Preplanned Studies

Potential Adverse Outcome Pathways of Chlorinated Organophosphate Flame Retardants

Meiyu Zhou[1,&]; Huilin Zhang[2,&]; Qi Xiao[1]; Kexin Li[2]; Xiaoting Li[3,#]; Haiyan Chu[1,2,#]

Summary

What is already known about this topic?

Chlorinated organophosphate flame retardants (Cl-OPFRs) are frequently detected chemicals in the environment and biological samples, yet there is a lack of systematic evaluation regarding the adverse effects and toxicological mechanisms of Cl-OPFRs.

What is added by this report?

This study utilizes the adverse outcome pathway (AOP) framework to assess the health implications and mechanisms of Cl-OPFRs, identifying multi-system toxicity, with a particular emphasis on reproductive issues and the possible toxic mechanisms.

What are the implications for public health practice?

These results enhance knowledge of the health hazards linked to Cl-OPFRs, supporting the creation of focused risk evaluations and suitable regulatory actions.

Chlorinated organophosphate flame retardants (Cl-OPFRs) have frequently been detected at high levels in environment and in human biological specimens. As a result, the toxic effects associated with Cl-OPFR exposure warrant increased attention. It is crucial to move beyond merely evaluating carcinogenic or non-carcinogenic risks (*1*) and engage in a comprehensive discussion on potential adverse health effects and toxic mechanisms of Cl-OPFRs. The adverse outcome pathway (AOP) concept comprises molecular initiating events (MIEs), key events (KEs), and adverse outcomes (AOs), offering a mechanistic understanding of crucial events and biological pathways leading to AOs, thereby enhancing the efficacy of toxicity risk evaluations. Recent studies have confirmed the practicality of this framework (*2*). This study utilizes the AOP framework to assess the health implications and mechanisms of Cl-OPFRs by integrating existing toxicity data. The findings suggest that Cl-OPFR exposure can result in multi-system toxicity, with a particular emphasis on reproductive issues. Through molecular investigations using tools such as Cl-OPFRs-gene-phenotype-AO framework and AOP-helpFinder, key molecular events (*IGF1, BAX, AR, MTOR*, and *PPARG*) linked to hormonal processes and reproductive system development were identified, indicating potential reproductive toxicity induction. This research enhances the understanding of the toxic effects, reproductive toxicity, and mechanisms associated with Cl-OPFRs.

Candidate genes, Gene Ontology (GO) terms, and pathways associated with Cl-OPFRs such as tris-(2-chloroethyl)-phosphate (TCEP), tris-(1-chloro-2-propyl)-phosphate (TCIPP), and tris-(1,3-dichloropropyl)-phosphate (TDCIPP), were identified from the Comparative Toxicogenomics Database (CTD, http://ctdbase.org) in February 2023 using the keywords "TCEP", "TCIPP", and "TDCIPP". Target genes were those with more than three interactions. Target phenotypes were determined by overlapping phenotypes obtained from GO enrichment analysis with a significance threshold of $P<1\times10^{-3}$ and relevant phenotypes in the CTD database. The target phenotypes were categorized into three levels — subcellular, cellular, and systemic — based on the hierarchical structure of GO terms (*2*). The study first identified AOs affecting individuals or populations as a result of exposure to Cl-OPFRs, prioritizing both biological importance and phenotype classification. Phenotypes that showed strong correlations with AOs were then selected as potential intermediate KEs. Additionally, genes associated with these KEs were identified as MIEs to construct an AOP framework. To prioritize MIEs and KEs, we developed chemical-gene-phenotype-disease frameworks utilizing Cytoscape software (version 3.9.1, Boston, MA, USA). We utilized AOP-helpFinder (http://aop-helpfinder-v2.u-paris-sciences.fr/) to identify relevant knowledge linking stressors automatically and events within an AOP and to assess potential candidate genes simultaneously. The assessment was conducted according to Organization for Economic Co-operation and Development (OECD) guidelines utilizing Weight of Evidence (WoE) methodology based on Bradford Hill's causal considerations, composite score, and

confidence score. The aim was to bolster credibility by consolidating evidence from PubMed, Web of Science, and the AOP Wiki (https://aopwiki.org/). WoE criteria primarily focus on biological plausibility and empirical support, incorporating mode of action analysis in chemical regulatory practices. Biological plausibility underscores mechanistic relationships, while empirical support leans on experimental data, particularly dose-response concordance.

Given the widespread presence and long-lasting nature of Cl-OPFRs in the environment, the AOP framework extensively outlined the harmful effects of Cl-OPFRs. A flow diagram depicting this is presented in Figure 1A. Seventy-four interactive genes with Cl-OPFRs effects and 531 shared phenotypes (comprising 483 GO terms and 48 pathways) were identified. These phenotypes were categorized into three levels based on the GO ancestor chart. Notably, at the system level, reproductive toxicity-related phenotypes constituted 43.59% of the total GO terms, followed by organ growth and development at 28.21%, Motor system at 7.69%, and others, indicating potential multi-system toxicity from Cl-OPFRs exposure (Figure 1B). Moreover, an in-depth analysis highlighted that genes such as *IL1B*, *BAX*, and *BCL2* showed higher frequencies within toxic pathways related to reproduction, while the *IGF1* gene emerged as a crucial factor across all levels (Supplementary Table S1, available at https://weekly.chinacdc.cn/).

