Risk Assessment of Endocrine Disrupting Chemicals by Integrating

Adverse Outcome Pathway, Machine Learning and Zebrafish

Embryo Model: A Case Example of Bisphenol A

Riping Huang

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Acknowledgement

After a long period of preparation and hard work, I finally have completed my doctoral thesis. Looking back on these years of study, when did I become interested in the field of environmental risk analysis? It probably started when I first learned about environmental risk assessment and risk management cases in an environmental chemistry class in my second year of college. Since then, some questions began to appear in my mind and I was eager to find the answers to these questions. For example, the impacts of human activities on the environment, the risks posed to humans by environmental pollution, and ways to reduce the health risks of environmental pollution. Therefore, I started my doctoral study and research. Time flies, my three-year doctoral program is coming to an end. The end of this program is not the end of my road to finding answers, but the beginning of a new journey of discovery. I hope that in my future career I will have the opportunity to make a small contribution to solving environmental problems.

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Abstract

Concerned about the health risks of endocrine disrupting chemical (EDC), government agencies in various countries have introduced policies to regulate EDC. However, the complexity of EDC toxicity mechanisms, especially some unique properties, such as important windows of exposure, low dose effects and non-monotonic dose-response, pose challenges to traditional risk evaluation methods. Moreover, there is an urgent need of toxicological data for risk assessment of new chemicals entering the market worldwide every year. Therefore, the research objective of this study is to develop an efficient and systematic approach to evaluate the integrated risk of EDC for supporting the regulation of EDC.

First, a traditional risk assessment method was used to evaluate the risk of bisphenol (BPA) exposure. BPA exposure levels in the Chinese population were calculated based on BPA urine concentration data collected from the literature and a simple metabolic model. The risk of BPA exposure was evaluated by Hazard Quotient based on reference dose (RfD, 50 mg/kg bw/day) and temporary tolerable daily intake (t-TDI, 4 mg/kg bw/day). The results showed that the Chinese population is widely exposed to BPA but the risk is not high. Pregnant women and children are at higher risk of exposure compared to adults, suggesting that more attention needs to be given to the exposure of sensitive populations. The RfD and TDI value used in traditional risk assessment method is limited to provide a comprehensive overview of the adverse outcomes of a chemical, which bring uncertainty into the risk assessment.

To profile the EDC toxicity mechanism and evaluate the risk of different adverse outcomes caused by EDC, an *in silico* model combining ToxCast database, Adverse Outcome Pathway (AOP) and machine learning was developed. 40 EDCs were selected as research targets based on the physicochemical properties and applications. Molecular initiating events (MIEs) caused by 40 EDCs were collected and prioritized through the ToxCast database and ToxPi tools. The AOP information related to MIEs was collected

from the AOP-Wiki database to build EDC related AOP networks. The toxicity of adverse outcomes (AOs) was evaluated by AOP network and machine learning methods. The prediction results were validated by animal experimental data collected from ToxRefDB. Six AOP networks covering 48 AOPs, 22 MIEs, 39 AOs and 164 key events (KEs) were constructed for profiling the mode of action of 40 EDCs. The results showed that the MIEs affected by the 40 EDCs contained ER, AR, PPARA, THRB, TPO, AHR, NR1I2, etc. The AOs caused by 40 EDCs were liver lesions, reproductive system damage, immune system disorders, cancer, obesity, developmental defects, and neurodegeneration, etc. The results of the random walk analysis indicated that disturbances are more likely to occur in the liver and reproductive system. The predicted reproductive toxicity and hepatotoxicity threshold were 0.36 and 0.80. AUC of 0.73 (reproductive toxicity) and 0.86 (hepatotoxicity) indicated that this method is a promising method for predicting EDC toxicity.

To explore further applications of the AOP-related *in silico* risk assessment method, biomarkers of EDC were predicted using this method, and a zebrafish embryo model was used to validate the predictions. Using BPA as the study target, BPA-induced MIEs were collected from ToxCast database, and related AOP information was collected from AOP-Wiki database, and the collected information was used to construct a BPA-related AOP network. The MIEs of BPA were quantified and ranked using ToxPi score. The important KEs in the AOP network were identified using the random walk with restart method. The identified genetic biomarkers of MIEs and KEs were tested in zebrafish embryo model. The results showed that the MIEs caused by BPA included TPO, PPARA, NR1I2, NR1I3, ER, AR, THRB, etc. with TPO Antagonism has the greater ToxPi score. The AOs caused by BPA included immune system disorders, reproductive system cancers and other cancers, reproductive system disorders, growth and developmental disturbances, breast cancer, liver lesions, neurological developmental disturbances and population trajectory alteration. Random walk with restart results showed that important KEs were vitellogenin concentration, thyroxine concentration,

estradiol concentration in blood; hippocampal gene expression, fatty acid in liver, and testosterone in ovary, etc. Results of zebrafish embryo acute toxicity test indicated that the 96 h LC50 values and the 96 h EC50 values for hatching rate, malformation rate, heartbeat rate of BPA were 9.4, 5.9, 5.1, and 8.5 mg/L, respectively. BPA can cause bent spine, pericardial edema, and yolk sac edema in zebrafish embryos. Results of zebrafish embryo genotoxicity test indicated that ESR1, ESR2B, THRB may applicable as biomarkers for MIEs.

Overall, this study developed an efficient risk assessment method for EDC based on ToxCast data, AOP and machine learning. It was applied to assess the risk of adverse outcomes and predict biomarkers of important pathways for EDCs.

Contents

A list of abbreviation

	General Concept		Gene		Chemical
EDC	endocrine disrupting chemical	ER	estrogen receptor	TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
NMDR	non-monotonic dose-response	ESR1	estrogen receptor 1	ATZ	Atrazine
IATA	Integrated Approach to Testing and Assessment	ESR2	estrogen receptor 2	CHL	Chlordane
AOP	adverse outcome pathway	ESR2B	estrogen receptor 2b	p,p'-DDT	Dichlorodiphenyltrichloroethane
MIE	molecular initiating event	ESR2A	estrogen receptor 2a	ES	Endosulfan
KE	key event	ESRRA	estrogen related receptor alpha	p,p'-DDD	p,p'-Dichlorodiphenyldichloroethane
AO	adverse outcome	PGR	progesterone receptor	PCP	Pentachlorophenol
KER	key event relationship	AR	androgen receptor	TPT	Triphenyltin chloride
NOAEL	no-observed-adverse-effect-level	PPARA	peroxisome proliferator activated receptor alpha	VZ	Vinclozolin
LOAEL	lowest-observed-adverse-effect-level	PPARD	peroxisome proliferator activated receptor delta	ZI	Ziram
UF	uncertainty factor	PPARG	peroxisome proliferator activated receptor gamma	4-NP	4-Nonylphenol
RfD	reference dose	THRB	thyroid hormone receptor beta	PCB-153	2,2',4,4',5,5'-Hexachlorobiphenyl

TDI	tolerable daily intake	TPO	thyroid peroxidase	2,4-DHBP	2,4-Dihydroxybenzophenone
t-TDI	temporary tolerable daily intake	AHR	aryl hydrocarbon receptor	TBBPA	3,3',5,5'-Tetrabromobisphenol A
EDI	estimated daily intake	NR1I2/PXR	nuclear receptor subfamily 1 group I member 2	PNP	4-Nitrophenol
HQ	hazard quotient	NR1I3	nuclear receptor subfamily 1 group I member 3	BPS	4,4'-Sulfonyldiphenol
AC50	activity concentration at 50% of maximal activity	NR1H3	nuclear receptor subfamily 1 group H member 3	B(a)P	Benzo(a)pyrene
LC50	50% Lethal Concentration	NR1H4	nuclear receptor subfamily 1 group H member 4	BBP	Benzyl butyl phthalate
LC10	10% Lethal Concentration	NR3C1	nuclear receptor subfamily 3 group C member 1	BPF	Bis(4-hydroxyphenyl)methane
EC50	concentration for 50% of maximal effect	NFE2L2	nuclear factor, erythroid 2 like 2	BPA	Bisphenol A
EC10	concentration for 10% of maximal effect	CYP2E1	cytochrome P450 family 2 subfamily E member 1	BPAF	Bisphenol AF
ToxAO	endocrine disrupting chemical toxicity of each adverse outcome	CYP19A1	cytochrome P450 family 19 subfamily A member 1	DEHP	Di(2-ethylhexyl) phthalate
ROC	area under the receiver-operating curve	KDR	kinase insert domain receptor	PFOS	Perfluorooctanesulfonic acid

x

AUC	area under the receiver-operating curve metric	SREBF1	sterol regulatory element binding transcription factor 1	PFOA	Perfluorooctanoic acid
TPR	true-positive rate	β-actin	actin beta	TBT	Tributyltin chloride
FPR	false positive rate	P53	tumor protein p53	TCL	Triclosan
PCA	principal component analysis	GATA3	gata binding protein 3	TPPA	Triphenyl phosphate
PPR	personalized PageRank	SCD-1	stearoyl-Coenzyme A desaturase 1	DES	Diethylstilbestrol
PR	PageRank	CPT1AL	carnitine palmitoyltransferase 1a, liver	FLX	Fluoxetine
NA	not available	CPT1AM	carnitine palmitoyltransferase 1a, muscle	LNG	Levonorgestrel
IPCS	International Programme on Chemical Safety	CYP17A1	cytochrome P450 family 17 subfamily A member 1	SPF	Butylparaben
WHO	World Health Organization	BDNF	brain derived neurotrophic factor	D5-sil	Decamethylcyclopentasiloxane
UNEP	United Nations Environment Programme	GNRHR1	gonadotropin releasing hormone receptor 1	4-MBC	Enzacamene
EU	European Union	DIO1	iodothyronine deiodinase 1	MP	Methylparaben
OECD	Organisation for Economic Co-operation and Development	DGAT2	diacylglycerol O-acyltransferase 2	tOP	4-(1,1,3,3-Tetramethylbutyl)phenol
US EPA	United States Environmental Protection Agency	FOXA2	Forkhead box protein A2	ZEN	Zearalenone
EFSA	European Food Safety Authority	VTG1	vitellogenin 1	GEN	Genistein

Tox21	Toxicology in the 21st Century program	TES	Testosterone
ToxCast	Toxicity Forecaster	EE2	17alpha-Ethinylestradiol
ToxRefDB	Toxicity Reference Database	E2	17beta-Estradiol
ToxPi	Toxicological Priority Index		
GUI	Graphical User Interface		

Chapter 1.   Introduction

1.1. Research background

Endocrine-disrupting chemicals (EDCs) were defined as "*an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations*" by the International Programme on Chemical Safety of the World Health Organization (IPCS/WHO) in a 2002 report *Global assessment of the state-of-the-science of endocrine disruptors* [1]. In 2012, World Health Organization and the United Nations Environment Programme (UNEP) produced a report on EDCs, *State of the Science of Endocrine Disrupting Chemicals-2012*, that highlighted three strands of evidence increase concerns over EDCs: *the high incidence and the increasing trends of many endocrine-related disorders in humans; observations of endocrine-related effects in wildlife populations; the identification of chemicals with endocrine disrupting properties linked to disease outcomes in laboratory studies* [2]. The health of human and wildlife is tied to normal metabolism, growth and development which are inseparable from a healthy endocrine system. The endocrine system regulates a wide range of biological processes in the body, from cell differentiation in early life stage to organ functions in adulthood, by secreting hormones. Hormones are chemical substances that exert specific hormonal effects on target organs and cells. They are secreted in minute quantities from secretory organs (hypothalamus, pituitary gland, gastrointestinal tract, kidney, testes, ovaries, etc.), transported to target organ via blood [3]. Endocrine system is a sophisticated system, small changes in hormonal conditions can have profound and enduring impacts on exposure individual [4]. Considering the high potential risk to human health posed by EDC exposure, there is an urgent need for risk assessment and risk management of EDCs.

Until a tailored risk assessment method is developed for EDCs, traditional risk assessment techniques may be used to evaluate EDC risk after adjustment.

Conventional risk assessment processes, which are widely used by regulatory agencies worldwide, were developed based on the principles of toxicology [5-8]. Some important assumptions including: the response of an organism to a toxicant increase with increasing level and duration of exposure; and threshold hypothesis, where no adverse effects are detected below a certain exposure level [9]. However, some characteristics of EDCs challenge traditional risk assessment.

Complex mode of action. Hormone action consists of a series of complex processes, and EDCs can interfere with each stage of these processes. For example, EDCs can activate/inhibit hormone receptors, alter hormone receptor expression, alter signal transduction in hormone-responsive cells, and induce epigenetic modifications in hormone-producing or hormone-responsive cells. Besides, EDCs also can alter hormone synthesis, alter hormone transport across cell membranes, alter hormone distribution or circulating hormone levels, alter hormone metabolism or clearance, and alter the fate of hormone-producing or hormone-responsive cells [10]. The specificity and complexity of mechanism of EDCs resulted in varies outcomes across species [11]. Therefore, information extrapolation between different species based on traditional risk assessment methods is not easy [12]. More notably, the biological pathways of the endocrine system are not independent but are interconnected into networks [13]. Therefore, the outcomes caused by EDCs in the endocrine system may also be systemic [14]. Traditional risk assessment methods usually draw dose-response curves for one endpoint, and it is difficult to analyze the systemic risk. Besides, EDCs may cause multiple adverse outcomes in growth, development, reproduction, and behavior [15], it is difficult to decide which endpoints and how many endpoints are best used for EDC toxicological testing.

