Two persistent organic pollutants which act through different xenosensors (alpha-endosulfan and 2,3,7,8 tetrachlorodibenzo-p-dioxin) interact in a mixture and downregulate multiple genes involved in human hepatocyte lipid and glucose metabolism

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Abstract

Individuals, typically, are exposed to mixtures of environmental xenobiotics affecting multiple organs and acting through different xenosensors and pathways in species and cell-type specific manners. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and α-endosulfan are Persistent Organic Pollutants (POPs) and endocrine disruptors which act through different xenosensors and accumulate in the liver. Our objective in this HEALS study was to investigate the effects of the mixture of these POPs on gene expression in a human-derived hepatocyte cell line, HepaRG. We found that, in spite of having largely uncorrelated effects, TCDD and α-endosulfan, when mixed, alter the expression of genes. The combined effects of the mixture of the POPs significantly altered the expression of 100 genes (42 up- and 58 down-regulated) whereas the same concentration of either POP alone did not alter significantly the expression of these genes. For 32 other genes, selective inhibitory crosstalk between TCDD and α-endosulfan was observed. One of the POPs inhibited the effect, on gene expression, of the other in the mixture although, when used alone, that POP did not affect expression. The expression of another 82 genes was significantly altered (up- or down-regulated) by a single POP. The addition of the second POP either increased, in the same direction, the effect on gene expression or had no further effect. At low concentrations (0.2nM TCDD and 1μM α-endosulfan), the POPs still had significant effects and the levels of expression of the corresponding proteins were found to be affected for some genes. Particularly striking was the 80-90% inhibition, by the mixture, of the expression of a number of genes of several hepatic intermediary metabolic pathways (glycerolipid metabolism, FXR/RXR activation, glycolysis/gluconeogenesis, retinoid and bile acid biosynthesis), whereas each pollutant alone had only a moderate effect.

Keywords: dioxin, pesticides, HepaRG, mixture, metabolism, microarray

1. Introduction

Environmental exposure to toxic chemicals for most individuals involves mixtures of compounds and extended periods of exposure. Humans are exposed not only to pesticides (estimated use exceeding 3 billion tons per year worldwide), mainly through food consumption, but also to a variety of other environmental xenobiotics, many of them being endocrine disrupters, which may have a variety of detrimental effects on human health [1, 2]. Epidemiological studies often associate occupational exposure to these compounds with an increase in the incidence of various pathologies, including cancers, neuro-degenerative disorders, reduced fertility and the metabolic syndrome which can lead to type 2 diabetes [3-10]. Although concerns about the adverse effects of mixtures of chemicals and their potential interactions are increasing, especially for vulnerable populations, most studies on the effects of pollutants to date have focused on exposure to a single compound or to a mixture of compounds that act through the same signaling pathway. Only a few studies [11-19] have addressed the toxicity of mixtures of contaminants which act through different xenosensors, even though combinations of different chemicals, especially at low levels of exposure, probably have significant effects on human health [20, 21].

In this study, part of the HEALS exposome European project, we investigated the effects of a mixture of two endocrine disruptors, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and α-endosulfan, which act via different signalling pathways, accumulate in the liver and may have non-cancerogenic toxic hepatic effects in humans [22-25]. TCDD is one of the most potent ligands of the aryl hydrocarbon receptor (AhR) and a classical one with little metabolism and a half-life of about 7 years in humans, which triggers several biological responses [1]. Alpha-endosulfan is the major isomer of an organochlorine insecticide known to act through two different signalling pathways, the pregnane X receptor (PXR) [26, 27] or the estrogen receptor α (ER α) [12, 28]. Although endosulfan use has been banned in many

countries, it is still in use in China and India and decades of agricultural use have built up soil reservoirs [17]. Endosulfan sulfate, which is a major metabolite of α-endosulfan is as toxic as the parent compound and more persistent with a half-life of months to years in soils, sediments and water [22, 23]. Moreover, since dioxins are by-products of chlorinated compounds, both pollutants can be associated during pesticide manufacturing. *In vivo*, pollutant induced toxic effects on the liver arise from direct effects on hepatocytes and from extra-hepatic factors. Among extra-hepatic factors that may alter hepatocyte function, the disruption of the endocrine system [29, 30] or pollutant induced modification of the environment of the organ may affect hepatic physiology. This may be due to the release of pro-inflammatory cytokines into the systemic circulation, the activation of hormonal or oxidative stress responses and hypoxia [31-37].

The objective of this study was to investigate the effects, related specifically to hepatocyte cell functions, of the mixture of TCDD and α-endosulfan, by studying global gene expression in a human-derived hepatocyte cell line, HepaRG.

To our knowledge, few studies have explored the effects on global gene expression of a combination of two Persistent Organic Pollutants (POPs), which act through different xenosensors, using a human liver cell model. HepaRG cells were chosen for the study because, after differentiation, they express high levels of several xenobiotic metabolizing enzymes and xenosensors [38] and, to date, it is the human cell line that most closely resembles human hepatocytes [39, 40]. A recent transcriptomic study, using five carcinogens, revealed that the HepaRG model was better suited for understanding the biological effects of exposure to the chemicals as compared to the HepG2 hepatocarcinoma cell line which has a low metabolic capacity and reduced PXR level [41-43]. Several other studies also have concluded that the HepaRG cell line is an excellent *in vitro* model to study human drug metabolism [39, 44-46] as well as being a relevant model for studying glucose, lipid and

lipoprotein metabolism [47, 48]. The use of the HepaRG cell line, which also exhibits stable and inducible enzyme expression over long periods (weeks), and has reduced variability, thus avoids many of the difficulties associated with the use of human hepatocytes such as scarce availability, complicated isolation procedures, variability, rapid dedifferentiation precluding long term use and cost [49-52].

This study was designed to provide information on the combined effects of TCDD and the pesticide α-endosulfan on genome-wide gene expression in one of the most relevant human hepatic cell lines, and to provide new data on the hepatic perturbations which may be linked to exposure to mixtures of persistent organic pollutants.

2. Materials and Methods

2.1. Compounds, cell culture, viability and treatments

The chemical compounds that were used in this study, 2,3,7,8 tetrachlorodibenzo-p-dioxin or TCDD (PubChem CID: 15625) and α-endosulfan (PubChem CID: 3224), were obtained from LGC Standards (France). HepaRG cells, obtained from Dr. Guguen-Guillouzo [53], were differentiated as described previously except that 1.5% DMSO was used for cell differentiation [54]. DMSO was removed from the medium for twenty-four hours before treating the cells for 30 hours with 25nM TCDD, 10μM α-endosulfan, the mixture of the two pollutants (at the above concentrations) or the vehicle (0.15% DMSO). The viability of the cells was evaluated using the WST-1 kit (Roche Applied Science). For some experiments, the cells were treated for 8 days with lower concentrations of the compounds (0.2, 0.5, 1 or 5 nM TCDD and 1 or 3μM α-endosulfan) with only two changes of medium containing the compounds during this period (day 0 and day 4) to minimize any build up in the concentrations of the chemicals due to binding of the xenobiotics to the plastic of the culture dishes.

2.2. RNA preparation and microarray hybridization

RNA from the HepaRG cells was prepared using the RNeasy mini kit from Qiagen (France) as described previously [55] except that a DNase I step was included in the protocol. For the microarray studies, the quality of the RNA (RIN value) was assessed with a Bionalyzer (Agilent Technologies) [56].

ssDNA (sense single stranded DNA) was synthesized using the Affymetrix GeneChip Whole Transcript Sense Target Labelling Assay kit, according to the manufacturer's protocol. ssDNA samples were then fragmented according to the Affymetrix protocol. The purified ssDNA was quantified and its quality was assessed with a Bioanalyzer. Subsequent

labeling of the samples was performed by synthesis of Biotin-labeled ssDNA using the GeneChip WT Terminal Labeling kit (Affymetrix). ssDNA targets were hybridized onto high-density microarrays (Affymetrix Human Genome 1.0 ST GeneChip array) according to the Affymetrix Eukaryotic Target manual. The microarrays were then washed and stained using the Affymetrix fluidics station 450/250 and Genechip Operating Software and scanned with an Affymetrix GeneArray scanner. The raw affymetrix datasets (.CEL) are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) with the accession number (GSE46874).

2.3. Microarray analyses

Quality controls, including scaling factors, average intensities, background intensities, noise (raw Q) values were within acceptable limits for all the arrays. The twelve datasets obtained were processed and normalized using the plier program in R. Two different statistical analyses were performed: 1) we determined which genes were significantly differently expressed ($p \leq 0.05$ by t-test, >2fold difference) following exposure of cells to TCDD, α-endosulfan or the mixture of the two as compared to the vehicle (DMSO), 2) we used the Focus software [57], as described by Garcia-Ortiz [58] to select genes that had at least a 1.2-fold mean difference in expression and a default "interest score" of >5. Principal component analysis (PCA) was carried out using the svd module in R. Hierarchical clustering analysis was performed with the Genepattern software [59] on the statistical scores derived from the Focus analysis rather than on the expression levels in order to reduce data variability.

Gene Set Enrichment Analysis (GSEA, http://www.broadinstitute.org/gsea/index.jsp) was used to test (using a metric derived from the Kolmogorov-Smirnov statistic [60, 61])

whether the distribution of the genes in our study deviated from random in ranked lists of genes up or down regulated by TCDD or 17β-estradiol. Rank-rank hypergeometric overlap was also used to identify statistically significant overlap between gene expression signatures [62]. The lists of genes were derived from an analysis of publicly available (literature or public repositories) genome-wide expression data obtained from exposure of primary human hepatocytes [63, 64], HepaRG [42] or mouse primary hepatocytes [65] to TCDD or 17β-estradiol. Raw data were renormalized with the plier module in R and robust averages calculated with Tukey's Bi weight average algorithm [66]. One way analysis of variance (ANOVA) was calculated for each pair of treatment groups and for all treatment groups. FDR were calculated by the Benjamini-Hochberg approach [67]. Differentially expressed genes were those having a fold change >2 and $p<0.05$. The data from supplementary files 11 and 17 from Forgacs et al. [64] were filtered as indicated in the files. Murine expression data from Flaveny et al. [65] were also analyzed with Focus. Genes were ranked according to Focus scores and filtered to retain only those showing a differential expression with t-test p-values less than 0.20 on log ratios. The genes with the highest Focus scores (165 and 195 genes up- and down-regulated, respectively, by TCDD) were selected as the gene sets to be compared with our samples. Exact area-proportional Euler diagrams were calculated and drawn with eulerAPE [68].

2.4. Functional analysis

Biological functions and pathways were generated from the Focus lists of genes up- or down-regulated by TCDD plus α-endosulfan (as compared to either DMSO, TCDD or α-endosulfan alone) and analyzed using Ingenuity Pathway Analysis v.8.3-3003 (IPA, Ingenuity Systems, CA).

2.5. Quantitative reverse transcriptase PCR

A selected set of genes was analyzed by RT-qPCR using cDNA prepared from the 3 independent culture replicates used for the microarray experiments. The PCR primers used are listed in Table S1. Reverse transcription and quantitative PCR were performed as previously described [55].

2.6. Western blotting

Cells were scraped into 1X PBS buffer, containing 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS, protease and phosphatase inhibitors (Sigma). After freezing at -80°C, the cells were thawed and centrifuged 10 min at 9 000g at 4°C. The protein concentration in the supernatant was measured using the bicinchoninic acid method (Pierce) with BSA as a standard. The supernatant was aliquoted and kept at -80°C. Equal amounts of total proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blocking of the membrane was performed using Odyssey buffer (LI-COR, ScienceTec, Courtaboeuf, France) for 1 hour at room temperature followed by incubation overnight at 4°C with a primary antibody directed against ADH1 (Acris AP16311PU-N, 1/1 000) or β-actin (Abcam 8227, 1/10 000). After three washes with 0.1% Tween-20 in 1X PBS, the membrane was incubated with either an anti-goat (IRdye 800 number 926-32214 LI-COR, ScienceTec, 1/15 000) or an anti-rabbit (IRdye 800 number 926-32211 LI-COR, ScienceTec, 1/10 000) secondary antibody. After three washes (0.1% Tween-20 in 1X PBS), signals were quantified using the Odyssey infrared Imager (LI-COR, ScienceTec).