Reproductive toxicity phenotypes were notably prevalent and thus selected as the AO for the establishment of an AOP framework. Reproductive toxicology terms were classified into 14 categories across three levels of biological organization based on thematic similarities (Supplementary Table S2, available at https://weekly.chinacdc.cn/). At the cellular and subcellular levels, the principal categories included hormone-related phenotypes encompassing biological processes and hormonal stimulation, phenotypes associated with cell damage pertaining to the regulation of cell proliferation and cell cycle, and oxidative stress. At the systemic level, out of 17 reproductive system-related phenotypes, 6 were specifically linked to female reproductive health, whereas only 2 pertained to male reproduction. This discrepancy indicates a potentially higher reproductive toxicity risk from Cl-OPFRs for females. The interconnected phenotypes across the three organizational levels constituted the KEs, and 74 genes identified as interacting with these phenotypes were designated as MIEs, thus forming the foundational structure of the AOP (Figure 2).

To assign priority to MIEs and KEs, we utilized a

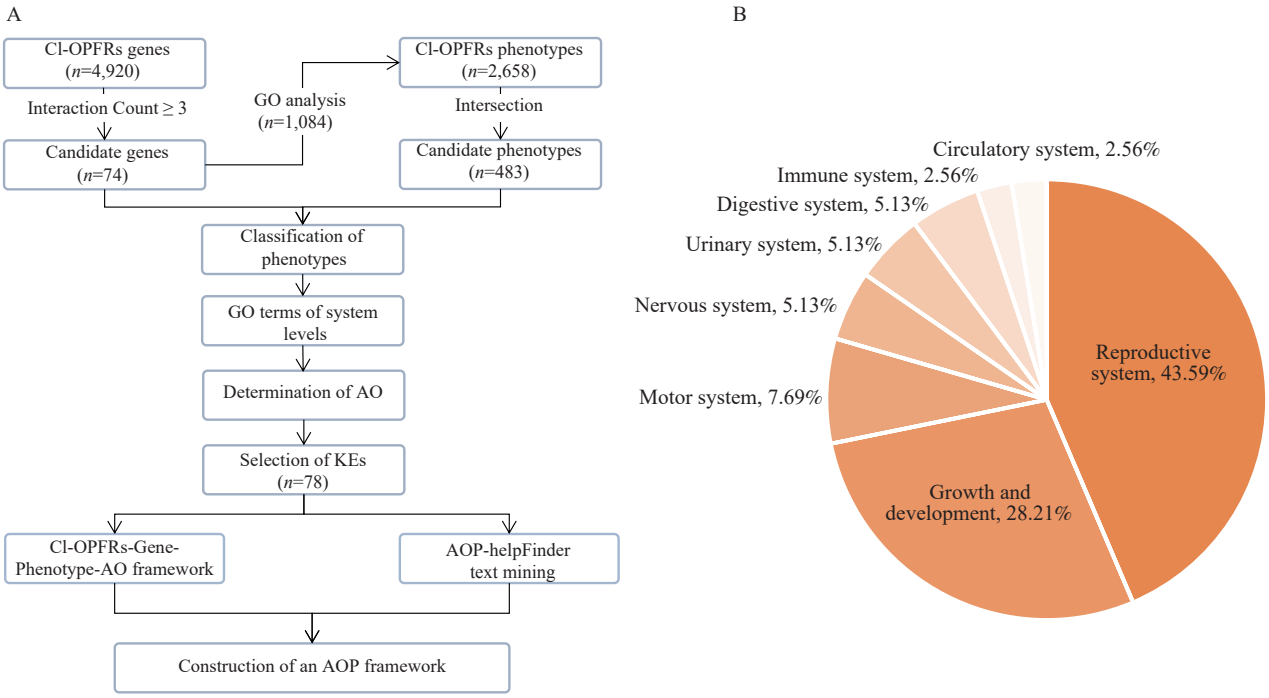

FIGURE 1. The strategy for the construction of the AOP framework. (A) The flow diagram for the construction of the AOP framework. (B) Percentage of GO terms at the system level.
Abbreviation: Cl-OPFRs=chlorinated organophosphate flame retardants; AOP=adverse outcome pathway; GO=geng ontology; AO=adverse outcome.

