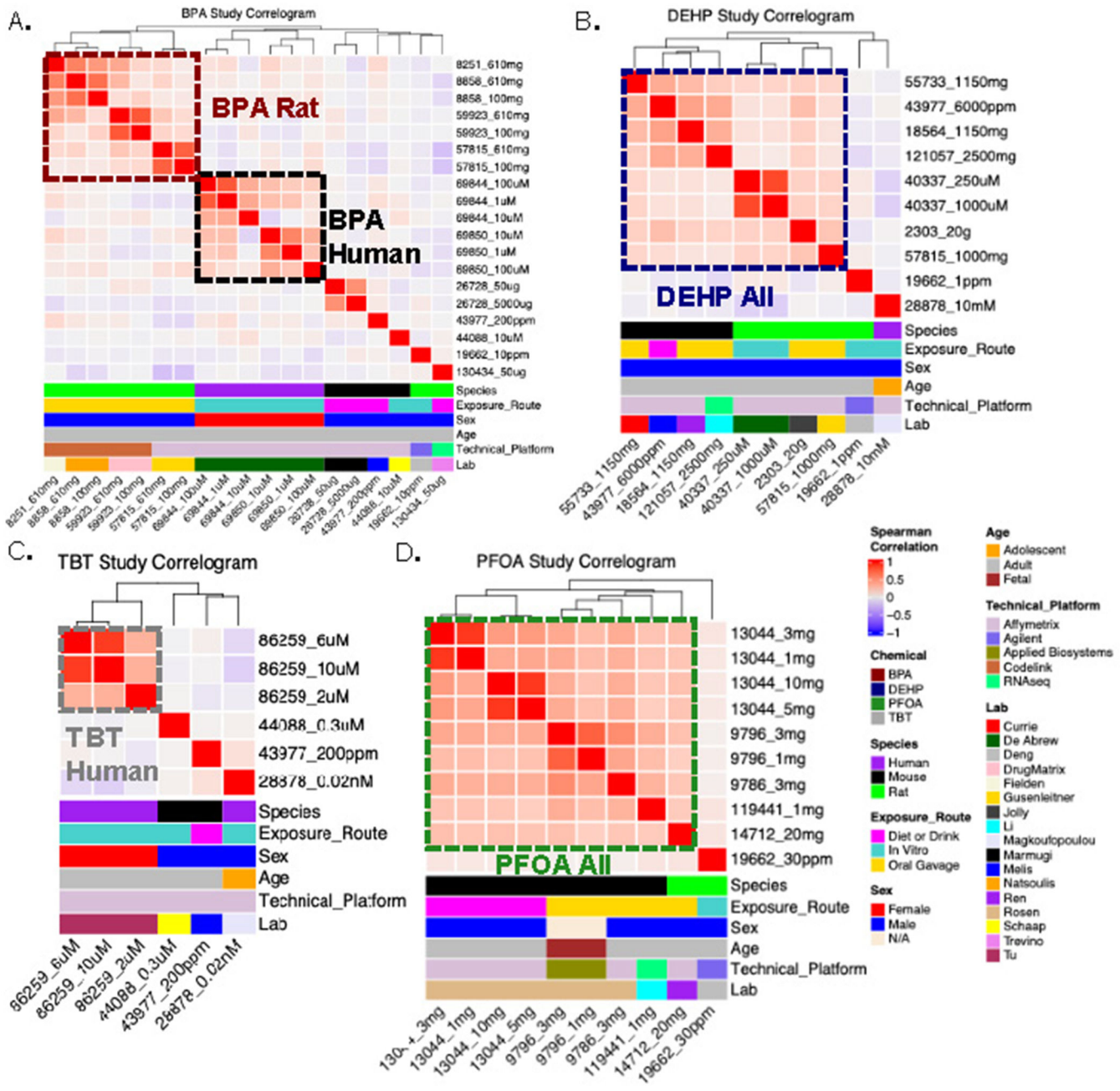

Fig. 3. Cluster analysis for each EDC based on similarity in gene signatures defines main EDC study clusters.