Important windows of exposure. While hormones regulate hormonal action at every stage of life, there are certain periods of specific tissue development that are critical (in utero, infancy and early childhood). For EDCs, the timing of exposure is as important as the dose of exposure. The effects of EDC exposure during adulthood may diminish

as EDCs are withdrawn. However, exposure to EDCs during sensitive periods can have permanent effects [16]. For example, if the body is exposed to EDC during tissue formation, resulting in changes in tissue structure, these changes are irreversible and may only become apparent in adulthood [17]. More seriously, some EDCs also show transgenerational effects, which means effects of EDCs can be transferred to the next generation and even persist over several generations [18]. This effect may occur through damaging DNA and altering epigenetic regulation [19]. However, the link between exposure and long-term/transgenerational effects may not be tested by traditional risk assessment.

Low dose effects and non-monotonic dose-response. Hormones in the endocrine system work at very low concentrations (at picomolar, nanomolar level). EDC mimics the effects of hormones, and some findings suggest that EDC also acts at such low levels [20]. Low dose effects were defined as that *a biological change, not limited to adverse effects, which occur either at human exposure levels or at doses below those routinely used in toxicity testing* [21]. The safety threshold identified in a traditional risk assessment may not applicable to EDC and poses unknown risks. For some EDCs, significant effects were detected below the safety threshold or reference dose or environmental exposure level in animal studies [22-23]. An extension of the low-dose-response study is the non-monotonic dose-response (NMDR) curve. NMDR was mathematically defined as *a nonlinear relationship between dose and effect where the slope of the curve changes sign somewhere within the range of doses examined* [24]. NMDRs are common in endocrinology and possible mechanisms including cytotoxicity, receptor down-regulation and desensitization, cell and tissue specific receptors and cofactors, receptor selectivity, receptor competition, negative feedback loops, tissue interactions [25]. The NMDRs of EDCs challenge the traditional concepts in toxicology that the lower the dose, the lower the toxicity. Therefore, for EDCs, extrapolation of information obtained from high exposure concentrations to low concentrations is not feasible.

Multiple exposure sources. EDCs cover a wide range of chemicals including natural or synthetic estrogens/androgens, phytoestrogens, mycoestrogens, and industrial chemicals [26]. They are found in a large variety of daily necessities, including pesticides, medicinal products, food packaging, personal care products, cosmetics, electronic equipment, construction materials, etc. EDCs are ubiquitous in the environment because of the global transportation of many known and potential EDCs through natural or commercial processes [27]. Human exposure routes to EDCs including ingestion, inhalation, and dermal uptake [28]. Exposure levels are higher in children because of their higher hand-to-mouth activities, higher metabolic rates, more ingestion per body weight, and more susceptible to environmental stressors [29]. In our daily lives, we have the chance to contact a variety of EDCs because of their wide range of applications. The knowledge about combination effects of human exposure to EDC mixtures are limited. The additive effect of some EDC mixtures was confirmed in animal studies [30]. Other studies reported that exposure to a mixture of EDCs can result in adverse effects while exposure to each EDC separately at same concentration used in mixture show no adverse effects [31-32].

There are other challenges to traditional risk assessment which are not only specific to EDCs but also other emerging chemicals are worth noting.

Incomplete data. Hundreds of chemicals were added to the EDC list. However, this is just the tip of the iceberg of emerging chemicals. There are still plenty of new chemicals waiting to be tested for safety [33]. The lack of toxicological data for these chemicals hinders the implementation of risk assessment. Besides, from the viewpoint of animal welfare, the trend toward the reduction of animal experiments is steadily progressing. From 2013, the European Union have banned the sale of cosmetics that use animals to test the safety of finished products or ingredients [34]. In response to these regulatory trends, developing animal alternative testing methods and improving computer predictive models for safety assessment are at a high priority.

Facing various uncertainties brought by EDCs, how can we regulate this kind of

chemicals? Whether to adjust the traditional risk assessment methods or to develop a new risk assessment paradigm, hoping we have enough wisdom to choose.

1.2. Research objective

Some decision-making organizations have attempted to apply the precautionary principle and take measures to regulate EDCs with the aim of reducing EDC exposure [35]. Some methodological guidelines were designed to assess the potential risks of EDCs [36]. However, there is still a knowledge gap regarding EDC that introduces uncertainty and unknowns into risk evaluation [37]. There is still an international discussion on the best way to apply scientific information to risk assessment of EDCs [25]. Because of the associated scientific gaps, testing difficulties, regulatory barriers, a well-defined, comprehensive and standardized risk assessment framework for EDCs has not yet been established [38]. But there is no time to waste. Currently, available information and techniques may be used properly to evaluate EDC risks. In the future, to face the needs of risk management for EDCs in the 21st century, time- and cost-saving, animal alternative, systematic and comprehensive risk assessment methods are needed. Therefore, the objectives of this study are:

1. to profile the network of mode of action of EDC.

2. to develop an efficient, informative approach for systematic assessing associated risks of EDCs. More specifically, EDC risks will be predicted by developing an Adverse Outcome Pathway-based risk assessment model. In addition, the model accuracy will be validated via *in vivo* experimental data. Also, the model feasibility will be validated through zebrafish embryo model using bisphenol A as an example.

1.3. The outline of thesis

Chapter 1 Introduction

In this chapter, the background, potential risks of EDCs, and the challenges that EDCs pose to traditional risk assessment are presented to motivate this study to be conducted.

Therefore, the objectives of this study are: to elucidate the mode of action for EDCs; to develop an efficient, informative approach for systematic assessing associated risks of EDCs.

Chapter 2 Review the development of risk assessment methods of EDCs

This chapter describes the current status of research on the risk assessment of EDCs, including actions taken by decision-making organizations and the development of assessment methods and tools. The methods of this study are decided after reviewing the available information and techniques. The novelty points of this study are mentioned.

Chapter 3 Exposure risk assessment of bisphenol A in the general Chinese population based on urinary levels

In this chapter, the exposure levels of bisphenol A, a representative EDC, in the general Chinese population are calculated via a simple excretion model. Exposure risks are calculated based on the Tolerable Daily Intake values obtained from traditional risk assessment methods. The limitations of traditional risk assessment methods are analyzed in this case.

Chapter 4 Risk assessment of EDCs by integrating ToxCast data, Adverse Outcome Pathway and machine learning

This chapter is divided into two parts. In the first part, the possible adverse effects of 40 common EDCs and the corresponding mode of action are analyzed. First, 40 representative EDCs are selected based on chemical properties and their applications. Then, the molecular initiating events of these EDCs are summarized through the ToxCast Database. Next, downstream biologically plausible key events of selected molecular initiating events are collected through the Adverse Outcome Pathway Database. Finally, a network of these events is established and the possible adverse outcomes caused by 40 EDCs and the corresponding biological pathways are predicted through network analysis.

The second part describes the use of a machine learning method to predict the risk of EDCs. The toxicological profiles of selected EDCs are compiled into a standardized

metric ToxPi score through ToxPi GUI tool, and then the probability of adverse outcomes and toxicity of EDCs is calculated by a machine learning method based on ToxPi scores and Adverse Outcome Pathway network. The results obtained are validated by *in vivo* animal experimental data from the Toxicity Reference Database.

Chapter 5 Health risk assessment of bisphenol A through adverse outcome pathway combining machine learning method and zebrafish embryo model

In this chapter, the feasibility of the established *in silico* method for predicting the risk of EDCs is tested, using bisphenol A as an example. First, a bisphenol A-related Adverse Outcome Pathway network is built, and the most important key events in the network and suitable toxicology endpoints of these key events are identified by machine learning method and network analysis. Then, four-day exposure experiments are performed to validate the changes in endpoints of key events using a zebrafish embryo model.

Chapter 6 Conclusion and perspectives

In this chapter, the main conclusions are summarized and emphasized. Limitations and future improvement plan of the developed approach are described.

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Chapter 2.   Review the development of risk assessment of EDCs

"*Endocrine disruptor*" became a named research area starting from a famous meeting held at the Wingspread Conference Center, Racine, Wisconsin in July 1991 and organized by Theo Colborn and colleagues [1]. After that, *Our Stolen Future* written by Colborn et al. has become a hot topic, bringing the widespread ecological and human health outcomes of EDCs into the public eye [2]. Over the past three decades, a large number of studies have provided new information on the mechanisms of EDCs acting on endocrine system, the distribution of EDCs in the environment, and the linkage between EDC exposure and adverse health outcomes in humans and wildlife.

Humans have created numerous chemical substances and have benefited from them to enjoy a comfortable and convenient life. It is an undeniable fact that many chemical substances have already been released into the global environment and the situation continues to this day. Close to 1000 chemicals are known or suspected to have endocrine disrupting properties [3]. However, only a small fraction of these chemicals has been tested for endocrine effects. These chemicals are useful in various aspects of modern life, but also have the potential to cause harm to humans or ecosystems [4]. Several government agencies begin to take action to minimize risks due to EDC exposure. The details of regulatory frameworks and policy initiatives regarding identified and potential EDCs through the world were documented in a UNEP report and summarized in Table 2-1 [5]. Due to the different scope and purpose of regulation, each government agency has adopted different hazard identification criteria and data requirements.

Table 2-1 Overview of the regulatory frameworks and policy initiatives regarding identified and potential EDCs [5]

Government body	Regulatory frameworks or policy initiatives	Target chemicals	Hazard identification criteria	Data requirement
China	13th Five-Year Plan of National Environmental Protection	unclear	No specific criteria are stated.	No specific data requirements are stated.
	Industry standard on evaluation methods of endocrine disrupting effects of pesticides	pesticides	A two-tiered approach with seven *in vitro* or *in vivo* testing guidelines included in industry standard NY/T2873-2015 "Evaluation Methods of the Endocrine Disruption Effects of Pesticides".	No specific data requirements are stated.
Japan	Japanese environmental regulation	chemicals detected in the ambient aquatic environment	A two-tiered testing strategy including *in vitro* and *in vivo* tests.	No specific data requirements are stated.
South Korea	Korean Regulation on the Registration and Evaluation of Chemicals (K-REACH)	industrial / commercial chemicals	No specific criteria are stated.	Data on hazard properties and risks (such as exposure scenarios, controls, and management actions over the course of the life cycle of the chemical substance).
Australia	National Industrial Chemicals Notification and Assessment Scheme (NICNAS)	industrial / commercial chemicals	For existing chemicals, definition of an EDC based on the list of priority substances developed under the EU-Strategy for endocrine disruptors.	No specific data requirements are stated.
Brazil	Federal Law 7802/1989 and Decree 4074/2002	pesticides and their components	The toxicological assessment of potential EDC is performed on a case-by-case basis using a weight of evidence approach.	Data from sub-chronic and chronic toxicity tests in rats, mice and dogs; *In vitro* tests or tests in other animal species; Academic publications and reports from other regulatory

				agencies or scientific organizations.
	Initiative to establish a national legislation on industrial chemicals	industrial chemicals	No specific criteria are stated.	No specific data requirements are stated.
Canada	Canadian Environmental Protection Act (CEPA)	industrial / commercial chemicals	No specific quantitative criteria are stated.	Various information is used: research results, peer-reviewed scientific literature, public or in-house databases, read-across information from structural analogues or quantitative structure-activity relationships (QSAR), data submitted by manufacturers and importers. For new chemicals: results from a 28-day repeated-dose toxicity study, e.g., OECD Test Guideline 407, 410 and 412.
	Pest Control Products Act (PCPA)	pest control products, including chemicals, devices, and organisms	No specific criteria are stated.	Data requirement in OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters and associated OECD Test Guidelines.
	Proposed regulatory framework under the Food and Drug Regulations	active pharmaceutical ingredients in new human and veterinary drugs	No specific criteria are stated.	Test data requirements for EDCs are still under development.
United States	Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Federal Food, Drug and Cosmetic Act (FD&C Act).	pesticide chemicals	Pesticides are identified as EDCs based on the results of the Endocrine Disruptor Screening Program (EDSP).	Test data from a two-tiered test battery and associated Endocrine Disruptor Screening Program Test Guidelines.
	Safe Drinking Water Act	drinking water contaminants	Chemicals are identified as EDCs based on the results of the Endocrine Disruptor	Test data from a two-tiered test battery and associated Endocrine Disruptor Screening Program Test Guidelines.

			Screening Program (EDSP).	
	Regulatory framework on new drug approval	new pharmaceutical drugs	Pharmaceuticals show adverse effects at clinically relevant exposures.	Test data from the standard non-clinical battery of toxicity tests including receptor-binding assays, pharmacology studies, repeat-dose toxicity studies, developmental and reproductive toxicity studies, and carcinogenicity studies. Test data from mechanistic studies, non-clinical juvenile studies, and clinical studies as a supplement.
European Union (EU)	European Chemicals Regulation (REACH)	industrial chemicals	No specific criteria are stated.	No specific data requirements are stated.
	Plant Protection Products Regulation (PPPR)	active substances, safeners, synergists used in plant protection products; basic substances	A draft of specific scientific criteria for the determination of endocrine disrupting properties of substances is under development.	Data requirements are laid out in the Regulation (EU). No. 283/2013 including many of the toxicological and ecotoxicological tests. Additional information or specific studies to elucidate the mode(s)/mechanism(s)-of-action and provide sufficient evidence for relevant adverse effects.
	Biocidal Products Regulation (BPR)	active substances used as biocides	A draft of specific scientific criteria for the identification of EDCs is under development.	Data requirements are laid out in the Regulation (EU). No. 528/2012. Additional information or specific studies to elucidate the mode(s)/mechanism(s)-of-action and provide sufficient evidence for relevant adverse effects.
	Water Framework Directive (WDF)	pollutants of water bodies	Refers to other relevant EU legislation, such as REACH.	Refers to other relevant EU legislation, such as REACH.

On the one hand, there is a need for EDC management by government agencies, and on the other hand, there is a lack of data for hazard identification of these chemicals. There is a trend to use novel, efficient, systemic methodologies and tools to conduct identification, prioritization, evaluation for the potential EDCs. In recent years, toxicological advancing methods have been applied.