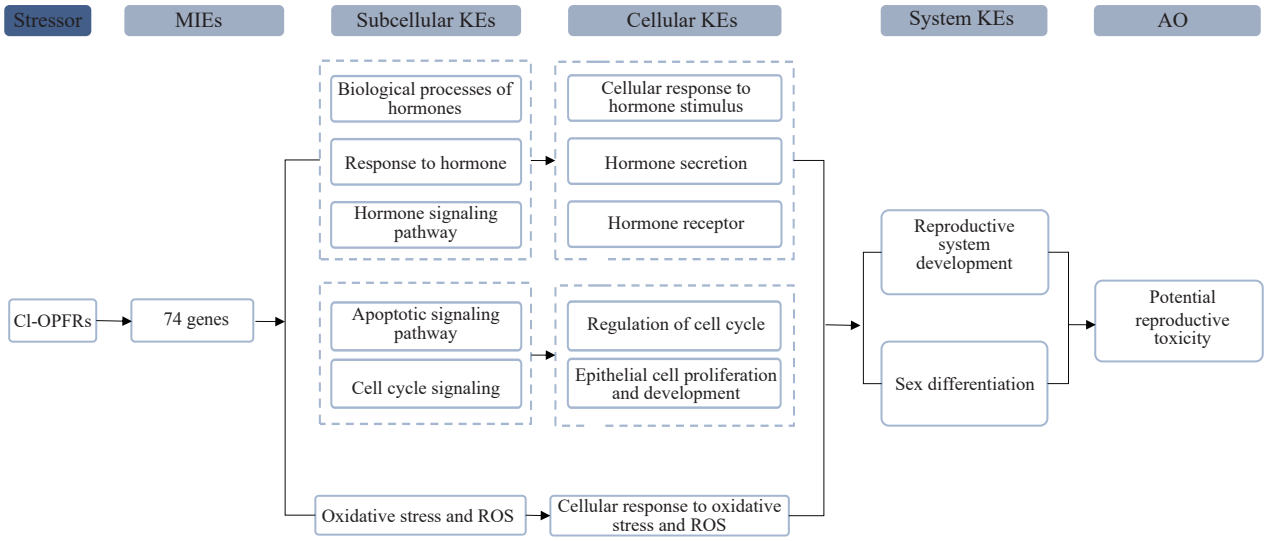

FIGURE 2. AOP framework of Cl-OPFRs-induced potential reproductive toxicity.
Abbreviation: AOP=adverse outcome pathway; Cl-OPFRs=chlorinated organophosphate flame retardants; MIEs=molecular initiating events; KEs=key events; AO=adverse outcome; ROS=reactive oxygen species.

dataset comprising 74 genes and 8 phenotypic metrics to develop a Cl-OPFRs-gene-phenotype-AO network, consisting of 84 nodes and 434 connections (Figure 3A). The relevance of each gene and phenotype within the network was determined by tallying the number of their connections. The AOP-helpFinder tool leveraged PubChem to compile alternate names for the three Cl-OPFRs identified as stressors, as well as the 11 genes that featured prominently in the gene-phenotype network analysis due to high connectivity. Notably, the genes *IGF1*, *BAX*, *AR*, *MTOR*, and *PPARG* showcased significant associations with Cl-OPFRs exposure (Supplementary Table S3, available at https://weekly.chinacdc.cn/). Subsequently, we crafted an AOP model delineating the putative role of Cl-OPFRs in reproductive toxicity, structured around the hierarchical and biological interplay between these components (Figure 3B). The proposed AOP model posits that the expression of the five aforementioned MIEs is disrupted upon exposure to Cl-OPFRs, which in turn perturbs biological processes and hormone-mediated pathways, potentially compromising reproductive development and culminating in reproductive toxicity. To evaluate the robustness of this AOP model, we conducted a WoE assessment in accordance with the OECD Handbook, which entailed scrutinizing the AOP Wiki and relevant literature. As indicated in Supplementary Tables S4 and S5 (available at https://weekly.chinacdc.cn/), the significance of the KEs and the validity of the inter-KE relationships were judged to fall within the "moderate" to "high" range, based on criteria such as biological plausibility and supportive experimental and epidemiological studies. In summary, the presented AOP model is characterized by a relatively high degree of credibility.

DISCUSSION

The study results indicated that Cl-OPFRs may lead to toxicity affecting multiple systems, with a focus on reproductive toxicity. The AOP framework suggested that Cl-OPFRs could impact the expression of crucial genes such as *IGF1*, *BAX*, *AR*, *MTOR*, and *PPARG*, leading to hormone-related effects that impact reproductive system development and indicating potential reproductive toxicity concerns.

Due to their high production volumes, extensive use, and environmental persistence, the toxic effects of Cl-OPFRs across different species have been under scrutiny (*3*). Despite this interest, a comprehensive assessment of Cl-OPFR toxicity and its underlying biological mechanisms remains elusive. The OECD has launched a project to create AOPs that consolidate existing toxicity data to enhance predictions of chemical toxicity, clarify the mechanisms of action, and inform regulatory decisions for hazardous substances (*4*). Utilizing the AOP framework, which includes data from the CTD, network-based strategies, and an extensive literature review, we investigated the connection between Cl-OPFR exposure and adverse health outcomes. Our AOP model revealed that Cl-