3. Results

3.1. Global gene expression in HepaRG cells treated with TCDD and α-endosulfan

The concentrations of TCDD (25 nM) and α-endosulfan (10μM) were chosen so as to maximize the activation of the corresponding signalling pathways and, thus, to increase the number of genes that are differentially expressed. To assess the overt toxicity of these concentrations, the viability of the differentiated HepaRG cells was measured and was found not to be significantly different following exposure for 72 hours to the mixture or to either POP alone as compared to the 0.15% DMSO vehicle (Figure S1, A) or for up to 8 days at lower concentrations of the POPs (Figure S1, B). These concentrations were, therefore, used to investigate the effects of the POPs on global gene expression.

Principal component analysis (PCA) of the global variation in transcription of the HepaRG genome following exposure to the mixture or to each pollutant alone leads to several conclusions. First, the samples clustered by condition, which demonstrates that there is a significant difference in the transcription profiles that resulted from exposure to either POP alone or the mixture (Figure 1). Further, the samples treated with TCDD (alone or in combination with α-endosulfan) consistently mapped to one side of the first principal component (55% of the variability). Alpha-endosulfan-treated samples mapped to one side of the second principal component (20% of the variability). The first principal component, PC1, thus accounts mainly for the effects of TCDD and the second principal component, PC2, for the effects of α-endosulfan. Second, the major effects of TCDD and α-endosulfan are largely uncorrelated because the corresponding PCA axes are orthogonal. This result is consistent with these compounds exerting their effects by different mechanisms (pathways) and with our current knowledge of the actions of these pollutants. Third, although the major effects of TCDD and α-endosulfan are largely uncorrelated, the principal component analysis also

clearly demonstrates that the effects of the mixture of α-endosulfan and TCDD are greater than those of TCDD alone as shown by the significant difference ($p < 0.05$, post-Anova Tukey's test) in the mean PC1 values of the corresponding groups. Thus, in general, α-endosulfan exacerbates the effects of TCDD in the same direction of change that is most strongly associated with TCDD.

We next analyzed the global transcription data for differentially expressed genes using stringent criteria ($p \leq 0.05$, >2-fold difference in expression for up-regulated genes and < 0.5-fold for down regulated genes). The mixture of pollutants significantly altered the expression of 182 annotated genes, whereas TCDD and α-endosulfan altered the expression of 98 and 23 genes, respectively, as compared to the DMSO vehicle. A Euler diagram shows that the 214 unique genes corresponding to the three treatments can be divided into 7 ensembles (Figure 2).

First, the two ensembles labelled A, B are remarkable in that 21 genes (ensemble A) were modified only following treatment of the cells with TCDD and 10 genes (ensemble B) were modified only following treatment with α-endosulfan (Figure 2, Table S2). Intuitively, one would expect that if the expression of a gene was altered following treatment of the cells with a pollutant individually, then treatment with a mixture containing that pollutant would also affect the expression of the gene. However, in this case, although each pollutant significantly alters the expression of certain genes when present alone, the presence of the other POP in the mixture abolishes this effect (that is, the expression of the gene is no longer found to be significantly differentially expressed by the mixture of the pollutants). This suggests that inhibitory cross-talk occurs between the pollutants at the level of gene expression. It is important to note that the POP that is inhibitory in the mixture is not identified as significantly altering the expression of the gene when used alone. This aspect distinguishes ensembles A and B from ensemble C. The single gene in this ensemble (serine

dehydratase) was significantly modified by either TCDD (61% decrease in expression) or α-endosulfan (6.37-fold increase in expression), individually, but in opposite directions, and, thus, the gene not being differentially expressed in the mixture (1.66-fold). Second, an ensemble of 100 genes (ensemble D) is identified only after treatment of the cells with the mixture of the POPs (Figure 2, Table S2). That is, the expression of each of these genes is not significantly altered following exposure of HepaRG cells to either TCDD or α-endosulfan alone but only to the mixture of the pollutants. Thus, although treatment with either POP alone may not result in a significant effect (according to the stringent criteria adopted for identifying differentially expressed genes), the mixture of the POPs does result in significant alteration in gene expression. Since the experiment was not designed to evaluate additivity or synergy of effects, no conclusions can be drawn in this respect although the fold changes in expression found for the genes altered by the mixture are not markedly different from the sums of the fold changes in expression obtained following exposure to each pollutant alone.

Third, two other ensembles are composed of genes which are differentially expressed by cells following exposure to either TCDD or the mixture of TCDD and α-endosulfan (70 genes, ensemble E, Figure 2, Table 1) or to α-endosulfan or the mixture (6 genes, ensemble F, Figure 2, Table 1). Thus, exposure to one pollutant results in a significant change in gene expression and the addition of the second pollutant does not modify this effect as in ensembles A, B and C. For most of the genes in ensembles E and F, TCDD and α-endosulfan exert their effects in the same direction (either both increasing or both decreasing gene expression). For some genes, TCDD seems to exert the major effect and α-endosulfan does not further alter the expression of the gene. Finally, for some genes, α-endosulfan diminishes somewhat the effect of TCDD and for one gene, GPX2, TCDD appears to diminish the effect of α-endosulfan (similar to the inhibitory cross-talk described for ensembles A, B and C). However, in all cases, the effect of the second pollutant is not sufficient to eliminate the gene

from the list of differentially expressed genes in contrast to what is observed for the genes in ensembles in A, B and C. Although the effect observed in ensembles E and F resembles that observed in ensembles A, B and C, the mechanisms involved are not necessarily the same.

The final ensemble, G, is composed of 6 genes (Figure 2, Table 2) which are differentially expressed ($p<0.003$-0.00001) following all of the treatments (TCDD or α-endosulfan alone or the mixture). Interestingly, as opposed to all the other ensembles in which both increases and decreases in gene expression are found, this ensemble is composed of genes in which only a decrease in expression is observed and bothTCDD and α-endosulfan exert their effects in the same direction.

In a second approach to identify differentially expressed genes, we ranked genes using a combination of multivariate and pairwise comparisons among all the conditions using somewhat less stringent conditions (a cutoff of 1.2 for the fold change and a Focus score of 5, followed by a two-tailed t-test, $p<0.05$). Exposure of HepaRG cells to the mixture of pollutants altered, to a greater extent, the expression of 662 annotated genes (558 upregulated and 104 downregulated) as compared to exposure to either chemical alone. An additional 289 non-annotated transcripts were also found (211 up- and 78 down-regulated). The top 10 up- and 10 down-regulated genes are listed in Table 3.

We then compared the list of those 20 genes (top 10 up- and down-regulated genes) with the list of genes identified with the stringent conditions (stringent t-test analysis). Five genes (indicated by bold type in Table 3) were found in the genes regulated following treatment with the mixture only (Table S1). Nine other genes are common to the list of genes whose expression is altered following exposure to TCDD alone and the mixture (Table 1 and * in Table 3) or to α-endosulfan alone and the mixture (CYP 2E1, see Table 1). Finally, 3 genes ($^{\$}$ in Table 3) are found on the list of genes that were found to be altered by exposure to all 3 treatments (Table 2). Since the expression of the remaining 3 genes of the Focus list

varied less than 2-fold, they were not present in the stringent analysis. The results of the Focus analysis were, thus, in good agreement with the more stringent analysis used to identify genes that discriminate the effect of exposure to the mixture from that of exposure to a unique POP.

Hierarchical clustering (HC) analysis of the Focus scores of the differentially expressed genes demonstrates that the gene expression profile for HepaRG cells exposed to the mixture of pollutants clusters with that of cells exposed to TCDD alone in agreement with the PCA analysis. The HC analysis also shows that large clusters of genes are more strongly up-regulated (Figure 3A, intense red color in black box) or down-regulated (Figure 3B, deep blue color in black box) in cells following exposure to the mixture as compared to exposure to a single POP.

3.2. Correlation between the microarray, RT-qPCR and immunoblotting technologies

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was used as an independent, confirmatory, technique to evaluate the expression of a subset of the genes found to be differentially expressed by global genome expression analysis. We measured the expression of 30 genes, 11 up-regulated genes and 19 down-regulated. These genes, taken together, exhibit a large range of fold changes, as assessed by whole genome analysis, following exposure of cells to the mixture of the POPs or to dioxin and α-endosulfan alone (Table S3). The values for the expression of the genes obtained by micrarray analysis and RT-qPCR are highly correlated (R^2 = 0.97, 0.90 and 0.97 for treatment by TCDD alone, α-endosulfan alone or the mixture, respectively, Figure S2) suggesting that the global gene results are trustworthy.

To determine whether exposure to lower concentrations of the POP mixture for longer periods of time exhibited the same effects, we exposed HepaRG cells for 8 days to lower concentrations of TCDD (0.2nM to 5nM) and a 10-fold lower concentration of α-endosulfan (1μM) and to the various mixtures. We examined the expression of two genes (ADH1B and G6PC), the expression of which was severely downregulated with the highest concentrations previously tested. ADH1B gene expression was markedly decreased after treatment with 5nM TCDD and G6PC gene expression was already decreased at the lowest TCDD concentration tested (0.2nM TCDD). Although a concentration of 1μM α-endosulfan did not affect significantly the expression of either ADH1B (no effect) or G6PC (40% decrease), the mixture of the POPs decreased the expression of both genes significantly more as compared to the effect of TCDD alone. (Figure 4A). Alteration in the expression of the gene may be followed by an effect on the level of the protein present in the cells, as shown by the decrease in the amount of ADH1 (Figure 4B), after 3 days of exposure to the POPs (5 or 25nM TCDD, 10μM α-endosulfan or their mixtures.

3.3. Pathways regulated by the mixture of TCDD and α-endosulfan

To elucidate the effects on hepatocyte function following exposure of HepaRG cells to the combination of the two pollutants, we used Ingenuity Pathway Analysis (IPA) to assign the biological pathways altered by the different treatments. The 4 top network functions associated with the genes regulated by the mixture of TCDD and α-endosulfan using the Focus analysis were: i) RNA post-transcriptional modification, genetic disorder, lipid metabolism; ii) cancer, gastrointestinal disease, genetic disorder; iii) molecular transport, RNA trafficking, cell cycle; iv) small molecule biochemistry, DNA replication, recombination, repair, cell cycle. The lipid metabolism, small molecule biochemistry and molecular transport networks were the top networks associated with genes that were down-

regulated whereas the other key words were mostly associated with up-regulated genes. The associated canonical pathways are given in Table 4. The farnesoid X receptor/retinoid X receptor (FXR/RXR), bile acid biosynthesis and glycerolipid metabolism pathway genes were down-regulated, as were genes in the glycolysis/gluconeogenesis pathway. The top down-regulated network by the mixture of TCDD and α-endosulfan (as well as the top molecular and cellular functions network), "lipid metabolism", derived by the Ingenuity network analysis ($p < 0.00001$) contains the genes shown in Table S4. Only the 11 genes exhibiting at least a two-fold change as compared to the control are shown in the table although another set of 13 genes belonging to the same network was also derived from the Ingenuity analysis with a cutoff of 1.2 for the fold change. For all of these genes, TCDD and α-endosulfan exert their effects in the same direction.

The pathways related to cancer and control of the cell cycle, as well as the AhR signaling pathway, were up-regulated. The GTPase RAN (Ras-related nuclear protein) signaling pathway was also up-regulated. RAN is involved in cell differentiation and transformation, as it acts on the assembly of the mitotic apparatus and in nuclear protein import/export and its overexpression is linked to a poor prognosis in cancer [69].