2.1. High throughput screening

In order to reduce the reliance on animal experiments, *in vitro* cell-based and cell-free assays are using to clarify toxicity mechanism of chemical. High throughput screening combines automated systems and *in vitro* assays to quickly and efficiently test the effects of chemicals at the molecular/cellular level. The ToxCast/Tox21 platform of the United States Environmental Protection Agency (US EPA) uses high throughput screening to test biological activity of approximately 8300 chemicals [6]. All ToxCast chemical data is publicly available through the EPA CompTox Chemicals Dashboard (https://comptox.epa.gov/dashboard/). Up to date, ToxCast data were used for identification of potential EDCs [7], for endocrine profiling and prioritization of environmental chemicals [8], and for calculation of a combined exposure risk for endocrine activity chemicals [9-10].

2.2. *In silico* methods

Computational tools are effective and animal alternative choices to screen, prioritize, and assess the potential risk of chemical exposures. The development of *in silico* models provides support for filling some of the knowledge gaps. One example is the creation of the physiologically based pharmacokinetic (PBPK) model, which quantitatively describes the absorption, distribution, metabolism, and excretion (ADME) of a chemical in a body, that facilitated the development of *in vitro* to *in vivo* extrapolation (IVIVE), which translate *in vitro* concentrations to *in vivo* equivalents [11]. IVIVE expands the application of *in vitro* assay data in hazard identification and

risk assessment. An online suite of tools (https://ice.ntp.niehs.nih.gov/) were developed by the US National Toxicology Program for supporting IVIVE calculation.

The rapid increase in both the quantity and complexity of data in chemical toxicity challenge conventional analytical methods. Advanced data analysis approaches, such as machine learning, have become promising tools to support data analysis [12]. Some machine learning algorithms were applied to predicted endocrine disrupting capabilities of chemicals, including AdaBoost Decision Tree, Bernoulli Naive Bayes, Random Forest, Support Vector Machine, and Deep Neural Networks [13].

2.3. Zebrafish embryo model

In addition to *in vitro* assay and computer models, the zebrafish embryo model has also become a popular animal alternative to link chemical exposure and effects. With a number of advantages, zebrafish (*Danio rerio*) embryos are an important model in the toxicity identification and risk assessment of novel compounds. Small size, ease of maintenance, high fecundity and less ethical problems favor its laboratory use. *In vitro* fertilization, rapid development, and transparent embryo allow easy observation of early embryonic development and detection of morphological endpoints [14]. Whole genome sequence availability and near about 70% similarities with human genome sequence make zebrafish an attractive option for molecular and genetic research [15]. Currently, the zebrafish embryo toxicity (FET) assay has been chosen by several regulatory agencies for environmental risk and hazard assessment. Germany has regulated the FET assay as a mandatory DIN (German Institute for Standardization) standard for whole effluent testing [16]. The Organization for Economic Co-operation and Development (OECD) has proposed FET assay (OECD test guideline 236) as a promising alternative for the adult fish toxicity test required for regulatory purposes [17]. The EU is discussing the application of FET assay to the EU REACH regulation framework [18]. Moreover, it is feasible to use this assay for detection of specific toxicity such as endocrine toxicity and genotoxicity by modifying the fish embryo test

protocol [19-20].

2.4. Adverse Outcome Pathway (AOP) framework

The emergence of novel testing techniques and analytical models has provided new ideas for safety assessment of the large number of emerging chemicals. However, the result obtained from these novel methods still need to be validated for applicability at the regulatory level. For this reason, a concept of Integrated Approach to Testing and Assessment (IATA) was proposed as practical solutions to explore the use of novel methods in a regulatory context. OECD guidance document define IATA as "*pragmatic, science-based approaches for chemical hazard or risk characterization that rely on an integrated analysis of existing information in a weight of evidence assessment coupled with the generation of new information using testing strategies*" [21]. AOP is an objective and systematic framework to support developing IATA. AOP is the causal links of biological events starting from the molecular initiating events (MIEs), in which a chemical first perturbs the biological system, through a series of key events (KEs) at the subcellular, cellular, tissue and organ level, and finally leading to the specific adverse outcomes (AOs) at the individual or population level, which is important for risk assessment [22]. AOP provides the basis for expanding the application of mechanistic toxicology data and provides sufficient information to guide animal alternative method development as well as support regulatory decisions. OECD initiated adverse outcome pathway knowledge base project (AOP-KB) and maintained the basic module of AOP-KB, AOP-Wiki (https://aopwiki.org) to integrate AOP related knowledge and manage AOP development [23]. In EU, the OBERON project was conducted according to IATA concept to detect ED-related metabolic disorders by developing, improving and validating a battery of test systems, including *in vitro* assays, *in vivo* assays, high throughput omics technologies, epidemiology and human biomonitoring studies, and *in silico* models. Novel AOPs will be developed based on data obtained in this project [24]. Available AOPs in AOP-Wiki may be used to construct AOP networks which were designed to address specific problems or

applications via connecting sharing MIEs, KEs or AOs of each AOP. For example, a comprehensive AOP network specific to endocrine disruption were developed to reveal several mechanistic insights on endocrine-mediated perturbations upon chemical exposure [25].

While many novel methods have been developed to meet the need for risk assessment of a large number of emerging chemicals. There are still knowledge gaps regarding the practical application of these methods to regulatory. Until now, most of the studies on AOP network-based risk assessment are in a step of *in silico* prediction, but information on *in vivo* validation of AOP networks is inadequate. Besides, established AOP networks mainly focus on a few specific MIEs or AOs, rather than systematically analyzing the overall toxicity of chemicals.

In this study, an AOP network for systematic assessment of EDC integrated risks was established by combining ToxCast data and AOP-Wiki data. A machine learning approach was developed to predict the toxicity of EDCs by analyzing quantitative MIEs and AOP networks. An approach combining AOP networks and machine learning was developed to determine the important endpoints of EDC risk.

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Chapter 3. Exposure risk assessment of bisphenol A in the general Chinese population based on urinary levels

3.1. Introduction

Bisphenol A (BPA) is a high-volume industrial chemical primarily applied to manufactured polycarbonate plastic and epoxy resins, which are widely used in a variety of products such as food and beverage packaging, toys, water pipes, sports equipment, medical equipment, and consumer electronics [1]. The wide range of applications of BPA has resulted in the detection of the presence of BPA in a variety of environmental media [2]. It has been shown that human exposure to BPA was widespread [3]. Although BPA has been forbidden in some infant and food products [4], it remains extensively used in other products. In 2014, approximately 14.0 million tonnes of BPA were used in China, with an increased rate of approximately 0.8 kg BPA/capita/year [5]. However, the nationwide exposure level and potential risks associated with BPA in China remain uncertain.

BPA is a chemical of concern because it is a famous endocrine disruptor that binds to estrogen receptors [6]. Animal studies have reported that BPA adversely affects inter alia, reproductive, nervous, cardiovascular, and immune systems [7-10]. To date, many epidemiological studies have indicated an association between BPA exposure and human diseases, such as obesity, diabetes, reproductive disorders, and cardiovascular diseases [11-14]. To estimate the adverse effects of BPA on humans, it is necessary to elucidate human exposure levels.

Regional scientific committees and national regulatory agencies generally use the no-observed-adverse-effect-level (NOAEL) or lowest-observed-adverse-effect-level (LOAEL) and uncertainty factor (UF) methods to establish acceptable or tolerable intakes of substances that exhibit toxicity. For BPA, a reference dose (RfD) of 50 μg/kg bw/day was recommended by the United States Environmental Protection Agency. A

tolerable daily intake (TDI) of 50 μg/kg bw/day was suggested by the South Korea Ministry of Food and Drug Safety, the Food Safety Commission of Japan, and the European Food Safety Authority (EFSA). A TDI of 25 μg/kg bw/day was recommended by Health Canada [15-19]. According to EFSA, TDI was established based on a NOAEL of 5 mg/kg bw/day and a UF of 100, which was decided from the available, extensive database [18]. Some animal studies have shown that BPA exerts adverse effects at a dosage that is lower than the LOAEL of 50 mg/kg bw/day used to calculate a reference dose [20]. Therefore, the EFSA updated the TDI to a temporary tolerable daily intake (t-TDI) of 4 μg/kg bw/day based on newly incorporated investigation and experimental data in 2015 [21].

The total urinary BPA concentration (conjugated and unconjugated forms) is generally considered a reliable measurement for estimating BPA exposure [22]. In humans, the routes of exposure to BPA include dietary, inhalation, and dermal exposure. And oral exposure by food was estimated to contribute to more than 90% of BPA exposure in all age groups without occupational exposure [23]. According to multiple toxicokinetic studies on BPA in humans, BPA is almost completely excreted in the urine in the conjugated form within 24 hours of exposure [24-26]. Widespread exposure to BPA causes spot samples to adequately reflect the average exposure of the population to BPA when the population investigated is sufficiently large and samples are randomly collected [27]. In addition, many studies have detected BPA urinary concentrations among different populations in different regions of China [28-30]. Estimations based on these related databases comprise an alternative method of nationwide sample collection and investigation, which is time-consuming and costly [31]. In this study, available data from published literature were compiled to estimate BPA daily intake among the Chinese population, allowing the potential risk of BPA exposure to be analyzed and discussed.

3.2. Materials and methods

A review of published literature was conducted to collect related data on urinary BPA concentrations in the Chinese population. Relevant published studies prior to September 15, 2020 were identified in Web of Science and Scholar Google. The following keywords were used: "bisphenol A (BPA)," "urine," and "China." Studies were included in the analysis when they fulfilled the following eligibility criteria: (1) published articles were peer-reviewed and original full articles; (2) study surveyed the general population (occupational exposure population was excluded); (3) total urinary BPA concentration was detected (free plus conjugated BPA, ng/mL). The data extracted from each study were listed in supplementary material Table S1.

Initially, 106 publications were selected for full-text reading. Of these 106 articles, 49 articles were excluded owing to duplicate study objects (25), additional exposure sources (2), lack of information about the study population (6) or analysis method (2), inappropriate calculation methods (12), and occupational exposure (2). Finally, 57 studies reporting urinary BPA concentrations in the general Chinese population were deemed eligible for our exposure level estimation. In total, the included studies published between 2009 and 2020 covered 31811 urine samples from 23 regions with sampling times ranging from 1998 to 2019. To compare urinary BPA data with other counties, we also retrieved data from different countries from published literature, and the results are listed in supplementary material Table S3.

The biotransformation and toxicokinetic studies of BPA illustrated that orally administered BPA is excreted from the human body rapidly (half-life < 6 h) and efficiently (almost 100% of the administration dose) via urinary elimination within 24 h, allowing urinary BPA to be considered a general measure for estimating total daily intake [24-26]. Therefore, in this study, the estimated daily intake (EDI, ng/kg bw/day) of BPA was calculated based on human urinary concentration data according to the follow equation [32].

EDI = total BPA concentration in urine samples (C, ng/mL)×daily urine excretion volume (V, mL/day)/body weight (W, kg)

Average EDI = Σ(Ci×Vi/Wi×Ni)/ΣNi

Here, average EDI is the average EDI of a city or a subgroup. Ci is the average urinary total BPA concentration of a study in the relevant literature, and Ni is the sample size of a study. Vi and Wi are the average daily urinary excretion volume and average body weight of study subjects in a study.

The population were categorized into four subgroups depending on their sensitivity to BPA exposure and metabolism level: infants (0–1 year), children (2–17 years), adults (≥ 18 years), and pregnant women. Daily urine excretion data were obtained from published literature, and body weight data were obtained from the General Administration of Sports of China (http://www.sport.gov.cn/n315/n329/c216784/content.html). Data for BPA daily intake estimation are shown in supplementary material Table S2.

For risk assessment, the hazard quotient (HQ) value was calculated by following equation [33].

HQ = exposure concentration (ng/kg bw/day)/reference concentration (ng/kg bw/day)

Here, the exposure concentration was the EDI of BPA. The reference concentration is the RfD proposed by the United States Environmental Protection Agency (50 μg/kg bw/day) or the t-TDI provided by the European Food Safety Authority (4 μg/kg bw/day). If the HQ value is calculated to be less than 1, adverse effects are unlikely to occur. If the HQ value is equal to or greater than 1, there may be concerns regarding potential human health effects [33].

We used Microsoft Excel 2010 for data collation and weighted average calculation and statistical analysis. BPA urinary concentrations below the LOD/LOQ were substituted with a value equal to LOD/LOQ divided by 2 to calculate the average EDI.

3.3. Results and discussion

3.3.1. Urinary BPA concentrations in China

After a review of related studies and calculation, average urinary BPA concentrations for infant, pregnant woman, child, and adult groups were 0.78 ± 0.71, 1.15 ± 0.43, 1.70 ± 1.05, and 1.03 ± 0.73 ng/mL, respectively. As shown in Table S2, the ranges of average urinary BPA concentrations in other countries were <LOD – 2.40 ng/mL (infant), 0.60 – 2.50 ng/mL (pregnant woman), 0.70 – 7.43 ng/mL (child), and 0.73 – 4.10 ng/mL (adult).

Urinary BPA concentrations in Chinese infants were higher than those in Canada and Germany but lower than or similar to those in the United States, Switzerland, and Korea. In the pregnant woman population, urinary BPA concentrations in China were higher than those in Canada and Mexico and close to those in Denmark and Korea but lower than those in the United States, Netherlands, Sweden, Spain, and France. For the child population, India had the highest urinary BPA concentration, followed by Spain, Australia, Germany, Slovenia, Belgium, Denmark, Brazil, the United States, Korea, Greece, Mexico, Canada, Japan, and Egypt. The urinary BPA concentration in Chinese children was similar to that in Denmark and Brazil. For the adult population, urinary BPA concentration in China was relatively lower, and the results of this study were similar to those of Canada and Korea. The urinary BPA concentration in other counties was higher than that in China.