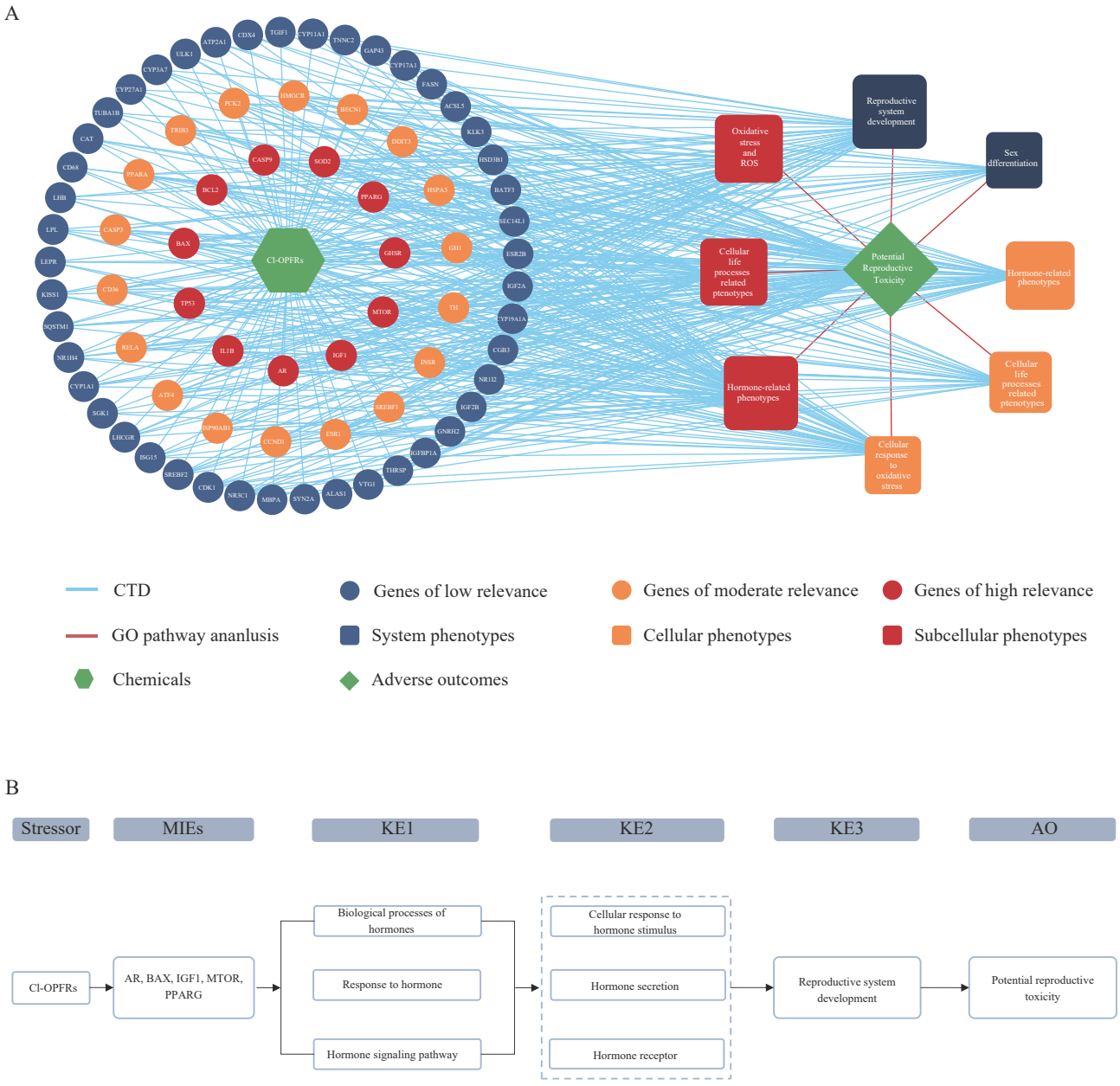

FIGURE 3. Cl-OPFRs-gene-phenotype-AO framework. The green hexagon node represents Cl-OPFRs; the green diamond node represents potential reproductive toxicity; the round nodes represent target genes, with their proximity to the center of the circle indicating their relative contribution to the framework; the blue rectangle nodes represent system phenotypes, while orange rectangle nodes represent cellular phenotypes, and red rectangle nodes represent subcellular phenotypes. The size of the rectangle reflects the magnitude of their impact on the framework. In total, 434 links extracted from the CTD, and GO and KEGG pathway enrichment analyses results are presented as different connections among the nodes.
Abbreviation: Cl-OPFRs=chlorinated organophosphate flame retardants; CTD=Comparative Toxicogenomics Database; MIEs=molecular initiating events; KEs=key events; GO=geng ontology; AO=adverse outcome; ROS=reactive oxygen species.

OPFRs are linked to multiple systemic toxicities, including those affecting growth and development, motor function, neurology, and particularly reproduction. Empirical evidence supports these findings, such as research demonstrating the ability of TCEP to affect survival, growth, and induce histological alterations in juvenile fish (*5*). Additionally, instances of spinal curvature and muscle malformations in zebrafish have been associated with exposure to TDCIPP (*6*).