There is no other published study on whole genome expression using TCDD and α-endosulfan with which we can compare our results and thus the information provided here is novel. We, thus, compared our results following exposure of HepaRG to TCDD or α-endosulfan alone with results published in the literature. None of the studies employ conditions that are identical to our work, but a similar study evaluated the effect of exposure of HepaRG cells to 10 nM TCDD or 30μM 17β-estradiol for 12 or 48 hours [42] and two other analyses employed primary human hepatocytes exposed to several concentrations of TCDD for different times [63, 64]. Several of the up- and down-regulated gene sets derived from these studies were enriched in the most strongly up- and down-regulated genes in our

own ranked list when analyzed by the gene set enrichment analysis algorithm (Figure S3). For the only study employing 17β-estradiol [42], the down-regulated gene sets for both the 12 and 48 hour treatments with 30μM 17β-estradiol and the up-regulated gene set for the 12 hour treatment were enriched in the down- and up-regulated genes of our ranked list for α-endosulfan with FDRq (false discovery rate) < $1x10^{-5}$ and FWER (family wise error rate according to Benjamini and Hochberg [67] <$1x10^{-4}$. Rank rank hypergeometric overlap also identified statistically significant overlap between the expression signatures found by Jennen et al. [42] for 17β-estradiol (both 12 and 48 hour treatments) and our own for α-endosulfan (see Figure S3 for representative GSEA curves and Rank Rank Hypergeometric heat maps). For TCDD, most enrichment was found (based on the normalized enrichment scores) for the up and down-regulated gene sets of Jennen et al. [42] following exposure of HepaRG to 10nM TCDD for 12 or 48 hours. Significant enrichment was also found for the up- and down-regulated sets of Forgacs et al. [64] following exposure of primary hepatocytes to 10nM TCDD for 12 and 48 hours as well as the up-regulated gene set for exposure for 24 hours. Less significant enrichment was found for the gene set of up-regulated genes of Carlson et al. [63] following exposure of primary hepatocytes to 10nM TCDD, although the FDRq was < $1x10^{-5}$ and the FWER was <$1x10^{-4}$ (data not shown). Finally, we compared the lists of genes that were differentially regulated by treatment with TCDD alone in our samples with those of a microarray study performed on primary mouse hepatocytes [55]. Although the lists of genes that were most strongly up- and down-regulated by TCDD in the mouse hepatocytes mapped among the most strongly up- and down-regulated genes in our ranked list, when analyzed by the Gene SetEnrichment Analysis algorithm (GSEA, $p<0.001$; data not shown), the FDR and FWER values were not as significant as for the gene sets from the HepaRG cell line or the primary human hepatocytes.

Taken together, the results attest to an overlap in the regulations of gene expression by estradiol and α-endosulfan in HepaRG cells as well as to common effects of exposure to TCDD in the HepaRG cell line and primary human hepatocytes.

4. Discussion

Elucidation of the mechanisms by which mixtures of compounds, which act via different pathways or xenosensors, can affect cell function may be useful for further understanding how toxicants interact and how these interactions might affect toxicity. However, as concerns human hepatotoxicity, there is no one model system that is ideal for elucidating these mechanisms. Epidemiological studies may be incomplete as to exposure, may have small numbers of participants which limit the power of the statistical analyses for detection of effects, may have concomitant exposures to other counfouding factors that may have effects and, finally, may involve extrapolations over long periods of time that may lead to considerable errors in estimations of initial exposure. *In vivo* animal models are limited by the differences that exist between animals and humans and by the difficulty in extrapolating effects found in animals to humans [70, 71].

Primary cultures or permanent cell lines, *in vitro*, are not exposed to the local factors derived from the organ or to factors originating at a distance in the organism. Nevertheless, *in vitro* studies present advantages for studying cell type specific mechanisms and have been promoted by regulatory agencies as alternatives to animal studies [71, 72] and transcriptomics has proven useful in identifying pathways perturbed by toxicants [73]. However, species specific differences exist between animal and human primary hepatocyte cultures [63]. Further, human primary cultures exhibit variability due to differences in the donors with respect to genetics [74], demographics, disease and drug therapies [75-77] and this variability may be manifest following xenobiotic exposure [78]. Finally, there may also

be differences in the response to toxicants between human primary hepatocytes and established human hepatic cell lines [79-81] or among established human hepatocyte cell lines [42, 82, 83].

In this study, we have used a human liver-derived cell line, HepaRG, to provide novel information concerning the effects on hepatocyte function of a mixture of POPs that exert their effects via different xenosensors. TCDD, which binds to the AhR, and α-endosulfan, which acts via the ER and/or PXR signalling pathways, were chosen because they both accumulate in the liver in animals and humans, they are suspected of having non-carcinogenic hepatic toxicity and there is knowledge of their effects individually (at least for TCDD) that may help to understand the effects of the mixture. The concentrations that were employed maximize the activation of the corresponding signalling pathways without leading to overt toxicity during the course of the experiment.

To our knowledge, this is the first genome-wide study in a human hepatic cell model of the effects of α-endosulfan alone or in combination with TCDD thus providing novel information on the modulation of global gene expression by the ER and/or PXR xenosensors alone and in combination with the AhR. The results demonstrate that, for the most part, the effects of TCDD and α-endosulfan on HepaRG hepatocytes are uncorrelated, which is consistent with these compounds exerting their effects by different mechanisms (pathways). However, it is also clear that the combined effects of α-endosulfan and TCDD are greater than those of each POP alone. Thus, for concentrations at which TCDD or α-endosulfan, individually, do not alter gene expression significantly, the mixture does produce a significant modification in the expression of 100 genes, although it should be emphasized that no conclusions can be made regarding the additivity or synergy of effects given that only a single concentration of each pollutant was studied. Further, although inspection of Table S2 reveals that many genes appear to exhibit some modulation in the level of their expression

after treatment with one pollutant, these changes are not significant given the stringency of the statistical analysis and the low level of expression of those genes in many cases. This may illustrate the delicacy of establishing zero-effect levels.

We also found evidence for what appears to be selective inhibitory crosstalk within the AhR and ER/PXR pathways. TCDD, in the mixture, inhibited the effects of α-endosulfan on the expression of 10 genes and α-endosulfan inhibited the effects of TCDD on 21 genes (Table 1). Serine dehydratase, the expression of which is affected by both TCDD and α-endosulfan, but not the mixture, may be added to these ensembles although the mechanisms may not be the same. Several mechanisms for inhibitory AhR-ERα cross-talk, which may be gene/response and cell-context dependent, have been derived from the study of various non-hepatic models [84-86]. Our observations would appear to be novel for a human hepatic-derived cell line and the genes identified, if validated, might be useful targets to further understand these negative mechanisms of regulation in the liver.

Our investigation further revealed that the response to the mixture of these endocrine disruptors involves both increases and decreases in the expression of many genes which belong to several pathways. However, the drastic down-regulation of genes involved in several metabolic pathways, such as lipid metabolism, which is the top network for down-regulated genes, is particularly striking. mRNA expression is almost abolished for some of the genes by the mixture. Indeed, some genes, which were not identified as targets after treatment by a single pollutant because the fold change in their expression was below the cut off, are identified following treatment by the combination of pollutants as a result of a significant change in their expression.

Among the genes belonging to the lipid metabolism network (See Table S4) is ADH4, a member of the alcohol dehydrogenase family, which is organized as a cluster on chromosome 4 (ADH1A, B, C, ADH4 and ADH6). Interestingly, the drastic down-regulation

of the expression of several of these ADHs has been linked to increased aggressiveness and a lower survival rate in breast, liver or lung cancer [87, 88]. The expression of these genes, as well as those of RDH16 (retinol dehydrogenase) and CYP2E1, all of which metabolize ethanol and/or retinoids, was decreased 70 to 80% in HepaRG cells exposed to both pollutants. These changes in the level of mRNA were paralleled by changes in the amount of protein as shown for ADH1. Moreover, we observed a similar type of regulation using lower concentrations of pollutants and a longer time of exposure.

The members of the alcohol dehydrogenase family metabolize a wide variety of substrates such as ethanol and vitamin A, various aliphatic alcohols and lipid peroxydation products. Several studies have shown clearly that dioxins, through the AhR, disturb retinoid homeostasis, in particular by decreasing hepatic vitamin A and by increasing oxidation of the retinoids [89]. A few studies suggest a role for other pollutants, such as pesticides, in the deregulation of retinoid homeostasis [90].

The drastic down-regulation, up to 80%, of the expression of the alcohol dehydrogenase gene family by the combination of dioxin and α-endosulfan could thus exacerbate the modulation of vitamin A content and retinoic acid production induced by a single pollutant in the liver, thus further affecting signalling through the RAR-RXR pathway. In fact, hepatic vitamin A depletion in rodent models after treatment with TCDD has been linked to CYP1A1 induction and increased metabolism of this nutrient [91]. It has been suggested that alteration of retinoid metabolism could play a role in the wasting syndrome associated with dioxin exposure [92]. Our results also suggest that the almost complete inhibition of the expression of several enzymes involved in retinoid and/or alcohol metabolism by the combination of TCDD and α-endosulfan may profoundly alter the homeostasis of vitamin A and impair the protective role of retinoic acids in cancer, aging or immuno-stimulation.

The combination of pollutants also inhibited the genes for several enzymes which belong to the FXR/RXR axis, which plays a role in bile acid signalling. The expression of a key enzyme involved in bile acid biosynthesis from cholesterol (CYP7A1) was down-regulated by 80%, following treatment with the mixture of POPs (Table S4). TCDD also decreased CYP7A1 expression in a rat liver model [92]. The expression of the canalicular half-transporters ABCG5/G8, which associate at the plasma membrane to control the excretion of excess cholesterol from the liver, was inhibited by 70% following treatment with the combination of pollutants (Table S4). Thus, the down-regulation of these genes may contribute to increased cellular cholesterol and fatty liver disease [93] and, along with other factors, to the development of the metabolic syndrome [94]. The treatment with the mixture also resulted in a 90% decrease in expression of the gene for the liver catalytic subunit of glucose 6 phosphatase (G6PC). G6PC is the final enzyme in gluconeogenesis and glycogenolysis which provides energy and controls the serum glucose level in the blood during fasting. These results are in line with the 50% decrease in the expression of G6PC that is observed in both the human hepatoma cell line HuH7 treated with the PXR agonist rifampicin [95] and in chick embryo hepatocytes treated with TCDD [96]. Several epidemiological studies indicate an association between obesity and the metabolic syndrome and the concentration of POPs in serum [4, 6, 97] and our results concerning the effects on hepatocyte gene expression may be consistent with a role of POPs in this syndrome. Finally, exposure to numerous pollutants is often associated with an increase in cancer [98] and the up-regulation of the genes involved in AhR signalling, the molecular mechanisms of cancer and cell cycle checkpoint control that we observe is consistent with these studies.

In the future, comparison of acute and “chronic” exposures to lower pollutant concentrations will be important. The HepaRG cell line is a human model of choice for studying such sub-chronic treatments since the differentiated cells stably express (for two

weeks at least) several CYPs and receptors for xenobiotics [39]. Our results obtained with an 8-day treatment with low doses of TCDD and α-endosulfan indicate that low doses over longer periods of time can lead to changes in gene expression similar to those obtained with an acute treatment at higher concentrations of POPs.

Epidemiological studies have associated chronic exposure to low levels of POPs with metabolic disorders such as insulin resistance or disruption of lipid metabolism [97, 99, 100]. Indeed, there are major concerns about the fact that living organisms are exposed to complex mixtures of POPs throughout their lifespan and that the effects of combinations are not well characterized. Our transcriptomic study was thus designed to pinpoint which genes had their expression severely dysregulated by the combination used. Our findings emphasize that deleterious hepatocellular effects may be more pronounced with a mixture of POPs which act through different signalling pathways as compared to each POP alone. We are currently investigating the effects of the mixture on a number of metabolic pathways down-regulated in the study and deciphering which receptors are involved in these effects.