3.3.2. Estimated BPA daily intake of Chinese population

The results showed that the average EDI of infants, pregnant women, children, and adults were 30.92 ± 22.70, 24.85 ± 9.40, 34.13 ± 20.65, and 22.48 ± 16.21 ng/kg bw/day, respectively. The adult EDI was highest in Shenzhen (96.15 ng/kg bw/day) and lowest in Sandu (8.74 ng/kg bw/day). The EDI of eastern cities (Xuzhou, Nanjing, Shanghai, Hangzhou, Suzhou, and Kunshan) was relatively low compared with the southern (Qingyuan, Shenzhen, and Guangzhou) cities (Figure 3-1). In contrast, the lowest EDI

of children was in Shenzhen with 4.81 ng/kg bw/day, and the highest EDI of children was in Guangzhou (54.22 ng/kg bw/day), which is close to Shenzhen (Figure 3-2). In the pregnant woman group, Guiyu and Haojiang area had the highest EDI of 56.42 ng/kg bw/day and Laizhou Wan had the lowest EDI of 10.42 ng/kg bw/day. Only two studies investigated the urinary concentration data of infants. The highest EDI of 55.65 ng/kg bw/day was estimated for infants in Xiamen (Figure 3-3).
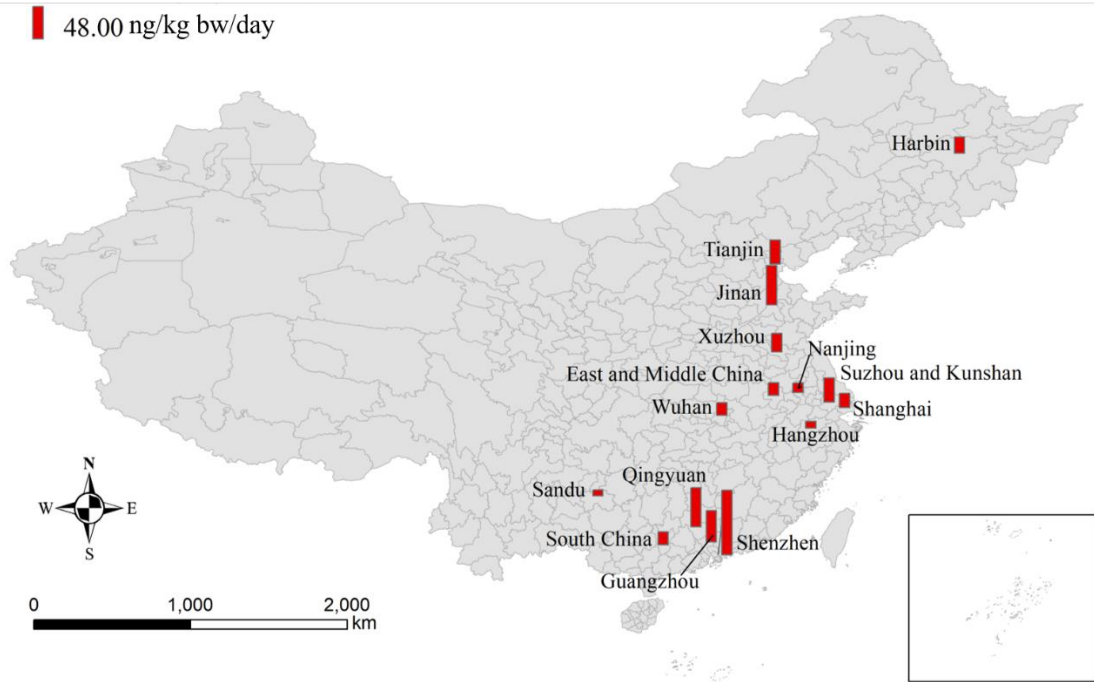
Figure 3-1 Average EDI of BPA for adults in different cities in China
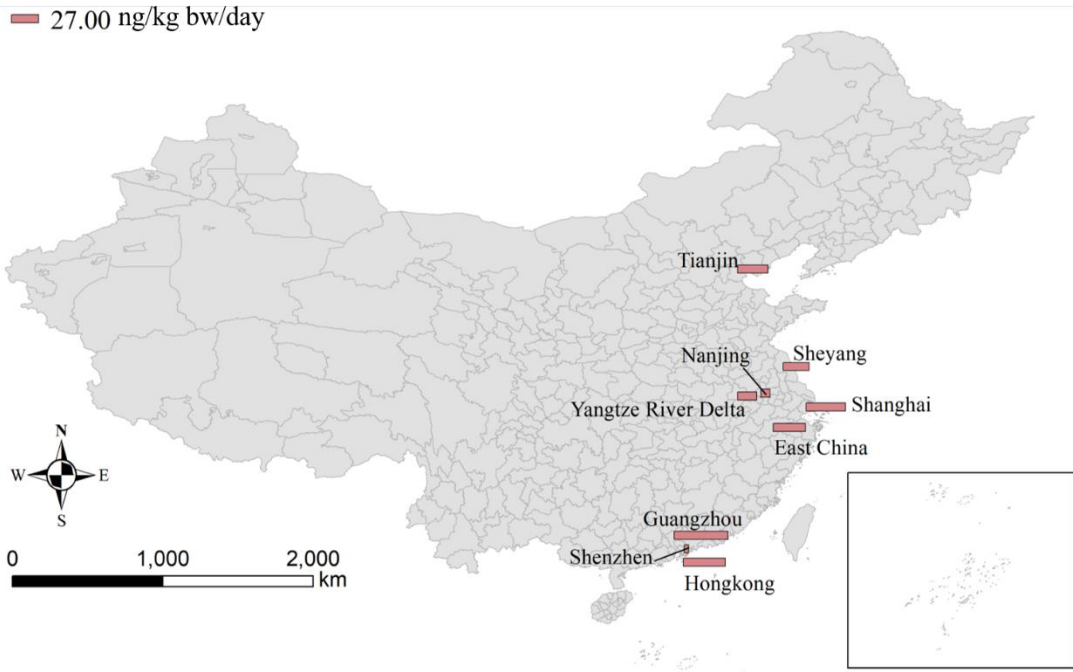
Figure 3-2 Average EDI of BPA for children in different cities in China
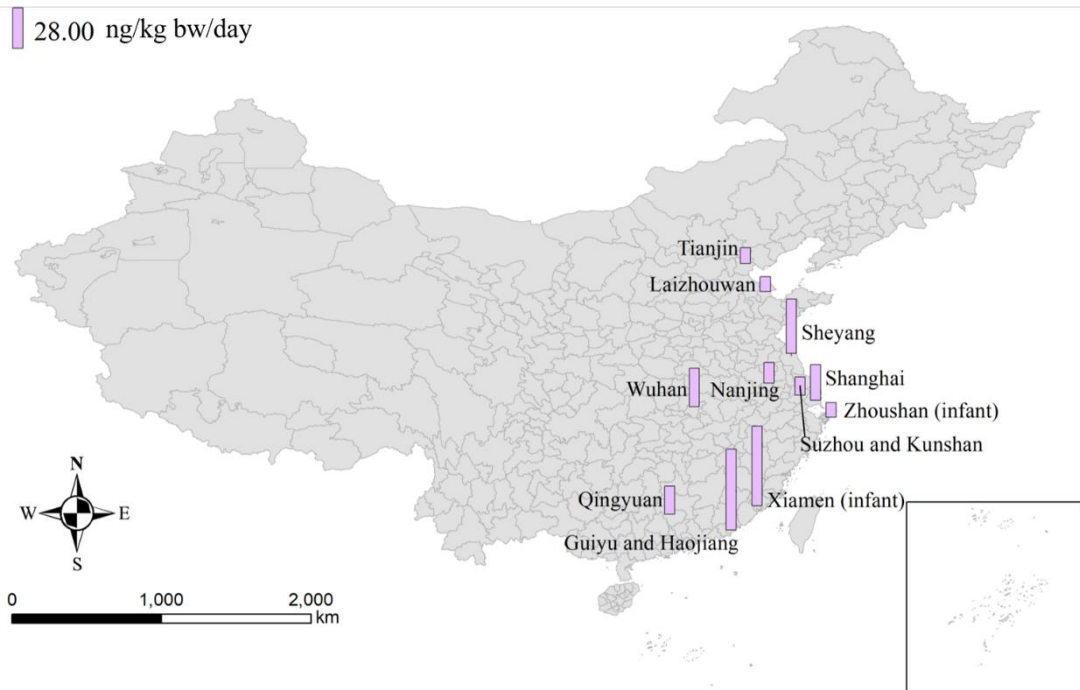
Figure 3-3 Average EDI of BPA for pregnant women and infants in different cities in China

There was a significant variation in BPA exposure between the different Chinese regions, and the reasons for this geographic difference are complicated. Dietary exposure is considered the main source of BPA exposure. A study investigated the BPA concentrations in foodstuffs from nine cities in China. The results showed that the average estimated dietary intake of BPA from food was 489 ng/kg bw/day for Chinese adults [34]. This result is higher than that estimated from the urinary data. Another study measured BPA concentrations in urine samples collected from residents living in and around e-waste dismantling facilities and people in a rural reference area and an urban reference area in China. The urinary BPA concentrations (geometric mean) of people from e-waste recycling, urban, and rural sites were 2.99, 0.952, and 0.589 ng/mL, respectively. The results indicated that e-waste dismantling increased BPA exposure levels in residents living in e-waste recycling areas [35]. Our results also showed that Sandu, a rural area in China, had the lowest BPA exposure for adults, and Guiyu, one of the largest e-waste recycling centers in China, had the highest BPA exposure among pregnant women. Exposure level is significantly related to the environment in which people live. To clarify the reasons for the differences in exposure levels in different regions, it is necessary to investigate the sources of BPA exposure to obtain more data.

3.3.3. BPA exposure risk of Chinese population

To assess BPA exposure risk in the general Chinese population, the HQ values of BPA exposure were calculated and are listed in Table 3-1. Overall, the ranges of HQ of different regions in China were 0.0001–0.0019 (results based on RfD) and 0.0012–0.0240 (results based on t-TDI). The HQ of BPA exposure among the four populations was two to four orders of magnitude lower than the recommended exposure limit prescribed by the United States Environmental Protection Agency and the European Food Safety Authority. This result indicated that the Chinese population was not at a high risk of BPA exposure.

However, the highest EDI for the adult, child, pregnant woman, and infant groups were

3419.47, 14807.69, 7710.60, and 2739.17 ng/kg bw/day, respectively, and the corresponding HQ values were 0.85, 3.70, 1.93, and 0.68, respectively (results based on t-TDI). It is worth noting that there is a risk of individual exposure to high levels of BPA, which may increase risks for children and pregnant women. Figure 3-4 shows the regions with the highest HQ exceeding one for children and pregnant women. Moreover, the average EDIs of the infant and child groups were relatively high compared with those of adults. Animal studies shown that exposure to BPA during the critical developmental period may cause irreversible effects and permanent damage in adults [36-37]. Therefore, special attention needs to be paid to the exposure of these sensitive populations, as they are more vulnerable to BPA exposure.
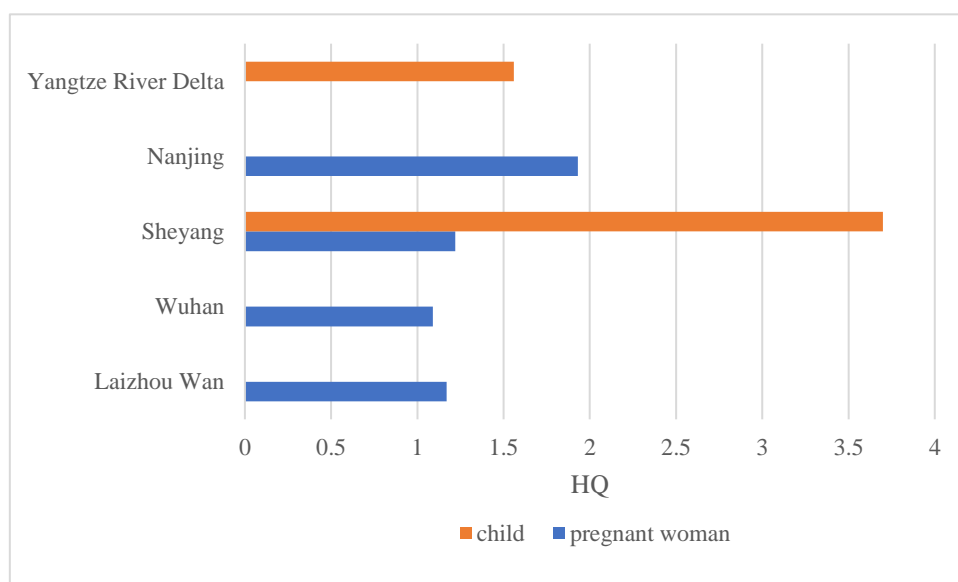
Figure 3-4 Regions with HQ values above 1 (HQ was calculated based on t-TDI)

Table 3-1 HQ of BPA exposure in China

Infant								
Region	Zhoushan	Xiamen						
HQ	**0.0002**/*0.0026* [a]	**0.0011**/*0.0139*						
Pregnant woman								
Region	Laizhou Wan	Suzhou and Kunshan	Wuhan	Sheyang	Qingyuan	Shanghai	Guiyu and Haojiang	Nanjing	Tianjin
HQ	**0.0002**/*0.0026*	**0.0003**/*0.0031*	**0.0005**/*0.0067*	**0.0008**/*0.0095*	**0.0004**/*0.0049*	**0.0005**/*0.0062*	**0.0011**/*0.0141*	**0.0003**/*0.0036*	**0.0002**/*0.0028*
Child								
Region	Guangzhou	Yangtze River Delta	Shanghai	Shenzhen	East China	Nanjing	Tianjin	Hongkong	Sheyang
HQ	**0.0011**/*0.0136*	**0.0004**/*0.0048*	**0.0008**/*0.0100*	**0.0001**/*0.0012*	**0.0007**/*0.0081*	**0.0002**/*0.0023*	**0.0006**/*0.0076*	**0.0008**/*0.0105*	**0.0005**/*0.0065*
Adult								
Region	Shanghai	Tianjin	Qingyuan	Suzhou and Kunshan	Harbin	Guangzhou	Wuhan	Jinan	Sandu
HQ	**0.0004**/*0.0052*	**0.0007**/*0.0089*	**0.0012**/*0.0146*	**0.0007**/*0.0090*	**0.0005**/*0.0060*	**0.0009**/*0.0116*	**0.0004**/*0.0047*	**0.0012**/*0.0146*	**0.0002**/*0.0022*
Region	East and middle China	Shenzhen	Nanjing	Xuzhou	Hangzhou	South China			
HQ	**0.0004**/*0.0048*	**0.0019**/*0.0240*	**0.0003**/*0.0035*	**0.0005**/*0.0069*	**0.0002**/*0.0026*	**0.0004**/*0.0049*			

a: Bold words indicate HQ calculated based on RfD, and italicized words indicate HQ calculated based on t-TDI.