Given the importance of reproductive toxicity, a comprehensive framework linking Cl-OPFRs with

genes, phenotypes, and AOs was developed. This framework is justified by previous studies demonstrating the endocrine-disrupting and reproductive toxicity potential of Cl-OPFRs. For example, Cl-OPFRs can interfere with the androgen receptor (AR) activity (*7*), leading to disruptions in hormone-related receptors and affecting genes involved in steroid hormone biosynthesis, ultimately causing adverse reproductive effects (*8*). These effects include decreased sperm concentrations and motility in males, increased risks of fetal chromosome abnormalities and spontaneous abortion in females post-Cl-OPFR exposure (*9*–*10*), with supporting evidence for KEs in this pathway.

Although potential adverse effects have been identified, the study has several limitations. First, this study only focuses on comprehensively analyzing the data of the CTD database by constructing the AOP. Second, the findings are not validated in the biological experiments. However, it is worth to noting that this study can enhance our understanding of the relationship between Cl-OPFRs and human reproductive toxicity, and it is advised that large multicenter national cohorts confirm our results.

Conflicts of interest: No conflicts of interest.

Funding: Supported by the Natural Science Foundation of Jiangsu Province (grant number 22KJA330002), Collaborative Innovation Center for Cancer Personalized Medicine, and the Priority Academic Program Development of Jiangsu Higher Education Institutions in the Field of Public Health and Preventive Medicine.

doi: 10.46234/ccdcw2024.105

[#] Corresponding authors: Xiaoting Li, xiaotingli@njmu.edu.cn; Haiyan Chu, chy_grape@njmu.edu.cn.

[1] Department of Environmental Genomics, Jiangsu Key Laboratory of Cancer Biomarkers, Prevention and Treatment, Collaborative Innovation Center for Cancer Personalized Medicine, School of Public Health, Nanjing Medical University, Nanjing City, Jiangsu Province, China; [2] Department of Genetic Toxicology, The Key Laboratory of Modern Toxicology of Ministry of Education, Center of Global Health, School of Public Health, Nanjing Medical University, Nanjing City, Jiangsu Province, China; [3] Department of Nutrition and Food Safety, School of Public Health, Nanjing Medical University, Nanjing City, Jiangsu Province, China.
[&] Joint first authors.

Submitted: January 06, 2024; Accepted: April 15, 2024

REFERENCES

1. Dou MS, Wang LJ. A review on organophosphate esters: physiochemical properties, applications, and toxicities as well as occurrence and human exposure in dust environment. J Environ Manage 2023;325:116601. https://doi.org/10.1016/j.jenvman.2022.116601.
2. Zhu HH, Zhang HL, Lu K, Yang S, Tang XY, Zhou MY, et al. Chlorinated organophosphate flame retardants impair the lung function via the IL-6/JAK/STAT signaling pathway. Environ Sci Technol 2022;56(24):17858 – 69. https://doi.org/10.1021/acs.est.2c05357.
3. Feng YX, Cui X, Yin J, Shao B. Chlorinated organophosphorus flame retardants-induced mitochondrial abnormalities and the correlation with progesterone production in mLTC-1 cells. Food Chem Toxicol 2022;169:113432. https://doi.org/10.1016/j.fct.2022.113432.
4. Wang XQ, Li F, Teng YF, Ji CL, Wu HF. Potential adverse outcome pathways with hazard identification of organophosphate esters. Sci Total Environ 2022;851(Pt 1):158093. http://dx.doi.org/10.1016/j.scitotenv.2022.158093.
5. Hu FX, Zhao YX, Yuan Y, Yin L, Dong FL, Zhang WN, et al. Effects of environmentally relevant concentrations of tris (2-chloroethyl) phosphate (TCEP) on early life stages of zebrafish (*Danio rerio*). Environ Toxicol Pharmacol 2021;83:103600. https://doi.org/10.1016/j.etap.2021.103600.
6. Rhyu D, Lee H, Tanguay RL, Kim KT. Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) disrupts zebrafish tail fin development. Ecotoxicol Environ Saf 2019;182:109449. https://doi.org/10.1016/j.ecoenv.2019.109449.
7. Rosenmai AK, Winge SB, Möller M, Lundqvist J, Wedebye EB, Nikolov NG, et al. Organophosphate ester flame retardants have antiandrogenic potential and affect other endocrine related endpoints *in vitro* and *in silico*. Chemosphere 2021;263:127703. https://doi.org/10.1016/j.chemosphere.2020.127703.
8. Kojima H, Takeuchi S, Itoh T, Iida M, Kobayashi S, Yoshida T. In vitro endocrine disruption potential of organophosphate flame retardants via human nuclear receptors. Toxicology 2013;314(1):76 – 83. https://doi.org/10.1016/j.tox.2013.09.004.
9. Siddique S, Farhat I, Kubwabo C, Chan P, Goodyer CG, Robaire B, et al. Exposure of men living in the greater Montreal area to organophosphate esters: association with hormonal balance and semen quality. Environ Int 2022;166:107402. https://doi.org/10.1016/j.envint.2022.107402.
10. Li YT, Wang X, Zhu QQ, Xu YQ, Fu QG, Wang T, et al. Organophosphate flame retardants in pregnant women: sources, occurrence, and potential risks to pregnancy outcomes. Environ Sci Technol 2023;57(18):7109 – 28. https://doi.org/10.1021/acs.est.2c06503.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. The frequency of genes occurring more than one time in each system.