(A-D) Correlograms generated via the hierarchical clustering of gene signatures of EDCs based on Spearman's rank correlation coefficients of transcriptome response (logFC) between treatment and control groups for BPA (A), DEHP (B), TBT (C), and PFOA (D). Studies were labeled using the GSE accession number and dose. Color of the heat map indicates Spearman's rank correlation coefficients where red tones represent positive correlations and blue tones represent negative correlations. Outlined signatures represent identified EDC clusters used for downstream analysis; 1) Green dashed lines represent PFOA All, 2) Navy dashed lines represent DEHP All, 3) Black dashed lines represent BPA

Human, 4) Brown dashed lines represent BPA Rat, 5) Gray dashed lines represent TBT Human. Below the cluster map, different factors such as chemical type, species, exposure route, sex, age, technical platform for transcriptome profiling, and lab/group (labeled by the last name of the first author) that carried out the studies are indicated by color bars.

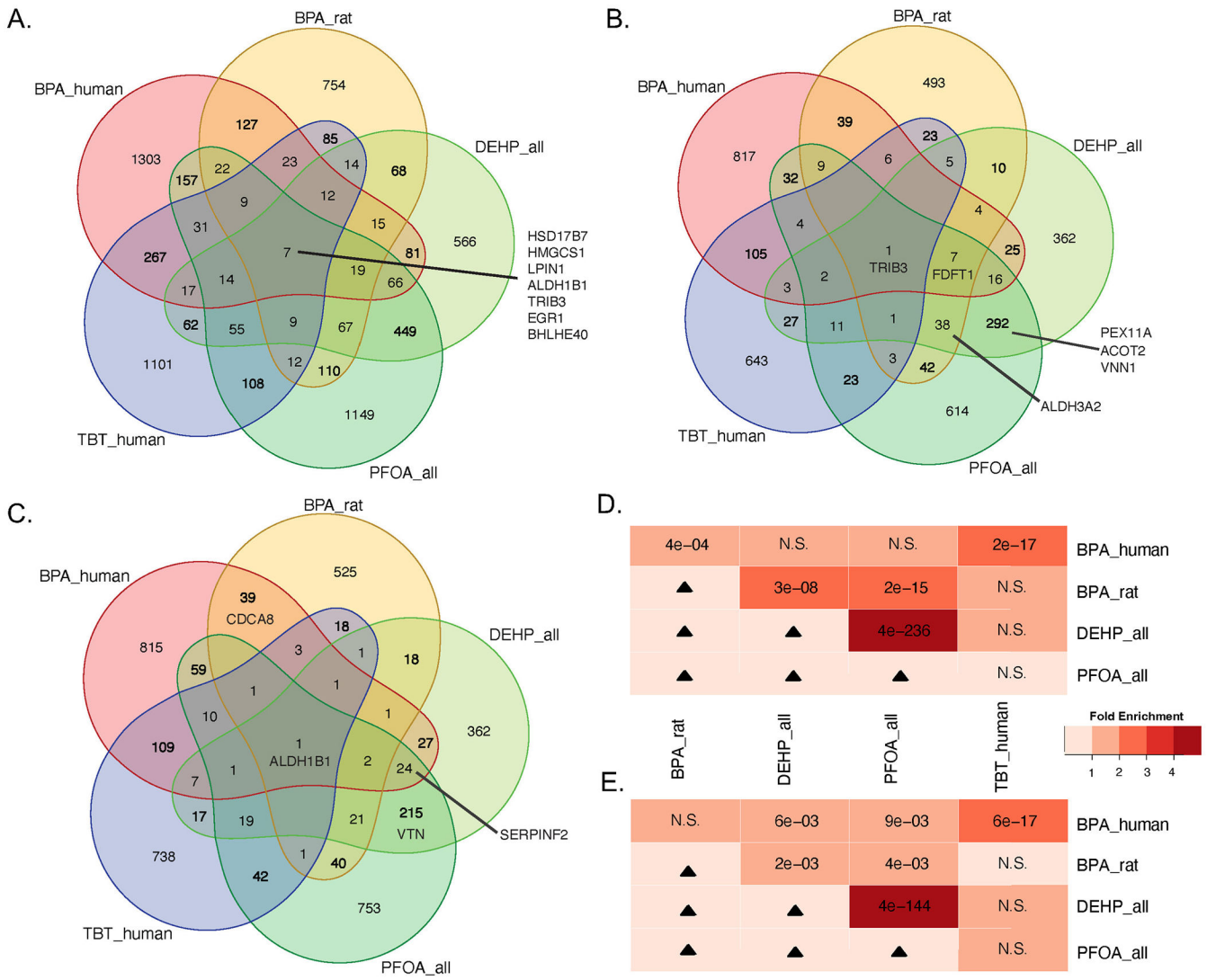

Fig. 4. Comparison of differentially expressed genes (DEGs) across different EDC clusters.

(A-C) Venn diagram of total DEGs (A), up-regulated DEGs (B), and down-regulated DEGs (C) at Bonferroni-adjusted p-value < 0.01. DEGs shared among all clusters (in the center) and top KDs shared are labeled. (D and E) Heatmaps illustrating the significance of DEG overlaps between clusters for up-regulated DEGs (D) and down-regulated DEGs (E). Fisher's exact test was used to calculate p-values. Color gradient is based on fold enrichment values between clusters. Number values indicate significant overlaps at p<0.05. N.S. indicates no significance and ▲ indicates comparison already performed.