3.3.4. Limitations and implications

Collecting and analyzing literature databases is a time- and labor-saving method for summarizing the profile of BPA exposure comparing with a nationwide survey. However, the small number of tested urine samples and the limitations of the sampling area hindered a comprehensive analysis. This may result in deviations from the actual situation. In this study, the sample size of eligible studies ranged from 15 to 3423, with sample sizes for infant, pregnant woman, child, and adult groups being 88, 9163, 5905, and 16655, respectively. The results obtained from a small sample size may be considered unrepresentative and should be interpreted cautiously. Some studies reported that a minimum sample size of 1000 was an acceptable criterion for obtaining human biomonitoring data that is nationally representative [38]. However, in this study, the main sampling locations of the eligible literature were concentrated in the eastern coastal area, especially in economically developed areas. In the central, southwest, northwest, and northeast regions, little or no data are available. To achieve an accurate analysis of BPA exposure in the whole country, additional BPA concentration data must be collected in these regions. Some countries such as the United States, Canada, and Korea have conducted screening studies to monitor BPA concentrations in the general population throughout the whole country [39]. These nationwide, continuous monitoring provides data not only for assessing the national exposure risk but also for evaluating the time changes in exposure level. Furthermore, we are able to analyze the impacts of BPA prohibition policies or actions using these data [31]. Besides, comprehensive testing of BPA in environmental media and human biomonitoring metrics with sensitive analytical techniques is required to define the BPA exposure profile as well as to identify and address the sources of BPA exposure in the general population. Continuous testing of BPA urinary concentration is especially important to evaluate long-term exposure risk. The decision-making agency should consider establishing systematic monitoring of BPA in both exposure sources and human biological samples.

The health-based guidance values of BPA were initially deduced based on the reduced mean body in rats (RfD) or the increased relative mean kidney weight in mice (t-TDI) [40]. However, the above toxic endpoints failed to include information on the toxic effects of BPA on other body system especially endocrine effects. There are animal studies showing the adverse effects of BPA on multiple body systems including reproductive, neurological, and endocrine systems even at exposure levels 1 – 4 magnitudes of order lower than the current LOAEL of 50 mg/kg bw/day [41]. The endocrine effects may be more sensitive than the effects on organ weights [42]. A single endpoint is limited to provide a comprehensive overview of the systemic toxic effect of a chemical, which introduces uncertainty into the risk assessment. In the future, an efficient, systematic assessment method is need to assess the associated risks of EDCs.

3.4. Conclusion

The Chinese population is widely exposed to BPA, and exposure levels vary greatly between regions. The average exposure levels in different regions indicate that the Chinese population is at a safe exposure level. It is worth noting that the average exposure level of children and pregnant women is higher than that of adults, and the highest HQ of children and pregnant women in some regions exceeds one. These findings indicate that it is important to focus on the risk of BPA exposure by sensitive and highly exposed populations.

Supplementary material

Table S1 Urinary concentration data of BPA in China.

Table S2 Daily urinary excretion volume and body weight used in calculation.

Table S3 Urinary concentration data of BPA in different countries.

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Chapter 4.   Risk assessment of EDCs by integrating ToxCast data, Adverse Outcome Pathway and machine learning

4.1. Introduction

EDCs are ubiquitous in various environmental media and have been reported to cause adverse human health outcomes such as obesity, diabetes, breast cancer, and infertility [1-3]. However, the complexity of the mechanism of EDCs (activate/antagonize hormone receptors, alter hormone receptor expression, alter signal transduction, etc.) makes it challenging to evaluate the holistic risk [4]. Besides, the necessity of emerging EDCs risk evaluations and the unavailability of data present challenges for EDC regulation as well [5]. Therefore, EDC risk evaluation in the 21st century requires more robust, informative and integrated evaluation methods to solve problems efficiently. Some organizations have introduced the integrated approaches for testing and assessment concept to provide a platform and methodology for chemical risk assessment, such as Toxicity Forecaster (ToxCast) program developed by the United States Environmental Protection Agency (EPA) and adverse outcome pathway (AOP) framework proposed by the Organization for Economic Cooperation and Development (OECD) [6-8].

An AOP is defined as a linear pathway composed of a Molecular Initiating Event (MIE), Key Events (KEs), and Adverse Outcomes (AOs) causally linked together [9]. The AOP network was applied in profiling the mechanistic toxicological relationships between chemical exposure and adverse outcomes, indicating alternative biomarkers for predicting diseases, screening and prioritizing contaminants [10-12]. However, there are many knowledge gaps regarding the application of AOP in chemical risk assessment. 1) a single MIE is difficult to integrated evaluate the overall risk of chemicals; 2) simple method is not available for quantifying the MIEs of chemicals; 3) there is no systematic analysis model for the prediction of AOs. Improving speed and accuracy of AOP-based

risk assessment can be achieved by combining *in silico* and *in vitro* models to meet the regulatory needs of large volumes of chemicals [13-14].

The development of toxicological databases and computational toxicology methods provides the basis and tools for chemical risk assessment. The ToxCast database, which contains toxicological pathway information of chemicals derived from high-throughput *in vitro* assays, provides a basic resource for predicting the MIEs of chemicals [15-16]. The Toxicity Reference Database (ToxRefDB), which stored legacy *in vivo* data from animal toxicity studies, provides an informative resource to validating the predicted AOs of chemicals [17-19]. Additionally, the Toxicological Priority Index (ToxPi), a tool for presenting toxicological profiles of chemicals based upon formal integration across multiple domains of information, makes it possible to quantify and rank the MIEs based on ToxCast data [20]. Machine learning has been advocated as a new idea in the field of environmental science and engineering to help analyze increasing data and overcome limitations of conventional analytical methods [21]. Recently, a knowledge-based deep neural network approach was applied in EDC identification by using AOP framework to mimic the signaling pathway initiated by ERα based on high-throughput screening data [22]. Random walk is one of machine learning methods that are widely used in mRNA-gene-disease network analysis and prediction [23]. The similarity of the structure and analysis purpose between mRNA-gene-disease network and AOP network makes it possible to apply random walk in AOP network analysis and prediction.

In this study, the MIEs of 40 EDCs were selected and quantified according to the ToxCast database and ToxPi score, the AOs of 40 EDCs were speculated based on AOP networks. For the first time, a holistic AOP network covering different MIEs and AOs of EDCs was established. The toxicities of different EDCs were predicted by AOP network, combined with random walk and quantification of MIEs and validated through *in vivo* assay results from the ToxRefDB database. The developed *in silico* risk assessment approach provided a powerful tool for health risk analysis of concern EDCs

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and has great potential in practical use for chemical regulation.

4.2. Materials and Methods

4.2.1. Study design

The framework of this study was described in Fig. 4-1. To build an AOP network for predicting the AOs of EDCs, we first analyzed the information of EDC-related bioassay through the ToxCast database to determine the EDC-related MIEs. According to the determined MIEs to collect downstream KEs and AOs information through AOP-Wiki and construct AOP network to predict the relationship between EDCs and AOs. In order to predict the toxicity of EDCs, first we depicted the effect of each EDC on MIEs using ToxPi score, then we analyzed the probability of each AO in AOP network using random walk method, and finally we predicted the toxicity of EDCs by combining EDC-MIE ToxPi matrix and MIE-AO probability vector. The predicted EDC toxicities were confirmed using the results of animal experiments obtained from ToxRefDB.

4.2.2. EDCs selection

In 2012, IPCS/WHO published a report about state of the science of endocrine disrupting chemicals including a table listed known or potential EDCs [2]. In 2017, IPCS published a report about worldwide initiatives to identify EDCs and potential EDCs, which concluded selection criteria and chemical information of EDCs for various stakeholders (governments, industry, civil society and academia) [24]. Based on these two reports, 40 EDCs were selected for the next step of the study considering their physicochemical properties and applications. The information of selected EDCs is summarized in Table 4-1.
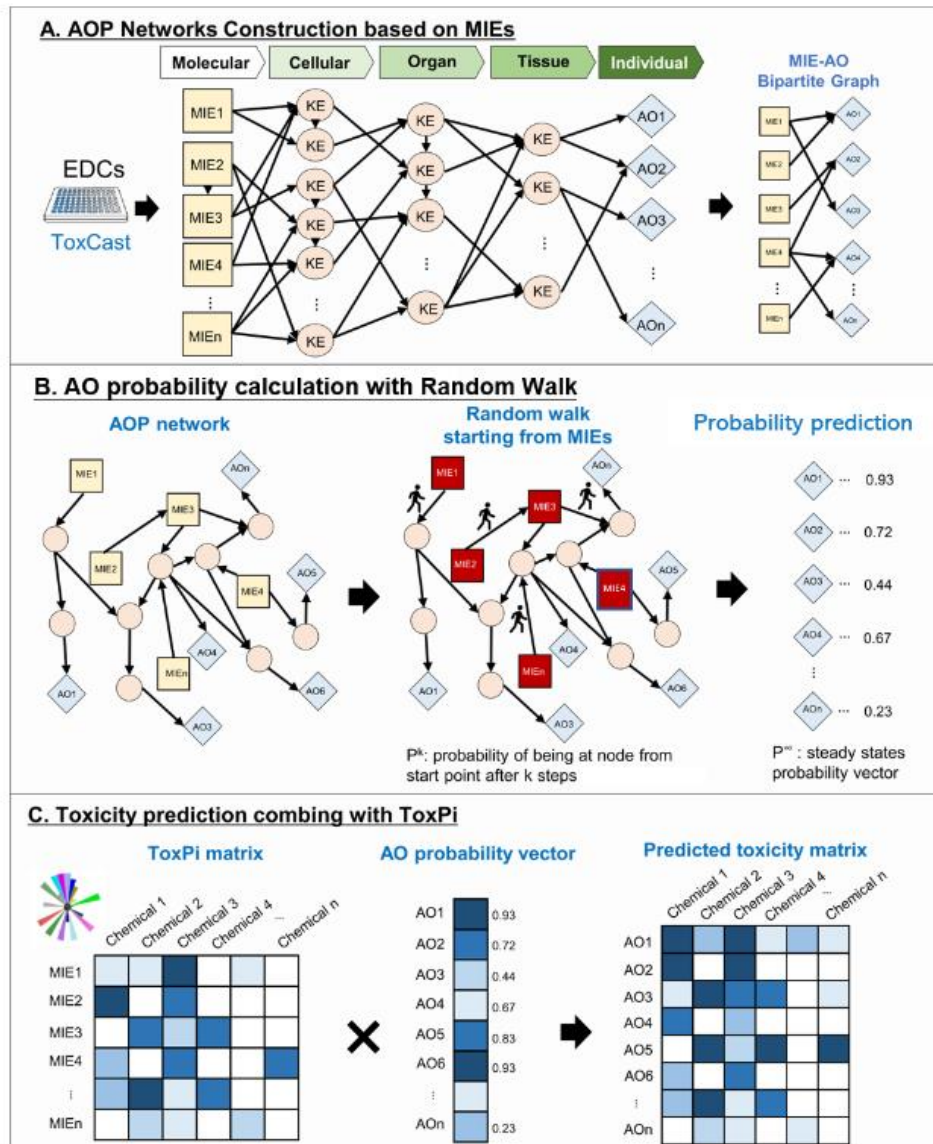
Figure 4-1 Study framework of this study

Table 4-1 Information of selected EDCs

Name	Abbreviations	DTXSID	CASRN	Application
2,3,7,8-Tetrachlorodibenzo-p-dioxin	TCDD	DTXSID2021315	1746-01-6	Pesticide
Atrazine	ATZ	DTXSID9020112	1912-24-9	Pesticide
Chlordane	CHL	DTXSID7020267	57-74-9	Pesticide
Dichlorodiphenyltrichloroethane	p,p'-DDT	DTXSID4020375	50-29-3	Pesticide
Endosulfan	ES	DTXSID1020560	115-29-7	Pesticide
p,p'-DDD	p,p'-DDD	DTXSID4020373	72-54-8	Pesticide
Pentachlorophenol	PCP	DTXSID7021106	87-86-5	Pesticide/Disinfectant
Triphenyltin chloride	TPT	DTXSID2040733	639-58-7	Pesticide
Vinclozolin	VZ	DTXSID4022361	50471-44-8	Pesticide
Ziram	ZI	DTXSID0021464	137-30-4	Pesticide
4-Nonylphenol	4-NP	DTXSID5033836	104-40-5	Industrial production
2,2',4,4',5,5'-Hexachlorobiphenyl	PCB-153	DTXSID2032180	35065-27-1	Industrial production
2,4-Dihydroxybenzophenone	2,4-DHBP	DTXSID8022406	131-56-6	Industrial production
3,3',5,5'-Tetrabromobisphenol A	TBBPA	DTXSID1026081	79-94-7	Industrial production
4-Nitrophenol	PNP	DTXSID0021834	100-02-7	Industrial production
4,4'-Sulfonyldiphenol	BPS	DTXSID3022409	80-09-1	Industrial production
Benzo(a)pyrene	B(a)P	DTXSID2020139	50-32-8	Industrial production
Benzyl butyl phthalate	BBP	DTXSID3020205	85-68-7	Industrial production
Bis(4-hydroxyphenyl)methane	BPF	DTXSID9022445	620-92-8	Industrial production
Bisphenol A	BPA	DTXSID7020182	80-05-7	Industrial production
Bisphenol AF	BPAF	DTXSID7037717	1478-61-1	Industrial production