With respect to the effects of TCDD, although manifestations of hepatotoxicity (hepatocellular tumors, cytoplasmic vacuolization, multinucleate hepatocytes, inflammation, steatosis, necrosis) have been noted [101-103] in several laboratory animals (mouse, rat, chicken, guinea pig, rabbit, zebra fish), the United States Environmental Protection Agency in its reanalysis of key issues related to dioxin toxicity [24] noted that “hepatic effects were evident in virtually all rodent studies that looked for them and are often severe, although not evident in humans”. Further, it is not clear whether toxicant-associated steatohepatitis develops in humans exposed to TCDD [104] and although there appears to be an association between dioxin and type 2 diabetes a causal link is not necessarily implied [5, 10, 105, 106]. Continued investigation in a variety of model systems clearly seems warranted to further elucidate the mechanisms of action of these pollutants in humans.

In conclusion, this study provides novel information on the increased effects on global gene expression of a mixture of environmental POPs, as compared to each one individually, in a human liver-derived model and, in particular, on the down-regulation of genes involved in metabolic pathways. It also demonstrates that in spite of their effects being largely uncorrelated, TCDD and α-endosulfan act together to affect the expression of a significant number of genes by several different mechanisms. Finally, the study provides novel information concerning the usefulness of the HepaRG cell line as a model system for studying human hepatocyte specific xenobiotic effects.

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Figure legends

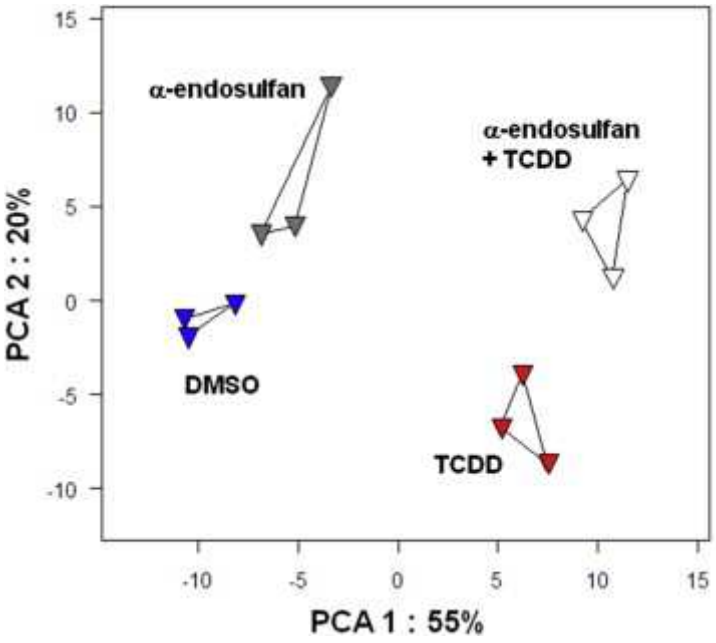


Figure 1. Principal Component Analysis (PCA) of the top 1,000 most variable genes. The expression profiles discriminate the experimental conditions. The total variance explained by either component is given in %. Triangles represent the samples and the lines connect the samples from the same experimental condition: DMSO (blue), α-endosulfan (grey), TCDD (red), mixture of TCDD and α-endosulfan (white).

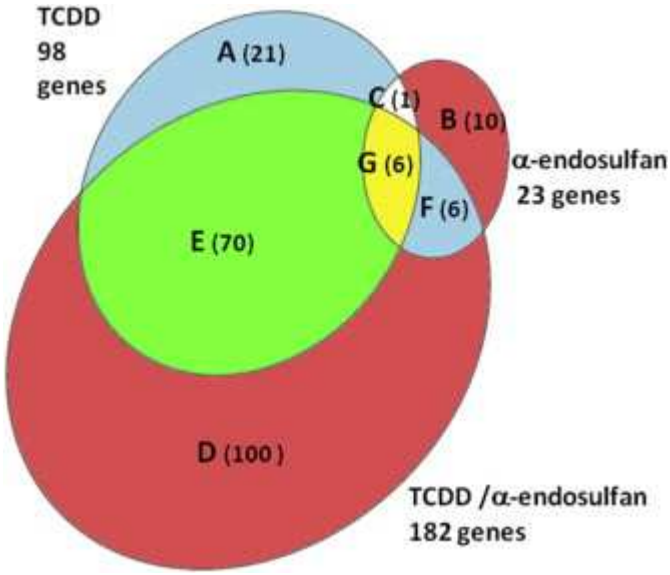


Figure 2. Exact-area proportional Euler diagram for the genes that are differentially regulated by the POP treatments. The total numbers of genes up- and down-regulated (p-value ≤ 0.05, >2-fold change, absolute value) following exposure of differentiated HepaRG cells to 25nM TCDD, 10µM α-endosulfan or their combination for 30H are shown.

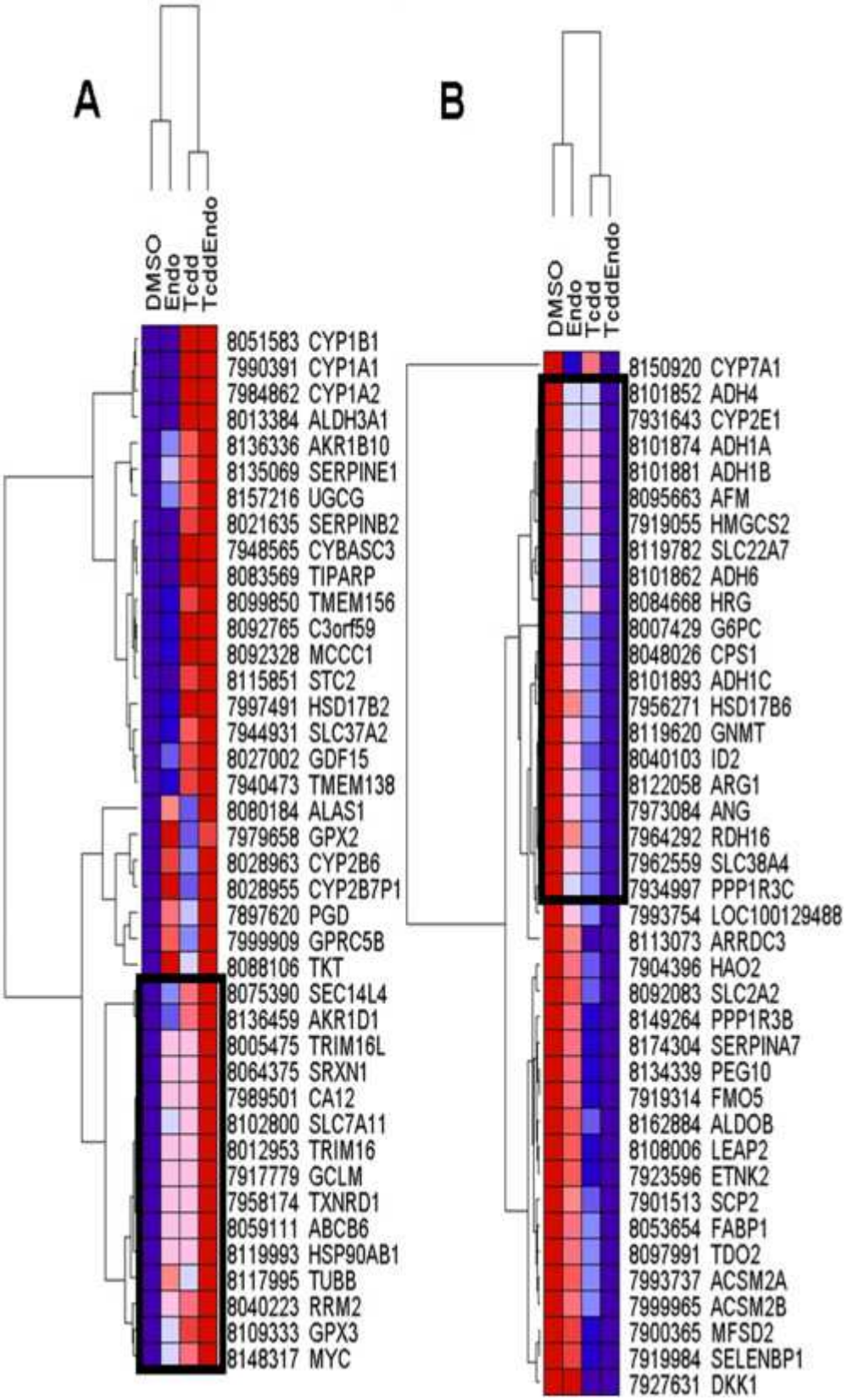


Figure 3. Hierarchical clustering analysis of 80 genes showing the greatest changes compared to vehicle (0.15% DMSO). Increased (N=40) and decreased (N=40) levels of

expression are shown in the left (A) and right (B) panels, respectively. The rows represent the genes. The columns represent the enrichment scores for each gene in the treatments. The black lined boxes indicate gene clusters that show strongly increased up-regulation (A) or down-regulation (B) after treatment of cells with the mixture as compared to the individual POPs. Red and blue colors indicate up- and down-regulation, respectively.