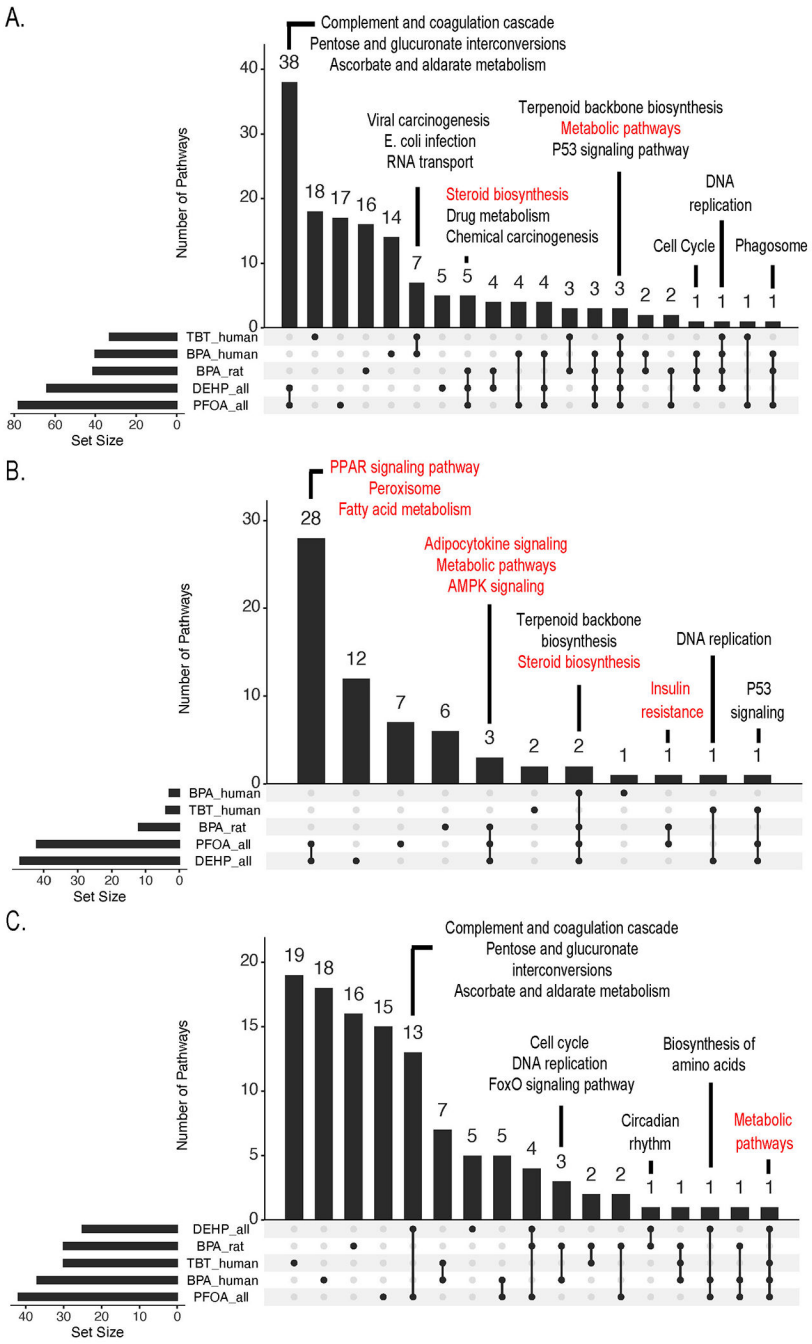

Fig. 5. Comparison of enriched KEGG pathways among EDC study clusters.

(A-C) Upset plot of significant KEGG pathways (FDR<0.05) enriched for total DEGs (A), up-regulated DEGs (B) and down-regulated DEGs (C). Horizontal bars (set size) indicate total number of pathways for each cluster in each plot. In the upset plots, dots point to the specific EDC clusters for which the vertical bars for pathway counts are shown, and vertical lines between dots represent the intersections between two or more clusters. Terms labeled in red indicate metabolic pathways of interest.