Di(2-ethylhexyl) phthalate	DEHP	DTXSID5020607	117-81-7	Industrial production
Perfluorooctanesulfonic acid	PFOS	DTXSID3031864	1763-23-1	Industrial production
Perfluorooctanoic acid	PFOA	DTXSID8031865	335-67-1	Industrial production
Tributyltin chloride	TBT	DTXSID3027403	1461-22-9	Industrial production
Triclosan	TCL	DTXSID5032498	3380-34-5	Industrial production
Triphenyl phosphate	TPPA	DTXSID1021952	115-86-6	Industrial production
Diethylstilbestrol	DES	DTXSID3020465	56-53-1	Pharmaceutical
Fluoxetine	FLX	DTXSID7023067	54910-89-3	Pharmaceutical
Levonorgestrel	LNG	DTXSID3036496	797-63-7	Pharmaceutical
Butylparaben	SPF	DTXSID3020209	94-26-8	Cosmetic
Decamethylcyclopentasiloxane	D5-sil	DTXSID1027184	541-02-6	Cosmetic
Enzacamene	4-MBC	DTXSID8047896	36861-47-9	Cosmetic
Methylparaben	MP	DTXSID4022529	99-76-3	Food additive
4-(1,1,3,3-Tetramethylbutyl)phenol	tOP	DTXSID9022360	140-66-9	Food additive
Zearalenone	ZEN	DTXSID0021460	17924-92-4	Phytoestrogen
Genistein	GEN	DTXSID5022308	446-72-0	Phytoestrogen
Testosterone	TES	DTXSID8022371	58-22-0	Natural Hormone
17alpha-Ethinylestradiol	EE2	DTXSID5020576	57-63-6	Natural Hormone
17beta-Estradiol	E2	DTXSID0020573	50-28-2	Natural Hormone

4.2.3. MIE identification and quantification

4.2.3.1. Data collection from ToxCast database

Currently, the ToxCast Phases I and II programme uses over 800 assays to test 1,858 chemicals. The assays include cell-free biochemical *in vitro* assays and cell-based *in vitro* assays with multiple human primary cells, human or rodent cell lines, and rat primary hepatocytes. A wide range of biological targets or effects of chemical interactions were tested, including cytotoxicity, cell growth, genotoxicity, enzymatic activity, receptor binding, reporter gene activity (mostly nuclear receptors), ion channels, and transcription factor activity [25]. The ToxCast program developed a novel R extension, ToxCast pipeline (tcpl), to identify potentially active compounds and to estimate the potency and efficacy through dose-response modeling [26]. All data is available at the ToxCast dashboard (CompTox Chemicals Dashboard. https://comptox.epa.gov/dashboard/). On the search results page of each chemical, the ToxCast: summary option in the bioactivity folder is available for exporting results about the *in vitro* assays. In this study, we focus on gene target assays which may link to MIE in AOP. The following information were collected: the names of active assays (Hit Call: active), related gene symbol, related AOPs and events, and AC50 (activity concentration at 50% of maximal activity) value of the search results of each selected chemical in June 2021. The details of each assay were obtained from details—annotations page including assay component endpoint name, analysis direction (fitting direction), tissue, cell format, cell short name. If an assay was labeled "use data with caution", the data from that assay are excluded from the analysis. After the data were compiled, the genes that can be searched in AOP database of 40 EDCs were identified as MIEs (supplementary material Figure S1). In order to analyze the risk of EDC comprehensively as possible, genes that were affected by more than 20% of 40 EDCs were selected as overlap MIEs for AOP network construction in this study.

4.2.3.2. ToxPi construction

In order to profile and quantify the effects of EDCs on MIEs, ToxPi score, a dimensionless index score, is calculated for each EDC as a weighted combination of all data by rational integration and normalization of *in vitro* information. Visually, ToxPi chart consists of a bunch of slices forming a unit circle, each slice represents a piece of information. The width of the slices represents the relative weight of this piece of information in the overall ToxPi calculation, and the distance from the center of the circle is proportional to the normalized value of this piece of information. A tool ToxPi Graphical User Interface (GUI) is developed to help data analysis and visualization and is freely-available (http://toxpi.org.) [27]. In this study, each slice of the ToxPi chart is composed of a collection of *in vitro* assays that share the same MIE and effect (agonism/antagonism that decided from each assay detail: analysis direction), such as ER-agonism. Scores and rankings were generated by summing and normalizing the AC50 values of the assays within each slice for each chemical as shown in equation below [20]. For any chemical where the AC50 was not applicable, the AC50 for that particular assay was set to NA (not available). All slices are equally weighted so that each slice has the same potential contribution to the overall ToxPi score.

For each chemical,

$$Normalized\ slide\ score = \left. \sum\nolimits_{MIE-effect} chemical \middle/ max \sum\nolimits_{MIE-effect} \right.$$

$$ToxPi\ score = \sum\nolimits_{i=MIE}(weight\ i \times slide\ score\ i) \times C$$

Where, $\sum_{MIE-effect} chemical$ means sum the AC50 across all component assays in that slice for each individual chemical. $max \sum_{gene-effect}$ means maximum $\sum_{gene-effect} chemical$ of that slice across all selected chemicals. Weight i and slide score i mean weighting and normalized slide score of a MIE i. C means a default value is provided by ToxPi GUI to normalize the ToxPi score to the [0, 1] interval.

4.2.4. Development of the Adverse Outcome Pathway

The AOP-Wiki contains AOPs describing toxicological and ecotoxicological pathway from stressor-induced events to outcome events. We conducted a data collection from the AOP-Wiki database in July 2021. The name and aliases of identified MIEs were searched and MIEs and KEs related to the search terms in the results were selected. The ToxCast database and the AOP-Wiki database have slightly different terminology for MIE. When match MIE between two databases, we take AOP-Wiki terminology as the standard. For example, ESR1, ESR2A and ESR2B in ToxCast were grouped in ER in AOP-Wiki. AOPs related to these selected MIEs/KEs were chosen to analysis. Information retrieved from AOP-Wiki includes: AOP identifier, AOP title, taxonomic applicability, sex applicability, each KE in AOP, KE identifier, KE name, KE type, biological organization, level of biological organization. For each key event relationship (KER) in an AOP, we captured information including the KER identifier, upstream KE, downstream KE, weight of evidence (WoE), and adjacency. The KE type can be either molecular initiating event (MIE), key event (KE) or adverse outcome (AO). Repeated AOPs and incomplete AOPs were removed from the analysis. Information that is not available is marked as not available (NA). Each AOP can be viewed as a network wherein the nodes are KEs and directed edges are KERs linking upstream KEs with downstream KEs. Several AOP networks were created by connecting the KEs according to the KERs and WoE of KERs (low = 1, median = 2, high = 3, NA = 1) starting from the MIEs and ending at the AOs. In this study, only mammalian AOPs were selected for the construction of AOP network for reducing species differences and evaluating human health risks.

4.2.5. ToxRefDB data collection

In order to validate new *in vitro* and *in silico* approaches, the Toxicity Reference Database (ToxRefDB) were developed to capture information from *in vivo* toxicity

studies. Currently, ToxRefDB version 2.0 was developed. ToxRefDB contains over 400 endpoints derived from over 5900 *in vivo* toxicity studies including chronic, subchronic, subacute, developmental, reproductive, multigenerational reproductive, developmental neurotoxicity, and other toxicity studies. Study species cover rats, mice, dogs and rabbits. Benchmark Dose (BMD) Modeling Software was used to generate results of dose-response modeling of nearly 28,000 datasets. The critical effect levels (LOAELs, NOAELs) of toxicity studies were extracted and stored [28]. All data is available at the ToxRefDB MySQL database (https://github.com/USEPA/CompTox-ToxRefDB). We collected information on *in vivo* assays of 40 EDCs in August 2021. Assays with AO-related endpoints and corresponding LOAELs (with unit mg/kg/day) were extracted. To maintain consistency of data, values were collected only from studies conducted with mice and rats. Due to the limitation of the data, not all chemicals were available for LOAELs. The unavailable LOAEL for particular endpoint was set to NA (not available).

4.2.6. Data calculation

4.2.6.1. AO probability calculation

After constructing the AOP network, we use a machine learning method called random walk to predict the possibility of AOs. The idea of the random walk in AOP network is that a walker begins at the set of seed nodes (MIEs) and moves by a succession of random steps to KEs in AOP network, traveling via the MIE-KE-AO interaction and KER edges. Random walk calculates the probability of a walker reaching each node in the network. In this case, we applied one of the classical random walk algorithms, PageRank algorithm to calculate the probability of events in AOP network. AO with a high PageRank (PR) score indicates a high probability of AO occurrence in AOP network. The algorithm is shown in the following equation.

$PR(i)=(1-d)/N+d\Sigma(PR(Ti)/C(Ti))$

Where PR(i) is the PageRank score of node i. PR(Ti) is the PageRank score of node Ti, which is a node among all nodes pointing to node i. C(Ti) is the out-degree of node Ti, that is, the number of edges of Ti pointing to other nodes. N is the total number of nodes. d is the damping factor, i.e., the probability that the walker will continue to travel after reaching a node, and usually d defaults to 0.85. The initial total PageRank scores of all nodes are set as 1 and each node has an equal PR score 1/N, and after a series of iteration processes, the PageRank scores of all nodes converge to a stable state PR^{∞}, that is the final PageRank score.

4.2.6.2. Normalization of PR scores

Note that the PR scores form a probability distribution over events in an AOP network, so the sum of all PR scores of an AOP network will be one. The PR score becomes smaller as the number of nodes in the AOP network increases. To make the results obtained from AOP networks with different sizes comparable, a min-max data scaling method was used to normalize the PR scores of the EDC-related AOP networks. The min-max algorithm scales PR scores in an AOP network in the interval of [Xmin, Xmax] to [0, 1].

V' = (V – Xmin)/(Xmax – Xmin)

Here, Xmin and Xmax are the minimum and the maximum PR score in an AOP network. V and V' are the PR score of each event in an AOP network before and after scaling.

4.2.6.3. Toxicity prediction

The toxicity of AOs for EDCs was predicted by combining the potential of EDCs to affect MIEs and the possibility of the induced AOs. In this study, the potential of EDCs to affect MIEs were compiled into the EDC-MIE ToxPi score matrix and the possibility of AOs were compiled into the MIE-AO PR score vector. Therefore, EDC toxicity of each AO (ToxAO) was calculated by equation below.

$$\begin{bmatrix} ToxAO\ 11 & ToxAO\ 12 & ToxAO\ 1y \\ ToxAO\ 21 & ... & ... \\ ToxAO\ z1 & ... & ToxAO\ zy \end{bmatrix} = \begin{bmatrix} ToxPi\ 11 & ToxPi\ 12 & ToxPi\ 1y \\ ToxPi\ 21 & ... & ... \\ ToxPi\ x1 & ... & ToxPi\ xy \end{bmatrix} \times \begin{bmatrix} PR\ 11 \\ ... \\ PR\ xz \end{bmatrix}$$

$x \in X$, $a\ set\ of\ MIEs$; $y \in Y$, $a\ set\ of\ EDCs$; $z \in Z$, $a\ set\ of\ AOs$.

If the data is not available it was set to NA. To validate the results of toxicity prediction, ToxRefBD *in vivo* assay data were used to compare with predicted results. Due to limitations of data, only data for 26 EDCs are available in ToxRefDB. It is difficult to link the AOs and the endpoints of *in vivo* assays individually. Therefore, AOs were integrated to match with endpoints of *in vivo* assay. Here, the toxicity of EDC is classified as development toxicity, reproductive toxicity, immunotoxicity, hepatotoxicity, pulmonary toxicity, neurotoxicity, cancer and tumor, and others. The information of the AOs and endpoints integrated in each toxicity is shown in supplementary material Table S4. And the integrated toxicity was calculated by following equation.

For each EDC y,

Toxicity score $_m$ = $\sum(ToxAO\ z')$

$z' \in Z'$, a set of AOs including in each toxicity m.