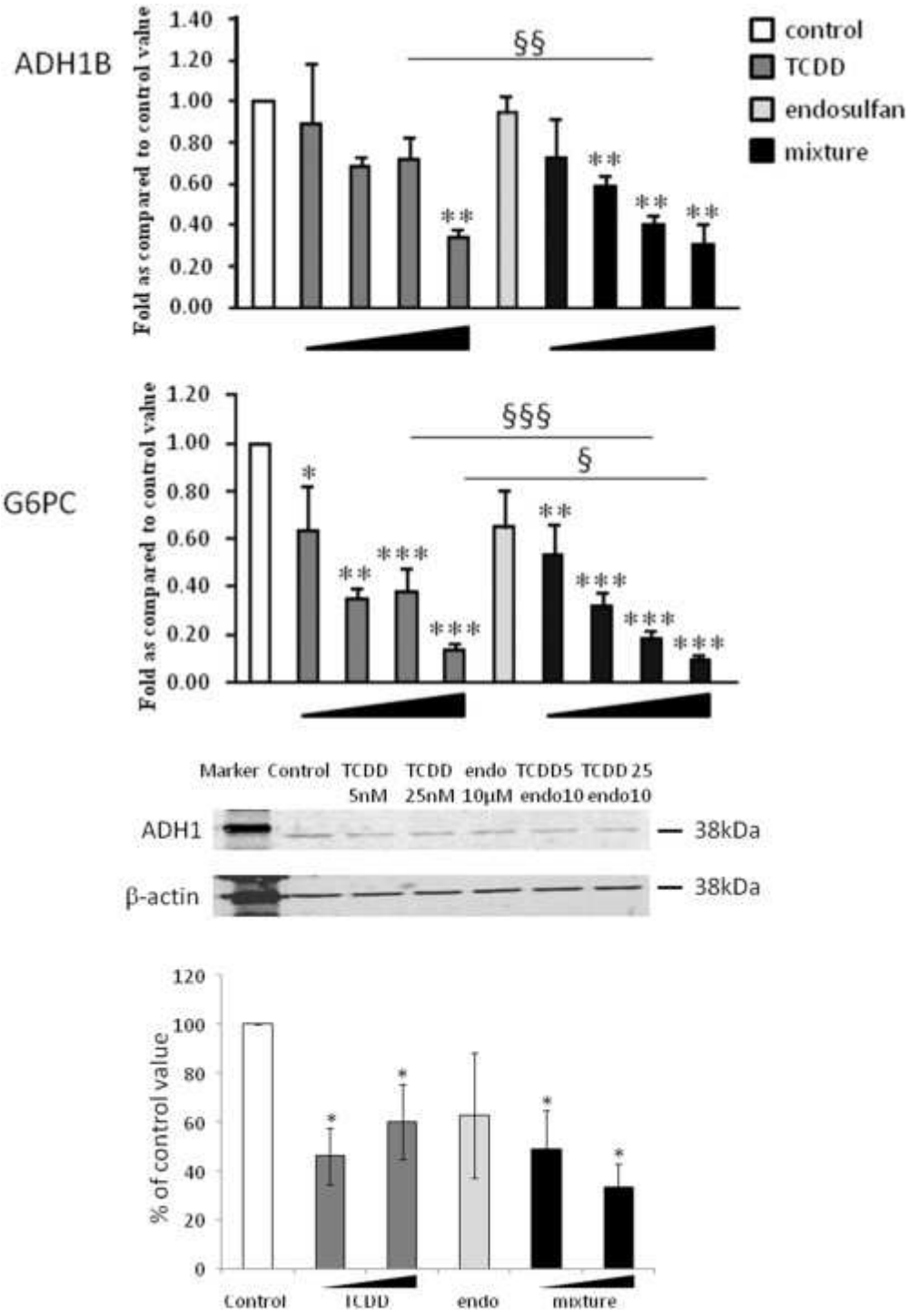


Figure 4. mRNA and protein levels of ADH1B and G6PC after pollutant exposure. A. HepaRG cells were exposed for 8 days to DMSO (0.15%), to a range (0.2, 0.5, 1, and 5 nM) of TCDD concentrations, to 1µM α-endosulfan or to the combination of 1µM α-endosulfan with the various concentrations of TCDD. The levels of mRNA, as measured by RT-qPCR, are expressed as the fold-change as compared to the control value. * represents the level of significance of the fold-change as compared to the control (*, $p<0.05$, **, $p<0.01$, ***, $p< 0.001$) and $ represents the the level of significance of the fold-change of the mixture as

compared to TCDD treatment alone (§, $p<0.05$, §§, $p<0.01$, §§§, $p<0.001$). The values are the

results of 5 independent experiments performed in triplicate. **B.** HepaRG cells were exposed for 72H to DMSO (0.15%), TCDD (5 or 25nM), α-endosulfan (10µM) or the mixtures. A typical Western blot analysis of the ADH1 protein is shown above and, below, the quantification of 3 independent experiments. *, $p<0.05$.

Tables

Table 1
Genes regulated in cells following exposure to either TCDD or α-endosulfan alone and to their combination

Gene symbol	Gene name	mRNA fold-change by	
Genes regulated by either TCDD or the combination TCDD plus α-endosulfan		**TCDD**	**combination**
CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2	51.40	34.27
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	49.38	48.21
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	37.55	38.48
STC2	stanniocalcin 2	29.30	43.20
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	26.07	38.3
TMEM156	transmembrane protein 156	13.37	16.72
SLC37A2	solute carrier family 37 (glycerol-3-phosphate transporter), member 2	8.70	16.36
ALDH3A1	aldehyde dehydrogenase 3 family, memberA1	6.49	5.88
C3orf59	chromosome 3 open reading frame 59	6.42	6.41
MBL2	mannose-binding lectin (protein C) 2, soluble (opsonic defect)	6.07	5.33
IGFBP1	insulin-like growth factor binding protein 1	4.28	3.51
SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	3.98	4.79
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	3.90	4.19
CYBASC3	cytochrome b, ascorbate dependent 3	3.89	4.01
IL17RB	interleukin 17 receptor B	3.70	3.70
IER3 [a]	immediate early response 3	3.25-3.27	4.08-4.12
RAP1GAP	RAP1 GTPase activating protein	2.84	3.26
GDF15	growth differentiation factor 15	2.81	3.55
SYT12	synaptotagmin XII	2.66	3.26
HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2	2.63	2.49
PAPPA	pregnancy-associated plasma protein A, pappalysin 1	2.51	2.64
GRIA3	glutamate receptor, ionotrophic, AMPA 3	2.40	2.56
SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	2.38	3.75
ASAM	adipocyte-specific adhesion molecule	2.36	2.67
VWCE	von Willebrand factor C and EGF domains	2.32	2.39

BMF	Bcl2 modifying factor	2.31	2.38
EREG	epiregulin	2.31	2.40
RUNX2	runt-related transcription factor 2	2.26	2.14
MCCC1	methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)	2.18	2.14
AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)	2.18	2.57
PNMA2	paraneoplastic antigen MA2	2.15	2.45
STON2	stonin 2	2.11	2.23
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1	2.11	2.48
SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	2.07	2.46
TMEM138	transmembrane protein 138	2.07	2.32
PLEKHF1	pleckstrin homology domain containing, family F (with FYVE domain) member 1	2.05	2.48
NPTX2	neuronal pentraxin II	2.04	2.66
UGCG	UDP-glucose ceramide glucosyltransferase	2.04	2.38
GPR175	G protein-coupled receptor 175	2.03	2.21
SLCO2B1	solute carrier organic anion transporter family, member 2B1	0.50	0.44
PLGLA	plasminogen-like A	0.49	0.49
MCTP1	multiple C2 domains, transmembrane 1	0.49	0.50
DLEU2L	deleted in lymphocytic leukemia 2-like	0.49	0.44
SLC22A7	solute carrier family 22 (organic anion transporter), member 7	0.48	0.28
CYP4X1	cytochrome P450, family 4, subfamily X, polypeptide 1	0.48	0.36
ABCG8	ATP-binding cassette, sub-family G (WHITE), member 8	0.47	0.33
SLC38A4	solute carrier family 38, member 4	0.45	0.26
ACOT12	acyl-CoA thioesterase 12	0.45	0.43
ADRA1A	adrenergic, alpha-1A-, receptor	0.44	0.39
KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)	0.44	0.41
ABCG5	ATP-binding cassette, sub-family G (WHITE), member 5	0.44	0.30
FABP1	fatty acid binding protein 1, liver	0.43	0.27
LEAP2	liver expressed antimicrobial peptide 2	0.42	0.30
SERPINA7	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	0.42	0.33
FMO5	flavin containing monooxygenase 5	0.41	0.38
LOC100129488	hypothetical protein LOC100129488	0.41	0.27
GPD1	glycerol-3-phosphate dehydrogenase 1 (soluble)	0.39	0.31
SLC2A2	solute carrier family 2 (facilitated glucose transporter), member 2	0.38	0.21
SLC10A1	solute carrier family 10 (sodium/bile acid cotransporter family), member 1	0.38	0.47
PPP1R3B	protein phosphatase 1, regulatory (inhibitor) subunit 3B	0.36	0.33

THRSP	thyroid hormone responsive (SPOT14 homolog, rat)	0.36	0.39
CXCL13	chemokine (C-X-C motif) ligand 13	0.35	0.41
HAO2	hydroxyacid oxidase 2 (long chain)	0.34	0.17
CTGF	connective tissue growth factor	0.34	0.37
DNAJC15	DnaJ (Hsp40) homolog, subfamily C, member 15	0.32	0.41
RDH16	retinol dehydrogenase 16 (all-trans)	0.31	0.16
MFSD2	major facilitator superfamily domain containing 2	0.30	0.25
CYP4B1	cytochrome P450, family 4, subfamily B, polypeptide 1	0.28	0.36
PFKFB1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	0.26	0.31
DKK1	dickkopf homolog 1 (Xenopus laevis)	0.18	0.20
Genes regulated by either α-endosulfan or the combination TCDD plus α-endosulfan		**mRNA fold-change by α-endosulfan**	**mRNA fold-change by combination**
GPX2	glutathione peroxidase 2 (gastrointestinal)	2.36	2.04
CYP2B6	cytochrome P450, family 2, subfamily B, polypeptide 6	2.14	2.35
LOC221442	hypothetical LOC221442	2.08	2.11
CYP2E1	cytochrome P450, family 2, subfamily E, polypeptide 1	0.49	0.19
LOC441120	hypothetical LOC441120	0.35	0.41
CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1	0.28	0.21

The mRNA fold change for each gene corresponds to the ratio of mRNA expression for cells exposed to each treatment (25nM TCDD or 10µM α-endosulfan or their combination) versus that for untreated cells (0.15% DMSO). The values (fold change >2 for up-regulated genes or < 0.5 for down-regulated genes) are the means of three microarray experiments. All the genes are significantly differentially expressed with $p < 0.05$ by Fisher t-test analysis. [a] Values correspond to several probes in the microarray.

Table 2
Genes differentially down-regulated in HepaRG cells following exposure to either 25nM TCDD or 10µM α-endosulfan or their combination for 30 hours)

Gene symbol	Gene name	mRNA fold change		
		TCDD	α-endosulfan	combination
G6PC	glucose-6-phosphatase, catalytic subunit	0.23	0.38	0.09
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	0.38	0.40	0.13
HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	0.50	0.46	0.19
GNMT	glycine N-methyltransferase	0.33	0.43	0.20
CPS1	carbamoyl-phosphate synthetase 1, mitochondrial	0.35	0.47	0.22
AFM	afamin	0.50	0.48	0.27

All these genes exhibit at least a 2-fold change in expression for cells exposed to all 3 treatments as compared to the control condition. The values are the means of three microarray experiments ($p \leq 0.05$). Down-regulated genes show a fold change <1 as compared to the control condition.

Table 3

Focus analysis top regulated genes differentially expressed following exposure of cells to the combination of TCDD + α-endosulfan as compared to all other treatments

Gene symbol	Gene name / function	mRNA fold change by TCDD + α-endosulfan
Up-regulated genes		
STC2*	stanniocalcin 2 / oxidative stress	43.20
SERPINB2*	serpine peptidase inhibitor, member 2 / cell migration	38.30
SLC37A2*	solute carrier family 37, member 2 / stress response	16.36
SLC7A11*	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11 / oxidative stress	3.75
GDF15*	growth differentiation factor 15 / stress response	3.55
AKR1B10*	aldo-keto reductase family 1, member B10/ fatty acid biosynthesis	2.57
CA12	**carbonic anhydrase XII / hypoxic stress**	2.45
serpine 1	serpine peptidase inhibitor / cell migration	1.89
MT1A	metallothionein 1A / oxidative stress	1.83
ST3GAL5	ST3 beta-galactoside alpha-2,3-sialyltransferase 5 / cell differenciation & proliferation	1.78
Down-regulated genes		
G6PC $	glucose 6 phosphatase, catalytic chain / glucose metabolism	0.09
ADH4 $	alcohol dehydrogenase 4/ retinoid and alcohol metabolism	0.13
HAO2*	hydroxyacid oxidase 2 / fatty acid oxidation	0.17
HMGCS2 $	3-hydroxy-3-methylglutaryl-CoA synthase 2 /ketone bodies synthesis	0.19
CYP2E1	cytochrome P450 2E1/ alcohol metabolism	0.19
SLC2A2*	solute carrier family 7, (facilitated glucose transporter) member 2	0.21
ADH1A	alcohol dehydrogenase 1A/ retinoid and alcohol metabolism	0.28
ADH1B	alcohol dehydrogenase 1B / retinoid and alcohol metabolism	0.33
ADH1C	alcohol dehydrogenase 1C/ retinoid and alcohol metabolism	0.38
HSD17B6	hydroxysteroid (17-beta) dehydrogenase 6 homolog / retinoid metabolism	0.41

Genes in bold, *, and $, were found to be differentially expressed in cells exposed to the mixture, to TCDD alone or the mixture or to all 3 treatments, respectively by t-test analysis. CYP2E1 was found to be altered following treatment with α-endosulfan alone and the mixture by t-test analysis. The values are the fold change by the combination compared to the control condition. Up-regulated and down-regulated genes show fold changes >1 and <1, respectively, as compared to the control condition.