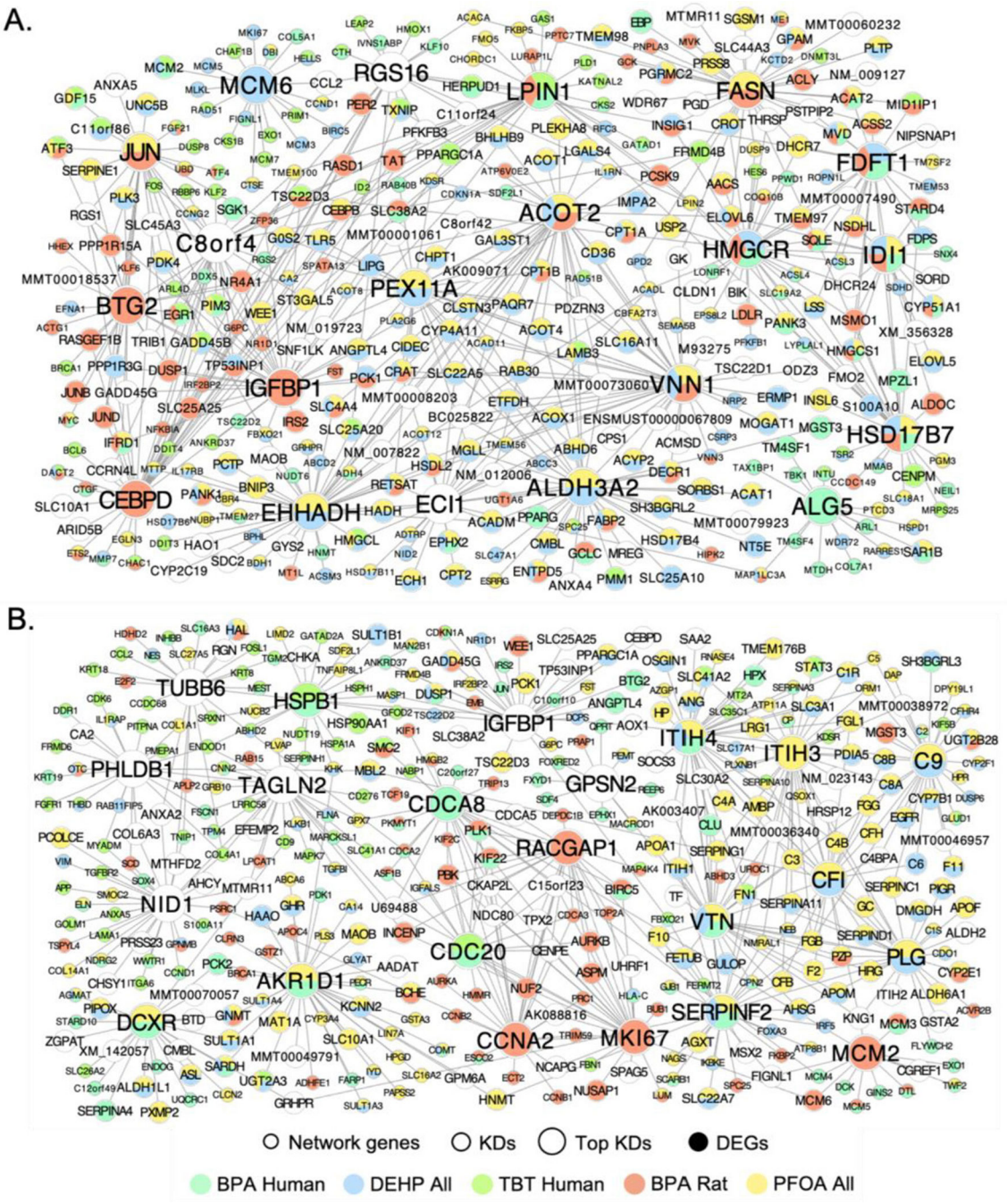

Fig. 6. Network analysis identified KDs potentially contributing to EDC-induced gene expression changes.

(A and B) Network of top 5 KDs (FDR < 0.05) derived from up-regulated DEGs (A) and down-regulated DEGs (B) for each EDC cluster. Liver Bayesian network was pre-constructed using a set of human and mouse liver datasets, as reported in Shu et al. 2016. Network genes are represented as small circular nodes, and KDs as medium/large circular nodes. Fill color indicates DEGs for specific EDCs at a Bonferroni-adjusted p-value < 0.01. (A) Top 5 KDs for BPA Human - *FDFT1, HMGCR, HSD17B7, IDI1, ALG5*; BPA Rat - *BTG2, CEBPDFASN, FDFT1, IGFBP;* DEHP All - *ACOT2, ALDH3A2, EHHADH,*

PEX11A, *VNN1;* TBT Human - *LPIN1, C8orf4, JUN, MCM6, RGS16;* PFOA All - *ACOT2*, *ALDH3A2*, *PEX11A*, *VNN1, ECI1.* (B) Top 5 KDs for BPA Human - *CDCA8*, *DCXR, GPSN2, IGFBP1, MCM2;* BPA Rat - *CCNA2*, *MKI67*, *RACGAP1, CDCA8, CDC20;* DEHP All - *C9*, *SERPINF2, VTN, AKR1D1, ITIH3;* TBT Human - *HSPB1, NID1, PHLDB1, TUBB6, TAGLN2;* PFOA All – *CFI, ITIH4, PLG, SERPINF2, VTN*.