System	Related genes (Counts)
Reproductive system development	*IL1B* (7), *BAX* (6), *BCL2* (5), *AR* (3), *GHSR* (3), *HMGCR* (3), *IGF1* (3), *MTOR* (3),*NR1H4* (3), *PCK2* (3), *PPARA* (3), *PPARG* (3), *SREBF1* (3), *TP53* (3), *ATF4* (2), *CASP9* (2), *CD36* (2>), *CYP11A1* (2), *CYP17A1* (2), *DDIT3* (2), *ESR1* (2), *KISS1* (2), *LHB* (2), *NR3C1* (2), *RELA* (2), *SOD2* (2), *SREBF2* (2)
Growth and development	*NR1H4* (4), *CD36* (3), *IGF1* (3), *IL1B* (3), *MTOR* (3), *PCK2* (3), *TP53* (3), *BAX* (2), *BECN1* (2), *CASP9* (2), *DDIT3* (2), *GHSR* (2), *HMGCR* (2), *PPARA* (2), *SREBF1* (2)
Motor system	*PPARA* (2), *TP53* (2)
Urinary system	*IGF1* (2)
Digestive system	*IGF1* (2)

SUPPLEMENTARY TABLE S2. Classification of phenotypes.

Classification	Ancestor terms	Phenotypes	Related genes
System	Reproductive system development	Gland development, Mammary gland development, Prostate gland growth, Reproductive structure development, Reproductive system development, Prostate gland development, Genitalia development, Urogenital system development, Developmental maturation, Mammary gland branching involved in pregnancy, Mammary gland duct morphogenesis, Gonad development, Female gonad development	*AR/ATF4/BATF3/BAX/BCL2/CASP3/CASP9/CCND1/CYP11A1/CYP17A1/CYP1A1/CYP27A1/CYP3A7/ESR1/FASN/GH1/GHSR/HMGCR/HSP90AB1/IGF1/IL1B/INSR/KISS1/LHB/LHCGR/MTOR/NR1H4/NR3C1/PCK2/PPARA/PPARG/RELA/SGK1/SOD2/SREBF1/SREBF2/TGIF1/TH/TP53/TRIB3*
	Sex differentiation	Sex differentiation, Development of primary sexual characteristics, Female sex differentiation, Development of primary female sexual characteristics	*ACSL5/ATF4/BAX/BECN1/CASP9/CD36/DDIT3/IL1B/LEPR/LPL/NR3C1/PCK2/PPARG/SREBF2/TP53*
Cellular	Cellular response to hormone stimulus	Cellular response to peptide hormone stimulus, Cellular response to steroid hormone stimulus	*ATF4/ATP2A1/BAX/BCL2/BECN1/CASP3/CASP9/CD36/CD68/DDIT3/GHSR/HSPA5/IL1B/RELA/SOD2/TP53/TRIB3*
	Hormone secretion	Hormone transport, Hormone secretion, Peptide hormone secretion	*ATF4/BAX/BCL2/BECN1/CCND1/CDK1/CYP1A1/HSPA5/IGF1/IL1B/INSR/PPARG/SQSTM1/SREBF1/SREBF2/TP53*
	Hormone receptor	Nuclear receptor binding, Nuclearreceptor activity	*AR/ATF4/BCL2/BECN1/CASP9/CAT/CD68/CDK1/CDX4/CYP1A1/DDIT3/ESR1/HMGCR/MTOR/NR1H4/NR1I2/NR3C1/PPARA/RELA/SOD2/SREBF1/TH/TP53*
	Regulation of cell cycle	Regulation of mitotic cell cycle, Negative regulation of cell cycle, Mitotic cell cycle phase transition, Negative regulation of the cell cycle process, Regulation of cell cycle phase transition, Regulation of mitotic cell cycle phase transition, Negative regulation of the mitotic cell cycle, Positive regulation of cell cycle, Negative regulation of cell cycle phase transition, Negative regulation of mitotic cell cycle phase transition, Positive regulation of the cell cycle process, G1/S transition of mitotic cell cycle, Cell cycle G1/S phase transition	*AR/ATF4/BAX/BCL2/BECN1/CASP3/CASP9/CD36/CDK1/CYP27A1/ESR1/GHSR/HMGCR/HSP90AB1/HSPA5/IGF1/IL1B/INSR/ISG15/LPL/MTOR/NR3C1/PCK2/PPARA/PPARG/SGK1/SOD2/SREBF1/TP53/TRIB3/ULK1*
	Epithelial cell proliferation and development	Epithelial cell proliferation, Epithelial cell development, Mammary gland epithelium development, Positive regulation of epithelial cell proliferation involved in prostate gland development, Positive regulation of epithelial cell proliferation	*AR/ATF4/BAX/BCL2/CASP3/DDIT3/GAP43/GHSR/HMGCR/HSP90AB1/HSPA5/IL1B/INSR/KISS1/LEPR/MTOR/NR1H4/PCK2/PPARG/SREBF1/TH/TP53*
	Cellular response to oxidative stress	Cellular response to oxidative stress, Cellular response to reactive oxygen species, Regulation of cellular response to oxidative stress, Negative regulation of oxidative stress-induced cell death, Cell death in response to oxidative stress, Cellular response to decreased oxygen levels	*AR/BCL2/BECN1/CASP9/CCND1/CD36/CDK1/GH1/GHSR/HSD3B1/HSP90AB1/IGF1/IL1B/INSR/MTOR/NR3C1/PCK2/PPARA/PPARG/RELA/SOD2/SREBF1/TH/TP53*