Table 4

Top canonical pathways regulated in cells exposed to the combination of TCDD and α-endosulfan

Name of pathway	p-value	ratio[a]
by Up-regulated genes		
Pyrimidine metabolism	2.05 E-07	18/231
Role of CHK proteins in cell cycle checkpoint control	2.05 E-06	8/35
RAN signaling	4.82 E-05	5/23
AhR signaling	6.96 E-05	13/154
Molecular mechanisms of cancer	1.10 E-04	22/372
by Down-regulated genes		
FXR/RXR activation	3.31 E-14	12/103
Bile acid biosynthesis	4.99 E-09	7/100
Metabolism of xenobiotics by CYPs	2.52 E-08	9/209
Glycolysis/Gluconeogenesis	4.90 E-08	8/142
Glycerolipid metabolism	8.42 E-08	8/156

[a] The ratio corresponds to the number of genes regulated by the combination of TCDD plus α-endosulfan as compared to the total number of genes in the specific pathway, found by the Ingenuity Pathway Analysis.

Supplementary Tables

Table S1
Primer sequences for RT-qPCR assays

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
ADH1A	GGCATCAGCACCTTCTCAC	GACCTTGGCAACATTGACTG
ADH1B	GGCAGAGAAGACAGAAACGAC	CAACCTCCACATCCTCAATG
ADH1C	CTGCTTCGCTCTGGAAAG	GAGGAGGCTGAAAACTGC
ADH4	GCATTGAAGAGGTTGAAGTAGC	GATAACAGTGGCATCAGTATGG
ADH6	GGAAGTTCGCATAAAGGTTG	CAAGATGGTGGGATACAAGAG
CYP2E1	ACTATGGGATGGGGAAACAG	GAGGATGTCGGCTATGACG
SERPINB2	GCTGTTTGGTGAGAAGTCTG	GCCTTTGGTTTGAGTCTTG
STC2	GGAAGAGGGGAGCACAAAG	CAGCGTTGACCAAACAGTG
SLC7A11	TGGCAGTGACCTTTTCTGAG	CCTGGAGACAGCAAACACAC
DTL	GTCCCAGTTCCTCCTTTTG	CCAGTGAGCCATCCATTC
SLC9A9	GCTGCTCCTCGTGTTCTTC	TGCTTCCTGGTGTTGTGAG
ABCG8	GCCTCCTTCTTCAGCAATG	TCAGCCCTTCAAAACACC
ALDH3A1	CAGAAGGTGGCTTATGGG	ACGCTGGTTGATGAACTG
AFM	ATGAAGTTGCCAGAAGGAAC	ATTGTGTGACAGGTATTGCC
AQP9	GCAGCTTAGCGAAAGAAACC	TGCAACTGCCATTGAAAATC
GPX2	TTTGGACATCAGGAGAACTG	TTCAGGTAGGCGAAGACAG
MBL2	ATGGTGGCAGCGTCTTAC	CCTGGTTCCCCCTTTTC
SLC22A7	CGTTGGGGGAAGAAAGG	CGGCGAAGAAGAAAGTGG
SLC38A4	GCAGTCCTTGTGGCAGTAAC	CCCCTATGAATCCGAAGATG
G6PC	TTGTGGTTGGGATTCTGG	CTGTGGATGTGGCTGAAAG
SDS	ATGAAGGTGCCACAGTCAAG	TCAAAGGGGGGAATGTAGAC
HMGCS2	CCCGTCTAAAGGTGTTCTG	AGCCCAGGACAGTGATTG
CYP1A1	GGTCAAGGAGCACTACAAAACC	TGGACATTGGCGTTCTCAT
CYP1A2	ACAGCACAACAAGGGACACA	TGCCAAACAGCATCATCTTC
CYP3A4	GATGGCTCTCATCCCAGACTT	AGTCCATGTGAATGGGTTCC
CYP2B6	TTCAGGAGGAGGCTCAGTGT	GGCCGAATACAGAGCTGATG
RPL13A	AAGGTCGTGCGTCTGAAG	GAGTCCGTGGGTCTTGAG
GDF15	GCTACGAGGACCTGCTAAC	ACTTCTGGCGTGAGTATCC
NEIL3	TTCCAGCCAGAATGTCTTGAG	CCGAAATGAATCCGTAAAGC
HAO2	CCTGAACTGTGGGTAGTGATG	GCCTGAAAGTCTGTCAAACAC
SLC2A2	CACTTGGCACTTTTCATCAG	AGGTATCTGGGGCTTTCTG
CA12	TCTTGGCATCTGTATTGTGG	GGCTGGCTTGTAAATGACTC
CYP7A1	CCATTCCAGCGACTTTCTG	AGCCTCAGCGATTCCTTG
PON1	CATAAAAGTGCTCAGGTCCCACAG	TGGAATTGGGGATCACTGGAAG
SULT2A1	CCTGAACTGTGGGTAGTGATG	GCCTGAAAGTCTGTCAAACAC

Table S2

Genes exhibiting at least a 2-fold change in expression in HepaRG cells exposed for 30 hours to either 25nM TCDD only or 10µM α-endosulfan only or the combination of TCDD plus α-endosulfan only

Gene symbol	Gene name	mRNA fold change by		
		TCDD	α-endosulfan	combination
Genes regulated by TCDD only				
KRTAP21-1	keratin associated protein 21-1	3.65	3.97*	3.51*
TRPV6	transient receptor potential cation channel, subfamily V, member 6	2.40	0.92	1.15
S100A8	S100 calcium binding protein A8	2.26	0.83	1.84
HK2	hexokinase 2	2.24	0.92	2.23*
UBE2U	ubiquitin-conjugating enzyme E2U (putative)	2.23	1.16	1.43
SCN1A	sodium channel, voltage-gated, type I, alpha subunit	2.11	1.17	1.62
SLC22A3	solute carrier family 22 (extraneuronal monoamine transporter), member 3	0.50	0.99	0.55
RASGEF1B	RasGEF domain family, member 1B	0.50	1.00	0.59
CLEC4E	C-type lectin domain family 4, member E	0.50	0.50*	0.37*
ABI3BP	ABI family, member 3 (NESH) binding protein	0.50	1.00	0.59
SLC16A12	solute carrier family 16, member 12 (monocarboxylic acid transporter 12)	0.49	1.44	0.65
MRC1	mannose receptor, C type 1	0.49	0.73	0.42*
ABCD2	ATP-binding cassette, sub-family D (ALD), member 2	0.49	0.98	0.54
MCF2	MCF.2 cell line derived transforming sequence	0.49	0.94	0.54
CIDEC	cell death-inducing DFFA-like effector c	0.48	1.10	0.63
OR10R2	olfactory receptor, family 10, subfamily R, member 2	0.48	0.83	0.39*
KCNB1	potassium voltage-gated channel, Shab-related subfamily, member 1	0.48	1.41	0.82
GHR	growth hormone receptor	0.48	1.03	0.56
OR7E18P	olfactory receptor, family 7, subfamily E, member 18 pseudogene	0.48	1.00	0.90
GYS2	glycogen synthase 2 (liver)	0.46	1.34	0.56

GZMK	granzyme K (granzyme 3; tryptase II)	0.43	0.63	0.81
Genes regulated by α-endosulfan only				
OR2T4	olfactory receptor, family 2, subfamily T, member 4	1.59	2.44	2.03*
SFRP4	secreted frizzled-related protein 4	0.79	2.19	1.65
NCRNA00052	non-protein coding RNA 52	0.63	0.50	0.68
CPA3	carboxypeptidase A3 (mast cell)	1.04	0.50	0.90
LIPI	lipase, member I	1.22	0.49	0.85
FAM99A	family with sequence similarity 99, member A	3.01*	0.27	0.62
CNGA1	cyclic nucleotide gated channel alpha 1	0.57	0.47	0.50*
FAM71D	family with sequence similarity 71, member D	1.08	0.44	0.69
LOC651503	seven transmembrane helix receptor	1.42	0.34	0.94
LOC441233	hypothetical gene supported by AK128010	0.76	0.25	0.43*
Genes regulated by the combination TCDD plus α-endosulfan only				
LOC100130904	similar to CD177 molecule	2.64*	2.39*	3.64
VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor	2.58*	1.26	3.38
LOC732275	similar to hCG1645603	0.98	1.31	3.04
DTL	denticleless homolog (Drosophila)	1.42	1.76	2.99
FAM111B	family with sequence similarity 111, member B	1.58	1.87	2.83
SLC9A9	solute carrier family 9 (sodium/hydrogen exchanger), member 9	2.11*	1.29	2.75
GPRC5B	G protein-coupled receptor, family C, group 5, member B	1.42	2.18*	2.72
E2F7	E2F transcription factor 7	1.65	1.61	2.71
EXO1	exonuclease 1	1.56	2.06*	2.68
DCLK1	doublecortin-like kinase 1	1.61	1.87	2.62
NEIL3	nei endonuclease VIII-like 3 (E. coli)	1.69	1.92	2.62
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2	1.51	1.66	2.57
RIBC2	RIB43A domain with coiled-coils 2	1.62	1.84	2.55

PTPRE	protein tyrosine phosphatase, receptor type, E	2.02*	1.35	2.47
SEC14L4	SEC14-like 4 (S. cerevisiae)	2.18*	1.41	2.46
RAD51	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)	1.41	1.86	2.45
CA12	carbonic anhydrase XII	1.73	1.61	2.45
PFKP	phosphofructokinase, platelet	1.87	1.30	2.35
FBXW10	F-box and WD repeat domain containing 10	1.51	1.59	2.31
C22orf9	chromosome 22 open reading frame 9	1.93	1.18	2.29
ORC1L	origin recognition complex, subunit 1-like (yeast)	1.42	1.49	2.27
HIST1H3A	histone cluster 1, H3a	1.28	1.64	2.26
G6PC2	glucose-6-phosphatase, catalytic, 2	2.13*	2.06*	2.22
HIST1H3B	histone cluster 1, H3b	1.31	1.64	2.21
CDRT1	CMT1A duplicated region transcript 1	1.46	1.68	2.19
CCL20	chemokine (C-C motif) ligand 20	1.57	1.35	2.14
SSH1	slingshot homolog 1 (Drosophila)	1.55	1.22	2.13
ARMC9	armadillo repeat containing 9	1.81	1.05	2.12
ALPP	alkaline phosphatase, placental (Regan isozyme)	1.44	1.40	2.12
ARSI	arylsulfatase family, member I	2.42*	0.92	2.12
MCM5	minichromosome maintenance complex component 5	1.39	1.56	2.11
FAM46C	family with sequence similarity 46, member C	1.86	1.21	2.11
ADAM12	ADAM metallopeptidase domain 12	1.72	1.54	2.08
CDC6	cell division cycle 6 homolog (S. cerevisiae)	1.27	1.50	2.07
MLLT1	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 1	1.87	1.20	2.06
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	1.74	1.40	2.06
NCF2	neutrophil cytosolic factor 2	1.32	1.41	2.06
CDCA7	cell division cycle associated 7	1.55	1.57	2.05
WDR76	WD repeat domain 76	1.22	1.54	2.05
MCM2	minichromosome maintenance complex component 2	1.45	1.35	2.05