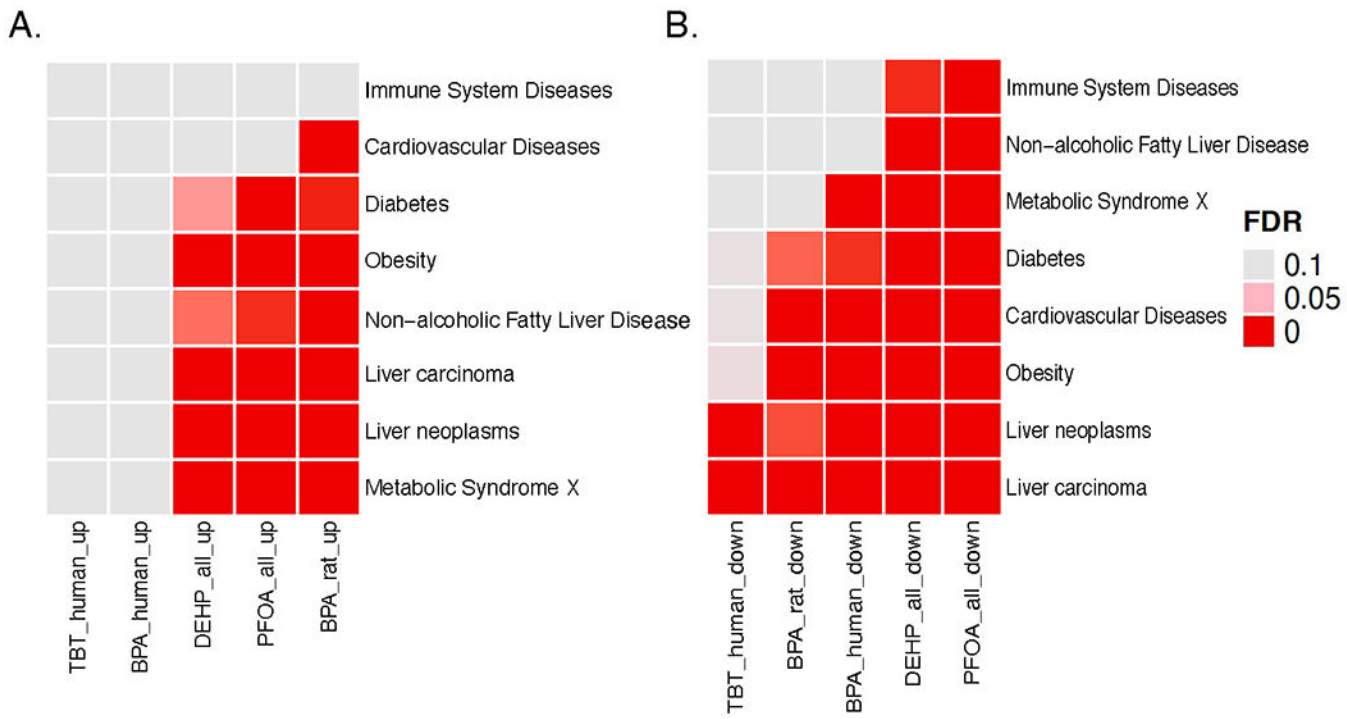

Fig. 7. Overlap between EDC cluster DEGs and CMD-associated genes

(A and B) Heatmap showing significance of overlap between up-regulated DEGs (A) and down-regulated DEGs (B) with disease-associated genes for CMDs in the DisGeNET database. Color indicates significance of associations, CMDs with FDR<0.05 were considered significant (pink and red colors).

Table 1.
Characteristics of datasets included in our meta-analysis.

GSE numbers in bold indicate RNA-Seq data. Species (Sp.) are abbreviated as follows human (Hs), rat (Rn) and mouse (Mm). Routes of exposure (Exp. Route) are abbreviated as follows *in vitro* (IV), oral gavage (OG), diet or drink (DD-C for chemicals added in the chow or DD-W when added in the drinking water). Italicized values in the dose per body weight (Dose/bw) column indicate in vitro studies for which no dose/bw are available. Exposure periods (Exp. Period) are denoted as hours (h), days (d), weeks (w), months (m). Sexes are abbreviated as male (M) and female (F). n is the study sample size/group.