Continued

Classification	Ancestor terms	Phenotypes	Related genes
Subcellular	Biological processes of hormones	Steroid metabolic process, Steroid biosynthetic process Hormone metabolic process, Steroid hydroxylase activity, Steroid hormone biosynthetic process, Androgen metabolic process, Steroid binding, Hormone biosynthetic process, C21-steroid hormone metabolic process, Estrogen metabolic process, Steroid delta-isomerase activity, C21-steroid hormone biosynthetic process, Regulation of peptide hormone secretion, Regulation of hormone secretion	*AR/ATF4/BAX/BCL2/BECN1/CASP3/CASP9/CAT/CCND1/CD36/CD68/CDK1/CDX4/CYP11A1/CYP17A1/CYP1A1/CYP27A1/CYP3A7/ESR1/GH1/GHSR/HMGCR/HSD3B1/HSP90AB1/HSPA5/IL1B/INSR/ISG15/KISS1/KLK3/LEPR/LHB/LHCGR/LPL/MTOR/NR1H4/NR1I2/NR3C1/PCK2/PPARA/PPARG/RELA/SGK1/SOD2/SQSTM1/SREBF1/SREBF2/TH/TP53/TRIB3/TUBA1B/ULK1*
	Response to hormone	Response to peptide hormone, Response to steroid hormone, Response toestradiol, response to corticosteroid	*AR/ATF4/ATP2A1/BAX/BCL2/BECN1/CASP3/CASP9/CD68/DDIT3/ESR1/GHSR/HSP90AB1/HSPA5/IGF1/IL1B/LPL/MTOR/NR1H4/NR3C1/PPARA/SOD2/SREBF1/SREBF2/TNNC2/TP53/ULK1*
	Hormone signaling pathway	Hormone-mediated signaling pathway,Steroid hormone-mediated signaling pathway, Intracellular steroid hormone receptor signaling pathway, ER-nucleus signaling pathway	*ATF4/CD36/CD68/DDIT3/GHSR/HMGCR/HSP90AB1/HSPA5/IL1B/INSR/KISS1/LPL/MTOR/NR1H4/PCK2/PPARA/PPARG/SREBF1/SREBF2/TP53*
	Apoptotic signaling pathway	Regulation of apoptotic signaling pathway, Intrinsic apoptotic signaling pathway, Negative regulation of apoptotic signaling pathway, Regulation of intrinsic apoptotic signaling pathway, Intrinsic apoptotic signaling pathway in response to oxidative stress,Intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress, Positive regulation of intrinsic apoptotic signaling pathway, Extrinsic apoptotic signaling pathway, Regulation of extrinsic apoptotic signaling	*AR/BAX/BCL2/CASP3/CAT/CCND1/CYP1A1/ESR1/GH1/GHSR/HMGCR/HSP90AB1/HSPA5/IGF1/IL1B/ISG15/LHCGR/MTOR/PPARA/PPARG/RELA/SOD2/SQSTM1/TP53/TRIB3*
	Cell cycle signaling	Cell cycle checkpoint signaling, Mitotic cell cycle checkpoint signaling	*CDK1/CYP1A1/CYP3A7/HMGCR/HSP90AB1/IGF1/IL1B/PCK2/SREBF2/TH*
	Oxidative stress and ROS	Response to oxidative stress,Response to reactive oxygen species, Positive regulation of reactive oxygen species metabolic process, Regulation of reactive oxygen species metabolic process, Reactive oxygen species metabolic process, Reactive oxygen species biosynthetic process	*AR/ATF4/BAX/BCL2/BECN1/CASP3/CASP9/CCND1/CD36/DDIT3/ESR1/GH1/GHSR/HMGCR/HSP90AB1/HSPA5/IGF1/IL1B/INSR/ISG15/LHB/LHCGR/MTOR/PPARA/RELA/SOD2/SQSTM1/SREBF1/TP53/TRIB3/TUBA1B*

Abbreviation: ROS=reactive oxygen species.

SUPPLEMENTARY TABLE S3. Top genes with the most linkages in Cl-OPFRs-gene-phenotype-AO framework and the top genes in the AOP-helpFinder text mining results.

Cl-OPFRs-gene-phenotype-AO framework, Gene (degree)	AOP-helpFinder, Gene (average score)
IL-1β (10), *TP53* (10), *AR* (9), *BCL2* (9), *BAX* (9), *MTOR* (9), *GHSR* (9), *PPARG* (9), *IGF1* (9), *CASP9* (9), *SOD2* (9)	*IL-1β* (-), *TP53* (-), *AR* (26.2), *BCL2* (-), *BAX* (29.6), *MTOR* (8.2), *GHSR* (-), *PPARG* (4.9), *IGF1* (31.1), *CASP9* (-), *SOD2* (-)

Abbreviation: Cl-OPFRs=chlorinated organophosphate flame retardants; AOP=adverse outcome pathway; AO=adverse outcome.