SRXN1	sulfiredoxin 1 homolog (S. cerevisiae)	1.55	1.48	2.03
CCNE2	cyclin E2	1.20	1.36	2.03
CFHR3	complement factor H-related 3	0.64	0.74	0.50
CYP2A6	cytochrome P450, family 2, subfamily A, polypeptide 6	0.54	0.92	0.50
AHSG	alpha-2-HS-glycoprotein	0.62	0.66	0.50
CYP2A13	cytochrome P450, family 2, subfamily A, polypeptide 13	0.55	0.91	0.50
DGAT2	diacylglycerol O-acyltransferase homolog 2 (mouse)	0.57	0.80	0.50
PDE8B	phosphodiesterase 8B	0.51	1.00	0.50
ABCC9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	0.55	0.92	0.49
C12orf27	chromosome 12 open reading frame 27	0.61	0.88	0.49
HFE2	hemochromatosis type 2 (juvenile)	0.56	0.65	0.49
CIDEB	cell death-inducing DFFA-like effector b	0.61	0.82	0.49
SLCO1B1	solute carrier organic anion transporter family, member 1B1	0.62	0.81	0.49
AQP9	aquaporin 9	0.53	0.97	0.49
C7orf45	chromosome 7 open reading frame 45	0.78	0.74	0.49
RORC	RAR-related orphan receptor C	0.61	0.72	0.48
SLC38A3	solute carrier family 38, member 3	0.64	0.77	0.48
ALDOB	aldolase B, fructose-bisphosphate	0.59	0.80	0.48
CCIN	calicin	0.87	0.68	0.48
LST-3TM12	organic anion transporter LST-3b	0.64	0.80	0.48
CYP2A7	cytochrome P450, family 2, subfamily A, polypeptide 7	0.51	0.91	0.48
SELENBP1	selenium binding protein 1	0.53	0.91	0.48
CALML4	calmodulin-like 4	0.56	0.82	0.47
ANG	angiogenin, ribonuclease, RNase A family, 5	0.60	0.74	0.47
CDC20B	cell division cycle 20 homolog B (S. cerevisiae)	0.67	0.64	0.47
PLA2G12B	phospholipase A2, group XIIB	0.60	0.75	0.46
DAB1	disabled homolog 1 (Drosophila)	0.52	090	0.46
CFHR2	complement factor H-related 2	0.57	0.90	0.46
SCGN	secretagogin, EF-hand calcium binding protein	0.51	0.92	0.46

GLYAT	glycine-N-acyltransferase	0.52	0.94	0.46
LOC441178	hypothetical LOC441178	0.67	0.52	0.46
OTC	ornithine carbamoyltransferase	0.57	0.70	0.45
PKLR	pyruvate kinase, liver and RBC	0.74	0.71	0.45
PON1	paraoxonase 1	0.6	0.75	0.45
GLYATL1	glycine-N-acyltransferase-like 1	0.51	0.75	0.45
SLC10A5	solute carrier family 10 (sodium/bile acid cotransporter family), member 5	0.59	0.75	0.44
SLC22A10	solute carrier family 22, member 10	0.66	0.53	0.44
AGXT2	alanine-glyoxylate aminotransferase 2	0.55	0.84	0.43
HRG	histidine-rich glycoprotein	0.64	0.61	0.43
ANGPTL5	angiopoietin-like 5	0.96	1.11	0.41
ARG1	arginase, liver	0.56	0.72	0.41
HSD17B6	hydroxysteroid (17-beta) dehydrogenase 6 homolog (mouse)	0.57	0.74	0.41
PCK2	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	0.51	0.73	0.41
SULT1E1	sulfotransferase family 1E, estrogen-preferring, member 1	0.48*	0.72	0.41
SLC17A4	solute carrier family 17 (sodium phosphate), member 4	0.53	0.83	0.40
SLC5A9	solute carrier family 5 (sodium/glucose cotransporter), member 9	0.53	0.83	0.40
LRRC31	leucine rich repeat containing 31	0.54	0.68	0.40
BDH1	3-hydroxybutyrate dehydrogenase, type 1	0.59	0.69	0.39
HSD3B1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	0.62	0.71	0.39
ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide	0.57	0.71	0.38
RANBP3L	RAN binding protein 3-like	0.51	0.79	0.38
PGLYRP2	peptidoglycan recognition protein 2	0.62	0.60	0.37
BBOX1	butyrobetaine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1	0.40*	0.81	0.36
ANXA13	annexin A13	0.50*	0.72	0.35
ADH6	alcohol dehydrogenase 6 (class V)	0.56	0.65	0.35
ADH1B	alcohol dehydrogenase 1B (class I), beta polypeptide	0.65	0.67	0.33
PSMAL	growth-inhibiting protein 26	0.54	0.58	0.33

FOLH1	folate hydrolase (prostate-specific membrane antigen) 1	0.55	0.58	0.32
UGT2B17	UDP glucuronosyltransferase 2 family, polypeptide B17	0.69	0.57	0.31
ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide	0.61	0.56	0.28

The mRNA fold change for each gene corresponds to the ratio of mRNA expression for cells exposed to each treatment (25nM TCDD or 10μM α-endosulfan or the combination) versus that for untreated cells (0.15% DMSO). The values (fold change >2 for up-regulated genes or < 0.5 for down-regulated genes) are the means of three microarray experiments. All the genes are significantly differentially expressed with $p < 0.05$ by Fisher t-test analysis for the treatment designated for each sub-group. For the other treatments, the fold change was either below two-fold change or not statistically significative (labeleld with an asterisk *****, if p-value >0.05). The genes underlined in the first two sections of the table are those retained after filtering with increased stringency (fold change>2.2 or <0.46, p-value<0.05), see text for discussion.

Table S3
Comparison of the changes in the expression of selected target genes by microarray and RT-qPCR analysis

		mRNA fold change					
		TCDD		α-endosulfan		combination	
Gene symbol	Gene name	µarray	RT-qPCR	µarray	RT-qPCR	µarray	RT-qPCR
ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide	0.6	0.40	0.55	0.18	0.28	0.09
ADH1B	alcohol dehydrogenase 1B (class I), beta polypeptide	0.65	0.32	0.67	0.44	0.33	0.13
ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide	0.55	0.41	0.68	1.48	0.37	0.57
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	0.38	0.29	0.40	0.28	0.13	0.08
ADH6	alcohol dehydrogenase 6 (class V)	0.56	0.49	0.65	0.62	0.35	0.29
CYP2E1	cytochrome P450, family 2, subfamily E, polypeptide 1	0.51	0.41	0.49	0.34	0.20	0.10
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	37.55	69.13	1.30	0.86	38.48	54.10
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	26.31	55.03	1.48	1.26	39.25	56.18
STC2	stanniocalcin 2	29.56	47.08	0.63	1.09	43.20	80.76
SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	2.36	2.54	1.91	1.28	3.73	3.25
DTL	denticleless homolog (Drosophila)	1.39	1.82	1.73	1.69	2.97	2.87
SLC9A9	solute carrier family 9 (sodium/hydrogen exchanger), member 9	2.1	2.24	1.28	0.96	2.73	2.52
ABCG8	ATP-binding cassette, sub-family G (WHITE), member 8	0.47	0.53	0.78	0.88	0.33	0.36
ALDH3A1	aldehyde dehydrogenase 3 family, member A1	6.47	22.58	0.92	1.12	5.88	17.9
AFM	afamin	0.5	0.45	0.48	0.35	0.27	0.21
AQP9	aquaporin 9	0.53	0.44	0.97	0.87	0.48	0.4
GPX2	glutathione peroxidase 2 (gastrointestinal)	1.34	1.55	2.35	3.16	2.05	2.76
MBL2	mannose-binding lectin (protein C) 2, soluble (opsonic defect)	5.89	11.45	0.67	0.93	5.14	9.27
SLC22A7	solute carrier family 22 (organic anion transporter), member 7	0.48	0.41	0.61	0.49	0.28	0.20
SLC38A4	solute carrier family 38, member 4	0.44	0.44	0.59	0.46	0.26	0.20
G6PC	glucose-6-phosphatase, catalytic subunit	0.23	0.18	0.38	0.23	0.09	0.04
SDS	serine dehydratase	0.39	0.23	6.37	5.76	1.66	1.37
HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	0.50	0.40	0.46	0.28	0.19	0.08

GDF15	growth differentiation factor 15	2.81	3.43	1.38	0.94	3.55	5.01
NEIL3	nei endonuclease VIII-like 3 (E. coli)	1.69	1.56	1.92	1.34	2.62	2.25
HAO2	hydroxyacid oxidase 2 (long chain)	0.34	0.12	0.64	0.28	0.17	0.04
SLC2A2	solute carrier family 2 (facilitated glucose transporter), member 2	0.38	0.26	0.69	0.45	0.21	0.12
CA12	carbonic anhydrase XII	1.73	1.57	1.61	1.01	2.45	2.09
CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1	0.66	0.56	0.28	0.27	0.21	0.27
PON1	paraoxonase 1	0.64	0.53	0.75	0.54	0.45	0.33
SULT2A1	sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)- preferring, member 1	0.63	0.49	0.93	0.7	0.52	0.34

The mRNA fold change for each gene corresponds to the ratio of mRNA expression for cells exposed to each treatment (25nM TCDD or 10µM α-endosulfan or their combination) versus that for untreated cells (0.15% DMSO). The values (fold change >1 for up-regulated genes or < 1 for down-regulated genes) are the means of three microarray or three or more independent RT-qPCR experiments.

Table S4

Genes belonging to the top down-regulated network in cells exposed to the combination of TCDD and α-endosulfan treatment

Gene symbol	Gene name	mRNA fold-change		
		TCDD	α-endosulfan	combination
G6PC	glucose-6-phosphatase, catalytic subunit	0.23	0.38	0.009
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	0.38	0.40	0.13
RDH16	retinol dehydrogenase 16 (all-trans)	0.31	0.55	0.16
HAO2	hydroxyacid oxidase 2 (long chain)	0.34	0.64	0.17
CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1	0.65	0.28	0.21
CPS1	carbamoyl-phosphate synthetase 1, mitochondrial	0.35	0.47	0.22
ABCG5	ATP-binding cassette, subfamily G (WHITE), member 5	0.43	0.62	0.30
UGT2B17	UDP glucuronosyltransferase 2 family, polypeptide B17	0.69	0.57	0.31
ABCG8	ATP-binding cassette, subfamily G (WHITE), member 8	0.47	0.78	0.33
ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide	0.55	0.68	0.37
PON1	paraoxonase 1	0.63	0.75	0.48

All the genes listed belong to the top down-regulated network, lipid metabolism. The mRNA fold change for each gene (fold change >1 for up-regulated genes or < 1 for down-regulated genes) corresponds to the ratio of mRNA expression for cells exposed to each treatment (25nM TCDD or 10μM α-endosulfan or their combination) versus untreated cells (0.15% DMSO). The values are the means of three microarray experiments. All the genes are significantly differentially expressed with $p < 0.05$ by Fisher t-test analysis. The values for gene expression following treatment with TCDD or α-endosulfan alone are shown for comparison.