4.2.6.4. Toxicity validation and model performance evaluation

The model from this study computed a toxicity score that each EDC will show a specific toxicity. An EDC was considered toxic when its calculated toxicity score exceeded a certain threshold. The toxicity predictions were evaluated using the area under the receiver-operating curve (ROC) metric (AUC). In this case, to draw a ROC, within the range of predicted results, a series of toxicity score thresholds are set to distinguish toxic and non-toxic EDCs. After that, the results were compared with *in vivo* assay results to calculate true-positive rate (TPR, following equation) and false positive rate (FPR, following equation). The ROC for model performance is a plot of the TPR and

FPR obtain using various toxicity thresholds. After ROC curve analysis, the AUC represents the total model likelihood of correctly classifying compounds as toxic or non-toxic. An AUC value of 0.5 represents random classifications, and an AUC value of 1 represents 100% predictivity [29]. The Yorden index represents the ability of the classification model to find toxic and non-toxic EDCs. The larger the Yorden index, the better the classification model performance. On the ROC curve, the threshold corresponding to the maximum Youden index (following equation) is determined as the optimal threshold to distinguish toxic and non-toxic EDCs. The input data of ROC construction including a set of EDC-predicted toxicity scores and EDC-ToxRefDB toxicity values. Endpoints from ToxRefDB were classified based on AO integration (Table S4) for each toxicity. The LOAELs of related endpoints classified in the same toxicity group were averaged. An EDC was defined as non-toxic and labeled with N when the average LOAEL of a toxicity > 500 mg/kg/day. Otherwise, it was considered toxic and labeled as Y. These data constitute the set of EDC-ToxRefDB toxicity values. Due to limitations of data in ToxRefDB, only hepatotoxicity and reproductive toxicity with sufficient data for AUC calculation. Data that is not available is set to NA (not available).

$$TPR = \frac{true\ positives}{true\ positives + false\ negatives}$$

$$FPR = \frac{false\ positives}{false\ positives + ture\ negtives}$$

Youden index = TPR - FPR

4.2.7. Statistical analysis

The AOP network construction and ROC construction were performed using Orange 3 (Bioinformatics Lab at University of Ljubljana, Slovenia). The ToxPi score calculation and visualization were performed using ToxPi GUI (http://toxpi.org.). The random walk and Min-Max data scaling were performed using Python 3 (Python Software

Foundation). The principal component analysis (PCA) was performed using SIMCA 16 (Sartorius Stedim Biotech), and the graphs were constructed using Prism 9 (GraphPad Software, Inc.).

4.3. Results and discussion

4.3.1. MIEs prioritization for EDCs based on ToxCast data

By analyzing the *in vitro* assay data collected from the ToxCast database, we determined and prioritized the overlapping MIEs for 40 EDCs. In the ToxCast database, different in vitro assays (different exposure times, different cell lines, etc.) may have the same gene target, so a chemical may have several active *in vitro* assays testing the same gene target. Heatmap of active *in vitro* assays of 40 EDCs in ToxCast database is shown in supplementary material Fig. S1. The characteristics of the genetic impact of each EDC are different. Among them, PFOS, DES, TBT, PCP, BPA and BPAF affect more genes, compared to other EDCs. Some pesticides also show a higher potential to affect genes, for example ATZ, CHL, ES, ZI, DDD and DDT. Except for industrial chemicals and pesticides, other chemicals show lower genetic impact such as pharmaceuticals (FLX, LNG), cosmetics (SPF, D5-sil, 4-MBC), food additives (MP), phytoestrogens (ZEN), natural hormone (TES), etc. However, there are some exceptions, such as E2, EE2 and GEN. These characteristics is consistent with the distribution of the 40 EDCs on the PCA plot (Fig. 4-2 A). It is worth noting that some chemicals have limited total *in vitro* assays performed due to high volatility or low solubility and other reasons, resulting in limited active *in vitro* assay data (Fig. 4-2 B). This may influence the final MIE selection.

In total, 40 EDCs affected 355 genes (Fig. S1). 28 of these genes are included in the AOP-Wiki database (Fig. 4-3). We ranked these genes based on the active *in vitro* assay information and selected genes that affected by more than 20% of the 40 EDCs as overlapping genes for linking MIEs in AOP-Wiki database. 20 MIEs were selected to

build the AOP network. According to the terminology in the AOP-Wiki database, some genes with the same MIE name were grouped together, e.g., ESR1, ESR2, ESRRA were combined into ER. Notably, some of the overlapping genes affected by more than 50% of the 40 EDCs are not included in the AOP-Wiki database. Future investigation and development of MIE and AOP information related to these genes in the AOP-Wiki database could support a better understanding of EDC toxicity, including CYP2C19, TP53, CYP2B6, CCL2, CSF1, HLA-DRA, RARA, CXCL8, CYP3A4, PLAUR, VCAM1, CD40, CYP1A1, HIF1A, SELE, TNF and VDR (Fig. S1).
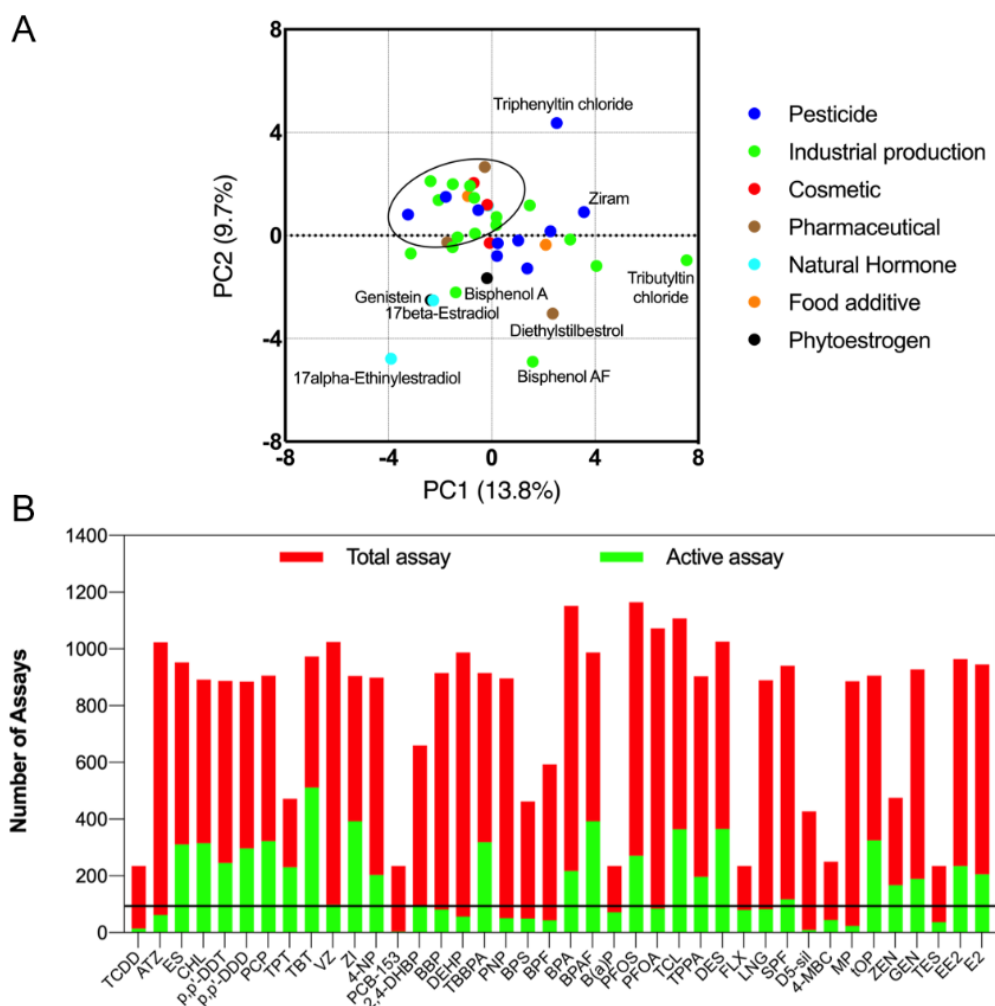
Figure 4-2 PCA of 40 EDCs based on active *in vitro* assays (A) and distribution of total assay and active assay of 40 EDCs (B) (A: The total number of *in vitro* assays for the chemicals in the black circles is less than 100.)

Figure 4-3 MIEs contained in the AOP-Wiki database extracted from the ToxCast database

4.3.2. AOP network construction for 40 EDCs

We created AOP networks for 40 EDCs by analyzing the data collected from the AOP-Wiki database. After data collation, a total of 48 AOPs met the data selection criteria, including 22 MIEs associated with overlapping genes, 39 AOs and 164 KEs associated with selected MIEs (Table 4-2). In the AOP-Wiki database, a stressor may promote or inhibit gene expression. Therefore, when genes identified from the ToxCast database are linked to the MIEs of AOP, the MIEs are classified into two directions, agonism and antagonism. The MIEs induced by 40 EDCs covers estrogen receptor, androgen receptor, peroxisome proliferator activated receptor, thyroid hormone receptor, thyroid peroxidase, aryl hydrocarbon receptor, genes of nuclear receptor subfamily, sterol regulatory element binding transcription factor, genes of cytochrome P450s family and kinase insert domain receptor, etc. The possible AOs caused by these MIEs cover tumors and cancers, developmental defects, immune diseases, liver lesions, obesity, pulmonary fibrosis, cognitive impairment, etc., involving multiple organs and biological systems of heart, liver, lung, breast, reproductive system, nervous system, and immune system, etc. (Table 4-3).

Table 4-2 AOP information related to selected MIEs of 40 EDCs

MIE(KE)	AOP No.	AO(KE) No.	Related organ	Number of event (MIE/KE/AO)								
				M*	C*	T*	O*	I*	U*	F*	M**	U*
ER_Agonism	314, 167, 200, 112	1714, 1070, 1193, 773	Immune system, Uterus, Breast	5	21	0	0	0	14	13	0	27
ER_Antagonism	165	1053, 1054	Ovary	0	2	0	0	0	8	10	0	0
AR_Agonism	117	719	Liver	0	2	0	0	0	2	0	0	4
AR_Antagonism	345, 344, 306, 19, 372, 111, 288, 307,305	972, 1786, 1688, 337, 1839, 745, 1616	Ovary, Breast, Penis, Reproductive system, Testis	3	6	0	0	0	18	0	23	4
PPARA_Agonism	166, 18, 37, 51	1063, 406, 348, 719	Liver, Pancreas, Penis, Reproductive system	2	12	0	7	1	0	0	11	11
PPARA_Antagonism	6, 36	864, 459	Liver, Development	4	4	0	5	1	0	0	0	14
PPARG_Agonism	72, 163, 34	1447, (1035, 1036, 1037), 345	Liver, (fibrous connective tissue, adipose tissue)	3	7	3	2	1	0	0	0	16
PPARG_Antagonism	206, 347	1276, 1458	Lung	0	5	0	3	0	0	0	0	8
THRB_Antagonism	300	402	Brain	0	1	0	3	1	0	0	0	5
TPO_Antagonism	42, 119	402, 741	Brain, Ovary	1	3	0	7	1	0	0	0	12
AHR_Agonism	57, 151, 41, 150, 131	455, 1893, 856, 947, 369	Liver, Placenta, Heart	5	10	0	12	1	0	7	0	21
NFE2L2_Agonism	61	459	Liver	4	1	0	0	0	1	0	0	6
NFE2L2_Antagonism	232	1418	-	7	0	0	1	0	0	0	0	8
KDR_Antagonism	43	1001	Development	2	0	0	0	0	3	0	0	5
SREBF1_Agonism	62	459	Liver	2	0	0	0	0	2	0	0	4
CYP2E1_Agonism	260, 220	1514, 1395	Liver, Brain	0	1	0	0	0	11	0	0	12
CYP19A1_Antagonism	7	405, 406	Ovary	0	2	0	0	0	3	5	0	0
NR1I2_Agonism	60	459	Liver	7	3	0	0	0	2	0	0	12
NR1I3_Agonism	107	719, 345	Liver	0	3	0	0	0	2	0	0	5
NR1I3_Antagonism	58	459	Liver	11	4	0	0	0	1	0	0	16
NR1H3_Agonism	34	345	Liver	7	4	0	0	0	1	0	0	12
NR3C1_Agonism	64, 318, 14	406, 459, 323	Liver, Testis, Immune system	4	5	0	0	0	9	0	7	11

M*: Molecular; C*: Cellular; T*: Tissue; O*: Organ; I*: Individual; U*: Unspecific; F*: Female; M**: Male.

Table 4-3 MIEs and AOs information

MIE	Organ	AO
AHR_Agonism	Heart	AO947: Increase, Early Life Stage Mortality
	Liver	AO455: Accumulation, Liver lipid
	Liver	AO856: Formation, Hepatocellular and Bile duct tumors
	Unspecific	AO1893: increase, Preeclampsia
	Unspecific	AO369: Uroporphyria
AR_Agonism	Liver	AO719: Increase, hepatocellular adenomas and carcinomas
AR_Antagonism	Breast	AO1786: increase, retained nipples in males
	Ovary	AO972: Decreased fertility, Reduced number of oocytes ovulated
	Penis	AO1688: short male AGD
	Penis	AO337: N/A, Impairment of reproductive capacity
	Testicle	AO1616: Malformation, cryptorchidism
	Testicle	AO745: Increase, Leydig cell tumors
	Testicle	AO1839: Testicular Cancer
CYP19A1_Antagonism	Ovary	AO405/406: impaired, Fertility/irregularities, ovarian cycle
CYP2E1_Agonism	Brain	AO1514: Neurodegeneration
	Liver	AO1395: Liver Cancer
ER_Agonism	Breast	AO1193: N/A, Breast Cancer
	Immune System	AO1714: Exacerbation of SLE
	Uterus	AO773: Increase, Endometrial adenocarcinomas
	Uterus	AO1070: Increased, adenosquamous carcinomas of endometrium
ER_Antagonism	Ovary	AO1053/1054: Promotion, ovarian granular cell tumors/Promotion, ovarian adenomas
KDR_Antagonism	Unspecific	AO1001: Increased, Developmental Defects
NFE2L2_Agonism	Liver	AO459: Increased, Liver Steatosis
NFE2L2_Antagonism	Liver	AO1418: Increased, steatosis
NR1I2_Agonism	Liver	AO459: Increased, Liver Steatosis
NR1I3_Agonism	Liver	AO345: N/A, Liver Steatosis
	Liver	AO719: Increase, hepatocellular adenomas and carcinomas
NR1I3_Antagonism	Liver	AO459: Increased, Liver Steatosis
NR3C1_Agonism	Immune System	AO323: Increased, Disease susceptibility
	Liver	AO459: Increased, Liver Steatosis
	Testicle	AO406: impaired, Fertility
NR1H3_Agonism	Liver	AO345: N/A, Liver Steatosis
PPARA_Agonism	Liver	AO719: Increase, hepatocellular adenomas and carcinomas
	Pancreas	AO1063: Increased, Pancreatic acinar tumors
	Testicle	AO406: impaired, Fertility/AO348: Malformation, Male reproductive tract
PPARA_Antagonism	Liver	AO459: Increased, Liver Steatosis
	Systemic	AO864: Decreased, Body Weight
PPARG_Agonism	Liver	AO1447: obesity

MIE	Organ	AO
	Liver	AO345: N/A, Liver Steatosis
	Unspecific	AO1035/1036/1037: Increased, hemagiosarcoma /Increased, Firbrosarcoma/Increased, liposarcoma
PPARG_Antagonism	Lung	AO1276: Lung fibrosis
	Lung	AO1458: Pulmonary fibrosis
SREBF1_Agonism	Liver	AO459: Increased, Liver Steatosis
THRB_Antagonism	Brain	AO402: Cognitive Function, Decreased
TPO_Antagonism	Brain	AO402: Cognitive Function, Decreased
	Ovary	AO741: Increase, Adenomas/carcinomas (follicular cell)

In this study, all the AOP events related to 40 EDCs are not connected into one network, but composed into six small networks. The AOP network is an information-rich network that illustrates the mechanism of how 40 EDCs cause adverse outcomes. For example, AOP 18 describes PPARA activation in utero leading to impaired fertility in males. Activation of PPARA leads to a decrease in steroidogenic acute regulatory protein resulting in a reduction in cholesterol transport in mitochondria, then cause a reduction in testosterone synthesis in Leydig cells and testosterone level, result in a decrease in translocator protein, and finally lead to malformation of the male reproductive tract and impairment of fertility.