GSE #	EDC	Sp.	Exp. Route	Dose/bw	Exp. Period	Sex	Age	n/group	Citation
69844	BPA	Hs	IV	*1,10,100μM*	6h	F	Adult	3	De Abrew et al., 2016
69850	BPA	Hs	IV	*1,10,100μM*	6h	F	Adult	3	De Abrew et al., 2016
59923	BPA	Rn	OG	100,610mg/kg/d	1,3,5d	M	Adult	3	DrugMatrix Database
8251	BPA	Rn	OG	610mg/kg/d	5d	M	Adult	3	Fielden et al., 2007
8858	BPA	Rn	OG	100,610mg/kg/d	0.25,13,5d	M	Adult	3	Natsoulis et al., 2008
57815	BPA	Rn	OG	100,610mg/ kg/d	1d	M	Adult	3	Gusenleitner et al., 2014
130434	BPA	Rn	DD-C	50μg/kg (3 exposures)	3d	M	Adult	3	Treviño et al., 2020
19662	BPA	Rn	IV	*10ppm*	24h	M	Adult	3	Deng et al., 2010
43977	BPA	Mm	DD-C	5,000mg/kg/d	7d	M	Adult	4	Melis et al., 2014
26728	BPA	Mm	DD-C	50,5000μg/kg/d	28d	M	Adult	6	Marmugi et al., 2012
44088	BPA	Mm	IV	*10μM*	24h	M	Adult	4	Schaap et al., 2015
28878	DEHP	Hs	IV	*10mM*	24h	M	Adolescent	3	Magkoufopoulou et al., 2012
2303	DEHP	Rn	OG	20g/kg	48h	M	Adult	5	Jolly et al., 2005
57815	DEHP	Rn	OG	1000mg/kg/d	3,5d	M	Adult	6	Gusenleitner et al., 2014
19662	DEHP	Rn	IV	*1ppm*	24h	M	Adult	3	Deng et al., 2010
40337	DEHP	Rn	IV	*250,1000μM*	24,48h	M	Adult	10	De Abrew et al., 2015
43977	DEHP	Mm	DD-C	6,000mg/kg/d	7d	M	Adult	4	Melis et al., 2014
121057	DEHP	Mm	OG	2500mg/kg/d	28d	M	Adult	6	Li et al., 2020
55733	DEHP	Mm	OG	1150mg/kg/d	2-72h	M	Adult	12	Currie et al., 2005
18564	DEHP	Mm	OG	1150mg/kg/d	4d	M	Adult	4	Ren et al., 2010
86259	TBT	Hs	IV	*2,6,10μM*	2h	F	Adult	3	Tu et al., 2016
28878	TBT	Hs	IV	*0.02nM*	12,24,48h	M	Adolescent	3	Magkoufopoulou et al., 2012
43977	TBT	Mm	DD-C	200mg/kg/d	7d	M	Adult	4	Melis et al., 2014
44088	TBT	Mm	IV	*0.3μM*	24h	M	Adult	4	Schaap et al., 2015
14712	PFOA	Rn	OG	20mg/kg/d	3d	M	Adult	3	Ren et al., 2009
19662	PFOA	Rn	IV	*30ppm*	24h	M	Adult	3	Deng et al., 2010
13044	PFOA	Mm	DD-W	1,3,5,10mg/kg/d	17d	M	Adult	5	Rosen et al., 2007
9796	PFOA	Mm	OG	1,3mg/kg/d	7d	NA	Fetal	4	Rosen et al., 2008
9786	PFOA	Mm	OG	3mg/kg/d	7d	M	Adult	4	Rosen et al., 2008
119441	PFOA	Mm	OG	1mg/kg/d	8w	M	Adult	4	Li et al., 2019

Table 2.

Study characteristics and number of differentially expressed gene (DEGs) at Bonferroni-adjusted p<0.01 of major EDC gene signature clusters.

In vitro (IV), oral gavage (OG), diet or drink (DD).