Supporting Information Legends

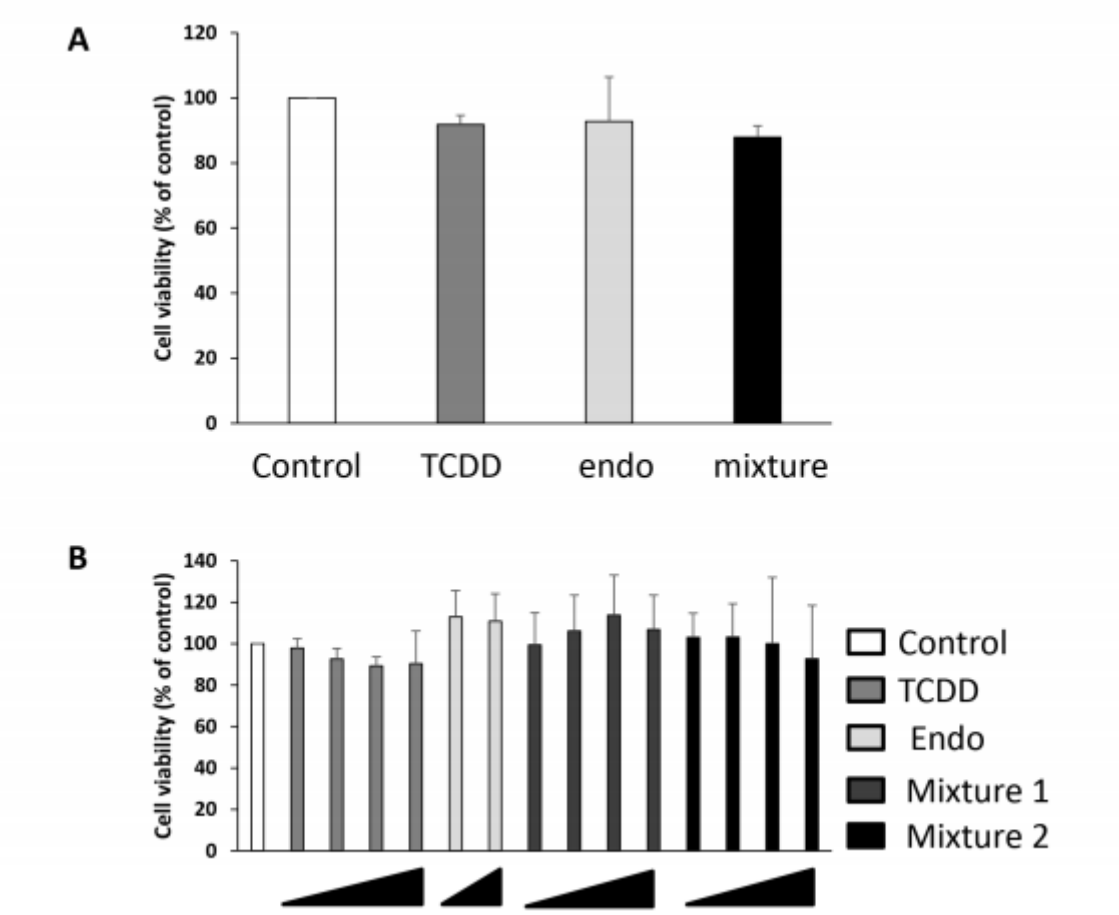
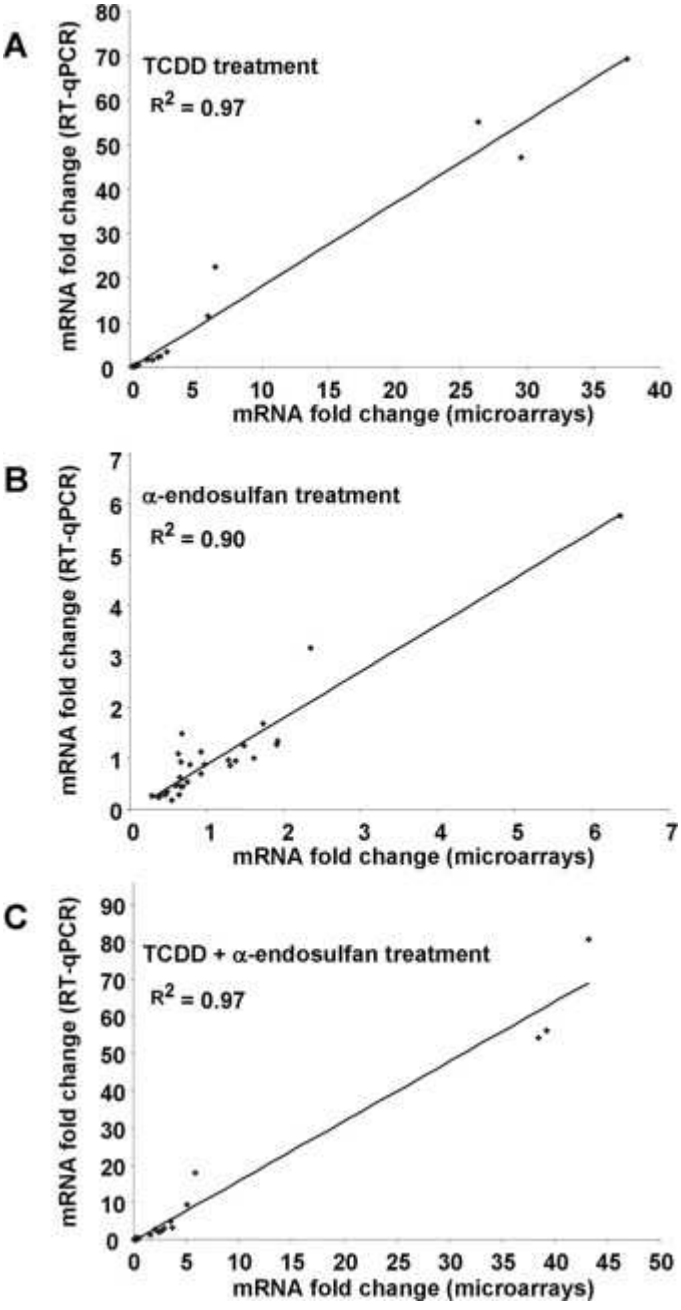


Figure S1. Cell viability after 3 or 8 days of exposure to pollutants. A. HepaRG cells (6 X 10^4 cells/well in a 96 well plate) were exposed for 72H to the control medium or TCDD (25nM), or α-endosulfan (10µM) or the mixture. **B.** HepaRG cells were exposed for 8 days to a range (0.2, 0.5, 1, and 5 nM) of concentrations of TCDD, to 3 or 10µM α-endosulfan and to the combinations of 3µM (mixture 1) or 10µM (mixture 2) α-endosulfan with the various concentrations of TCDD (lowest to highest from left to right). Cell viability was measured in 3 (A) or 4 (B) independent experiments in triplicate using the WST-1 kit (Roche Applied Science) according to the manufacturer's instructions. The viability is expressed as the % of the control condition (100%). No statistical difference in the viability of the cells after the various treatments was found.


A
TCDD treatment
R^2 = 0.97
mRNA fold change (RT-qPCR)
mRNA fold change (microarrays)
B
α-endosulfan treatment
R^2 = 0.90
mRNA fold change (RT-qPCR)
mRNA fold change (microarrays)
C
TCDD + α-endosulfan treatment
R^2 = 0.97
mRNA fold change (RT-qPCR)
mRNA fold change (microarrays)

F

Figure S2. Comparison between the levels of expression of genes as measured by microarrays and RT-qPCR. A. Comparison for the treatment with TCDD (25nM, 30H). **B**. Comparison for the treatment with α-endosulfan (10μM, 30H). **C**. Comparison for the combined treatment with TCDD (25nM, 30H) and α-endosulfan (10μM, 30H). The correlation coefficients R^2 are 0.97, 0.90, and 0.97 for the treatments with TCDD, α-endosulfan and the mixture, respectively.

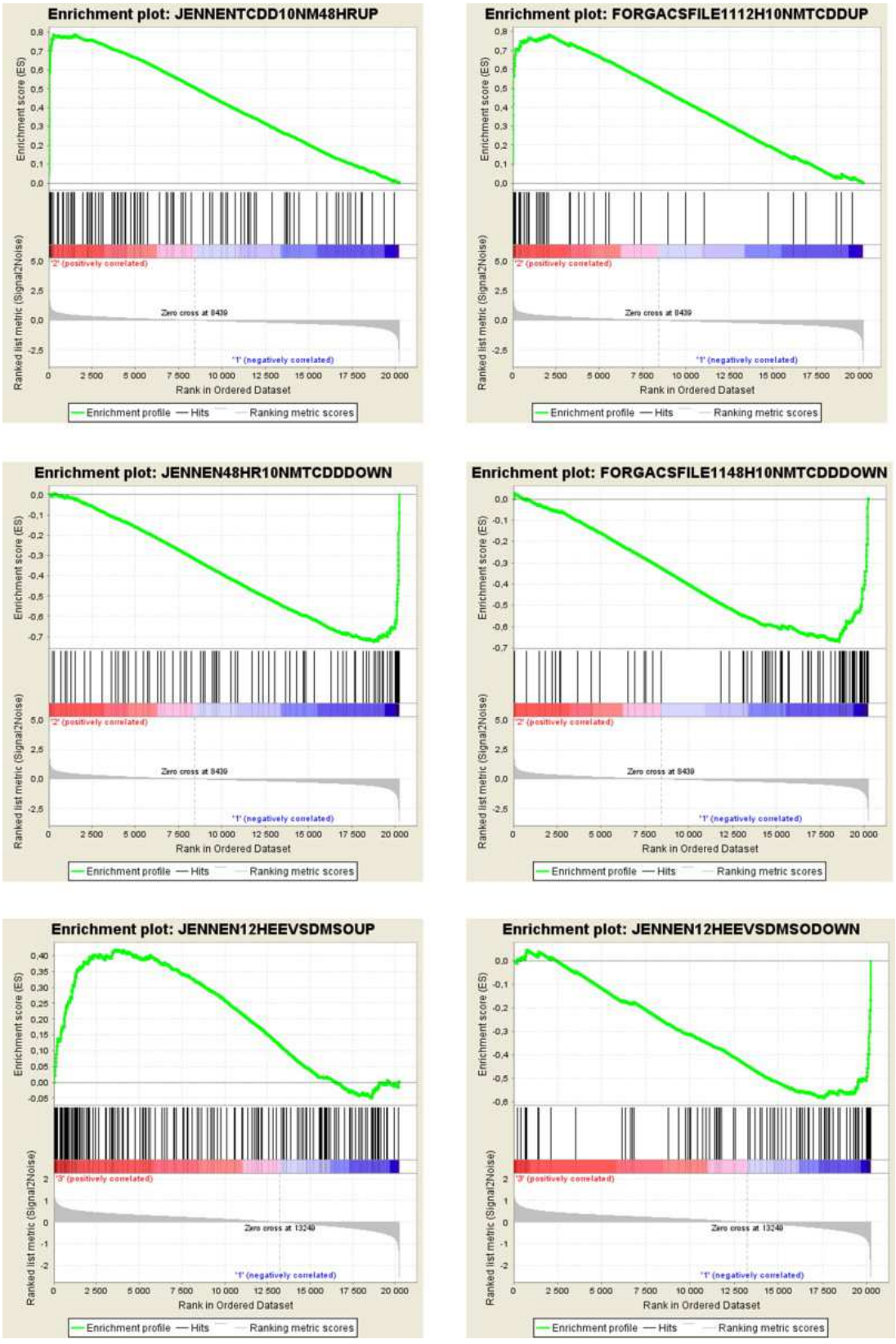


Figure S3. Gene Set Enrichment Analysis (GSEA) and Rank Rank Hypergeometric Overlap Analysis. **A.** GSEA plots for the gene sets up-regulated by TCDD (first row) derived

from the data of Jennen et al. [42] (left, 10nM TCDD, 48H treatment) and Forgacs et al. [58] (right, 10 nM TCDD, 12H treatment), for gene sets down-regulated by TCDD (second row) from the data of Jennen et al. [42] (left, 10 nM TCDD, 48H treatment) and Forgacs et al. [58] (right, 10 nM TCDD, 48H treatment), for gene sets up- and down-regulated by 17β-estradiol (third row) from the data of Jennen et al. [42] (left, up-regulated, right, down-regulated, 30 mM 17β-estradiol, 12H treatment). The abscissas correspond to the ordered list of genes that are differentially expressed for HepaRG cells treated or not by TCDD in this study. The ordinates represent the cumulative distribution (enrichment score, green line) for the cells (HepaRG or primary human hepatocytes, published data) treated or not with TCDD. The red and blue colors indicate enrichment and depletion, respectively, of genes. **B.** Rank rank hypergeometric overlap heat plots of expression data from this paper (abscissas) and that from Jennen et al. [42] (ordinates) for treatment with 10 μM α-endosulfan, 30H and 30 μM 17β-estradiol, 12H, respectively (top) and for treatment with 25 nM TCDD, 30H and 10 nM TCDD, 12H, respectively (bottom). The metric on the right represents the $\log_{10}$-transformed t-test P-value. Pixels with positive values (red) indicate a higher than expected number of overlapping genes and pixels with negative values (blue) indicate a lower than expected number of overlapping genes. Overlap is seen here by the orange areas.