SUPPLEMENTARY TABLE S4. Assessment of the essentiality of KEs.

Events	Evidence	WoE	PMID
MIE			
AR	*AR* inhibition induced malformation of the male reproductive tract.	High	16417039
	Reduced *AR* expression caused reproductive disorders in rats.	High	32392119
	pAOP-9: Disrupted *AR* activity leads to altered follicle growth and impaired fertility.	High	32638039
IGF1	Reduced *IGF1* expression caused testicular dysplasia.	High	31141788
	Decreased *IGF1*-induced ovarian developmental toxicity.	High	29370380
BAX	Elevated *BAX* expression inhibits follicle growth.	High	16081520
	Driving *BAX* expression caused premature ovarian failure.	High	12488331
	High expression of *BAX* induced Loss of ovarian follicles.	High	11455387
MTOR	Activation of the *MTOR* pathway improved ovarian function.	Moderate	35300716
	Inhibition of spermatogenesis by *MTOR* activation.	Moderate	27296223
	DNA damage in duck testes caused by *MTOR* activation.	Moderate	36351481
PPARG	Agonistic *PPARG*-induced ovarian toxicity.	High	19265280
	Upregulation of *PPARG* had a protective effect on rat testes.	High	34890588
	Testicular injury in male mice disrupted *PPARG* levels.	Moderate	26219505
KE1			
Biological processes of hormones	C21-steroid hormone metabolism affects organ morphology and the development and function of the reproductive system.	High	23717070
Response to hormone	Transcriptome aberration in mice uterus was associated with steroid hormone response.	High	33971472
Hormone signaling pathway	Disruption of steroid hormone-related signaling pathways induced ovarian toxicity.	High	32610232
	pAOP-4: Disrupted ESR and AHR signaling led to disturbed primordial follicle formation and impaired fertility in females.	High	32638039
	pAOP-5: Disrupted AHR signaling leads to follicle atresia and premature ovarian insufficiency.	High	32638039
KE2			
Cellular response to hormone stimulus	Primary co-cultures of epithelial and stromal cells from human prostate carcinoma can response to hormone.	Moderate	15679620
Hormone secretion	Testosterone secretion induced anovulation and ovarian characteristics of PCOS in WT mice.	High	10.1016/j.coemr.2020.03.001
Hormone receptor	Mechanisms of sex-dependent reproductive toxicity due to estrogen receptor and androgen receptor antagonism.	High	34638023
	AOP-Wiki-19 Androgen receptor antagonism leads to adverse effects in the male fetus.	High	-
	AOP-Wiki-167 Early-life estrogen receptor activity leading to endometrial carcinoma in the mouse.	High	-
KE3			
Reproductive system development	Delayed development and further induction of ovarian reproductive toxicity in Drosophila.	High	37080472
	Zebrafish reproduction is inhibited, with reduced female spawning and slow growth of offspring reflecting reproductive toxicity.	Moderate	34638023
	Reproductive toxicity due to damage to gonadal development.	High	31398636

Abbreviation: AOP=adverse outcome pathway; MIEs=molecular initiating events; KEs=key events.

SUPPLEMENTARY TABLE S5. Assessment of the evidence supporting KERs.

KERs	Evidence	WoE	PMID
MIE to KE1	Disruption of androgen signaling by *AR* antagonists in utero.	Moderate	20487044
	IGF1 perturbation causes abnormal steroid hormone response.	High	33628636
	MTOR disorder causes lower testosterone levels.	High	32026564
	Accumulation of *BAX* increased the release of testosterone in cultured granulosa cells.	High	31344364
	PPARG was involved in several estrogen metabolism pathways.	Moderate	36932403
KE1 to KE2	The gonadotropin receptor complex promotes testosterone production.	Moderate	1646778
	Blocking the classical testosterone-signaling pathway to exert antagonistic effects on *AR* receptors.	High	16169144
	Activation of the testosterone-signaling pathway mediated anti-androgenic secretion.	High	30974244
	Promotes the responsiveness of testicular-like organs to hormones and increases sensitivity to reproductive toxicants.	Moderate	36219953
	Disrupting steroid hormone-related signaling pathways to block steroid hormone secretion.	High	35990263
KE2 to KE3	Hormone secretion affects ovarian development.	High	23279460
	Feline gonads exhibit tissue-specific alternative splicing of estrogen receptors.	Moderate	20963760
	Antagonizing androgen receptors or inhibiting steroid hormone synthesis inhibits the normal development of the male reproductive system.	High	23525324
	Affected the development of the reproductive system of ovarian-intact experimental rats by regulating hormones and estrogen receptors.	High	21722617
KE3 to AO	Delayed seasonal gonad development in mature female cod reflecting reproductive toxicity.	High	20487044

Abbreviation: AOP=adverse outcome pathway; MIEs=molecular initiating events; KEs=key events; KERs=KE relationships.