Major signature clusters	BPA Human	BPA Rat	DEHP All	TBT Human	PFOA All
Species	Human (6)	Rat (7)	Mouse (4) Rat (4)	Human (3)	Mouse (8) Rat (1)
Technical platform	Affymetrix (6)	Codelink (5) Affymetrix (2)	Affymetrix (7) RNAseq (1)	Affymetrix (3)	Affymetrix (6) Applied Biosystems (2) RNAseq (1)
Exposure Route	IV (6)	OG (7)	IV (2) DD (1) OG (5)	IV (3)	DD (4) OG (5)
Dosages	1uM-100uM	100mg-610mg	250-1000μM (IV) 6000ppm (DD) 1000mg-20g(OG)	2-10uM	1-20mg
Sex	Female (6)	Male (7)	Male (8)	Female (3)	Male (7) N/A (2)
Age	Adult (6)	Adult (7)	Adult (8)	Adult (3)	Adult (7) Fetal (2)
Down-regulated DEGs	1100	672	717	969	1189
Up-regulated DEGs	1070	681	804	857	1095
Total DEGs	2170	1353	1521	1826	2284

Table 3.

Summary of top key drivers (KDs), CMD DisGeNET associations, and KEGG pathways of major EDC clusters.

Significance was determined using FDR < 0.05 as a cutoff. Specific sub-pathways for the "Metabolic pathways" are indicated in parenthesis.

Cluster	Top 5 KEGG Pathways	Top 5 KDs	Total KDs	CMD DisGeNET Associations
BPA Human				
Down	Metabolic Pathways (amino acid and carbohydrate metabolism) Biosynthesis of Amino Acids HIF-1 signaling pathway Cell cycle Carbon metabolism	*CDCA8* *DCXR* *GPSN2* *IGFBP1* *MCM2*	13	Obesity Diabetes CVD MetS
Up	Terpenoid backbone biosynthesis Ribosome Steroid Biosynthesis	*FDFT1* *HMGCR* *HSD17B7* *IDI1* *ALG5*	17	N/A
BPA Rat				
Down	Cell cycle DNA replication Steroid hormone biosynthesis Cytokine-cytokine receptor interaction HTLV-I infection	*CCNA2* *MKI67* *RACGAP1* *CDCA8* *CDC20*	48	Obesity Diabetes CVD
Up	Steroid biosynthesis Adipocytokine signaling pathway Terpenoid backbone biosynthesis Mineral absorption Metabolic pathways (lipid metabolism)	*BTG2* *CEBPD* *FASN* *FDFT1* *IGFBP1*	86	Obesity Diabetes CVD MetS NAFLD
DEHP All				
Down	Complement and coagulation cascades Steroid hormone biosynthesis Pentose and glucuronate interconversions Metabolic pathways (lipid metabolism) Chemical Carcinogenesis	*C9* *SERPINF2* *VTN* *AKR1D1* *ITIH3*	88	Obesity Diabetes CVD MetS NAFLD
Up	Metabolic pathways (amino acid, carbohydrate, and lipid metabolism) PPAR signaling pathway Peroxisome Fatty acid metabolism Fatty acid degradation	*ACOT2* *ALDH3A2* *EHHADH* *PEX11A* *VNN1*	223	Obesity Diabetes MetS NAFLD Hypertriglyceridemia
PFOA All				
Down	Complement and coagulation cascades Metabolic pathways (carbohydrate and lipid metabolism) Staphylococcus aureus infection Steroid hormone biosynthesis Starch and sucrose metabolism	*CFI* *ITIH4* *PLG* *SERPINF2* *VTN*	283	Obesity Diabetes CVD MetS NAFLD Hypertriglyceridemia
Up	Metabolic pathways (amino acid and lipid metabolism) Peroxisome Fatty acid degradation Fatty acid metabolism PPAR signaling pathway	*ACOT2* *ALDH3A2* *PEX11A* *VNN1* *ECI1*	226	Obesity Diabetes MetS NAFLD
TBT Human				
Down	Protein processing in ER Small cell lung cancer Proteoglycans in cancer AGE-RAGE signaling pathway in diabetic complications Measles	*HSPB1* *NID1* *PHLDB1* *TUBB6* *TAGLN2*	17	N/A

Cluster	Top 5 KEGG Pathways	Top 5 KDs	Total KDs	CMD DisGeNET Associations
Up	Systemic lupus erythematosus Alcoholism p53 signaling pathway DNA replication	*LPIN1* *C8orf4* *JUN* *MCM6* *RGS16*	6	N/A