Fig. 4-4 A shows the organ information of the AOP networks of 40 EDCs. Interestingly, blood is an important vehicle that may cause changes in several organs. For example, inhibition of thyroperoxidase (TPO) leading to decrease in thyroid hormone synthesis (KE 277) resulting in decrease of thyroxine (T4) in serum (KE 281). A decrease of T4 in the serum causes, on the one hand, a decrease of T4 in neuronal tissue, leading to alterations in hippocampal gene expression, hippocampal anatomy, hippocampal physiology, and ultimately, decreased cognitive function (AO 402). On the other hand, it causes an increase in thyroid-stimulating hormone, which leads to increase in hypertrophy and proliferation, hyperplasia of thyroid follicular cells, finally results in increase of adenomas/carcinomas of thyroid follicular cells (AO 741).

Figure 4-4 B illustrates the biological level information of the AOP networks of 40 EDCs. Compared to organ-level events, KEs are more frequent at the molecular and

cellular levels. For AOP 200, estrogen receptor activation leading to breast cancer (ER-agonism to AO 1193), a total of 22 events were included in this AOP, but 19 events were at the molecular and cellular level. The future development of AOP could focus more on tissue or organ level events. It provides comprehensive information not only for chemical risk assessment, but also for validation by linking to the apical endpoints of animal experiments. Since endpoints in animal studies are focused at the tissue and organ level.

Fig. 4-4 C depicts the gender information of the AOP networks. Gender-specific events are primarily associated with the reproductive system, such as alterations of the testis and penis in males and alterations of the placenta, uterus and ovary in females. unspecific means that gender information is not available. Mixed means that there is no gender specific difference, such as alterations in the immune system, heart, liver, brain and lungs.
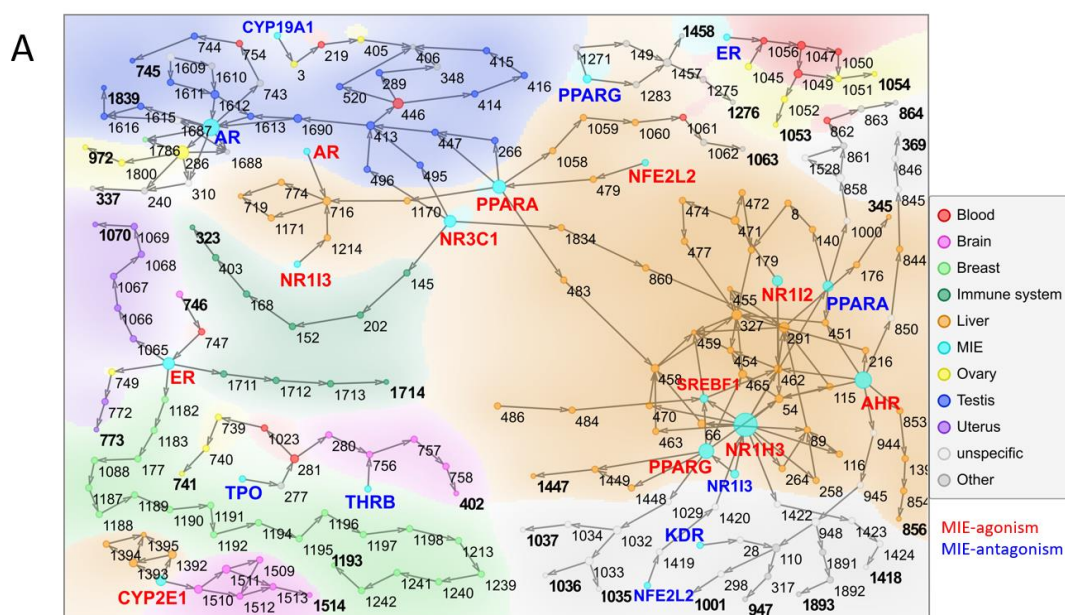
Figure 4-4 AOP networks for 40 EDCs (A: organ, B: biological level, C: gender. The number is the event ID in AOP-Wiki.)
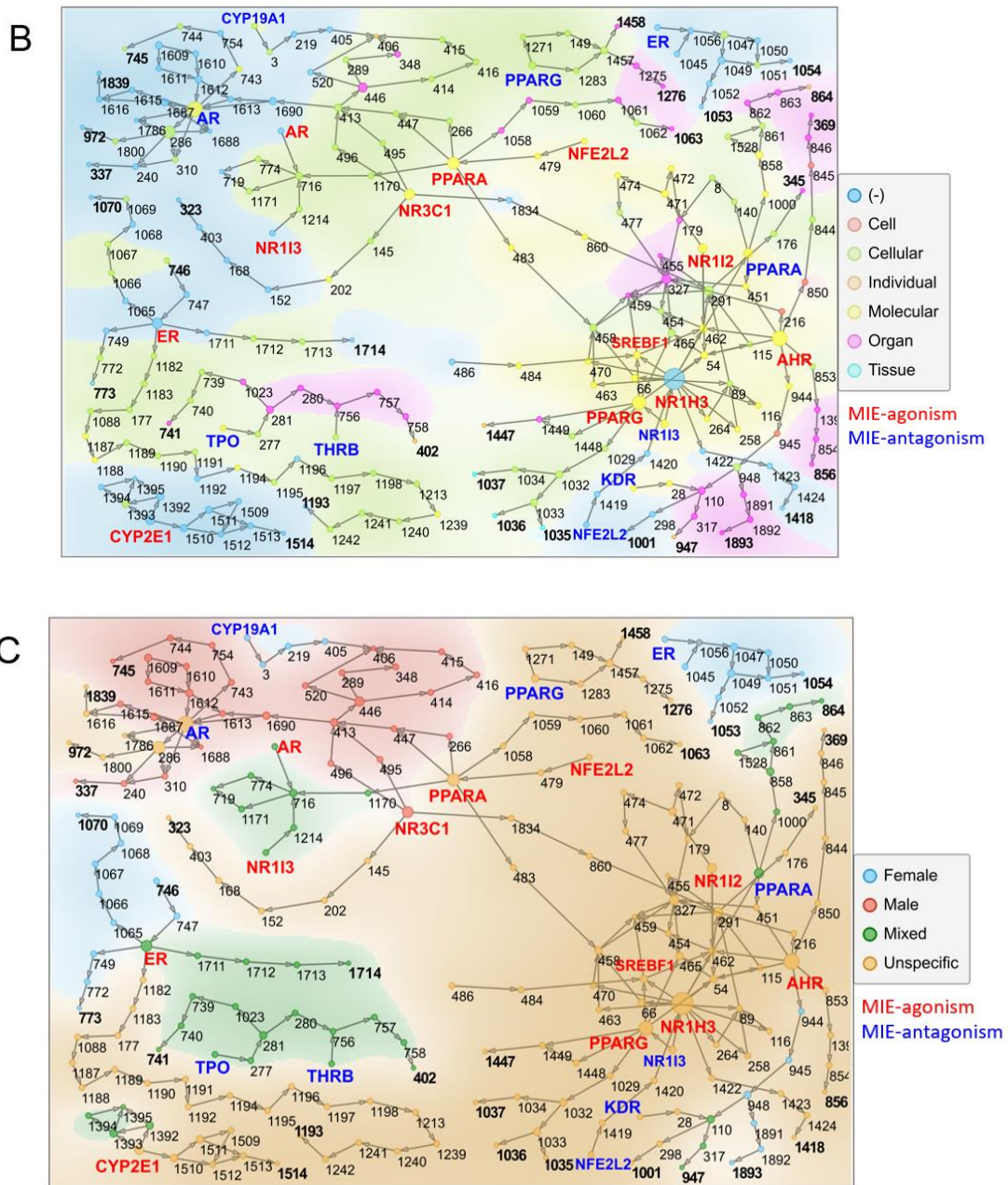
Figure 4-4 AOP networks for 40 EDCs (A: organ, B: biological level, C: gender. The number is the event ID in AOP-Wiki.)

4.3.3. MIE quantification using ToxPi score
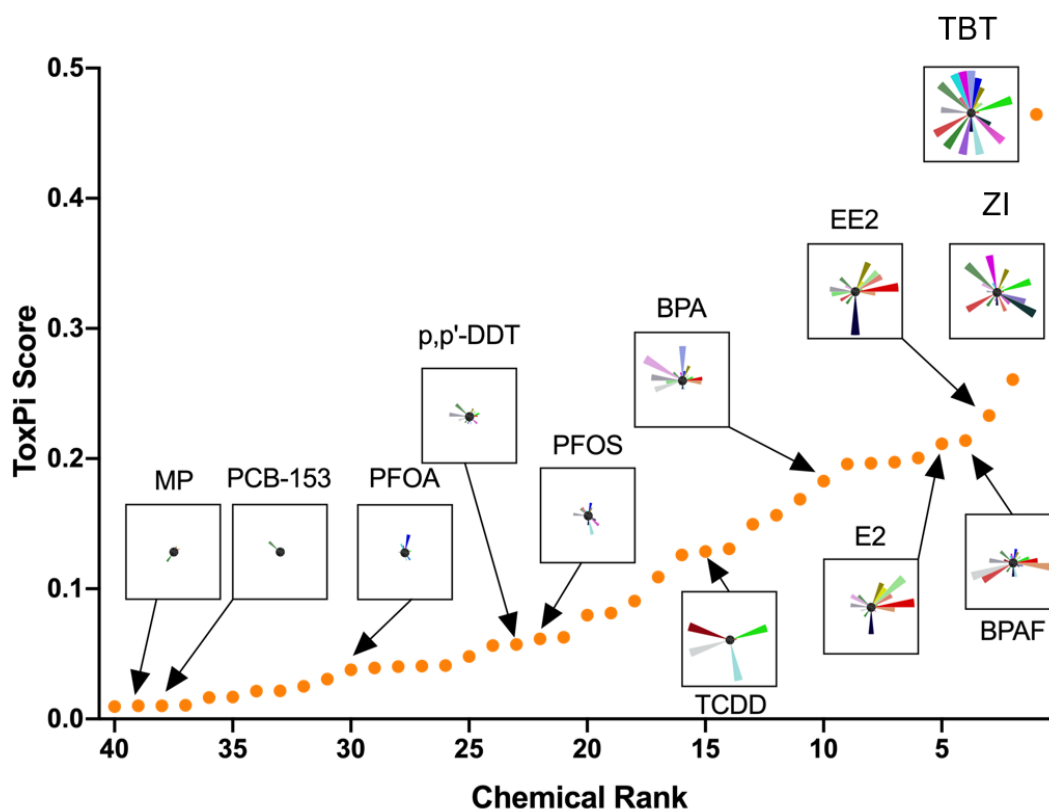
Figure 4-5 ToxPi scores of 40 EDCs

We quantified the potential of different chemicals to impact MIEs using *in vitro* assay AC50 data and ToxPi score. For the assays in the ToxCast database, the tested chemicals also have different effects on the genes, i.e. promotion and inhibition, so assays with the same genes and the same effects were combined into one ToxPi slice for analysis. Fig. 4-5 show that TBT has the highest ToxPi score, indicating that TBT has the highest potential to affect multiple MIEs, followed by ZI, EE2, and BPAF. MP, 4-MBC, PCB-153, and BPF show a relatively low potential to affect MIEs.

ToxPi scores of MIEs of 40 EDCs were listed in Fig. 4-6. Some chemicals exhibit unique properties. For example, TCL has high potential to affect MIE NR1H4 Antagonism, while TPT has a large effect on MIE PPARA Agonism, NR1I3 Antagonism, and KDR Antagonism. Although there are few *in vitro* assay data for TCDD in the ToxCast database, the results obtained from the limited data suggest that

it has a strong effect on ER Antagonism, AHR Agonism, NR1I3 Agonism, and NFE2L2 Agonism. DES and E2 have similar properties with high potential to affect MIE ER Agonism. The BPA analogue BPAF shows a higher potential to affect MIEs than BPA, while the other analogues BPF and BPS are lower than BPA.
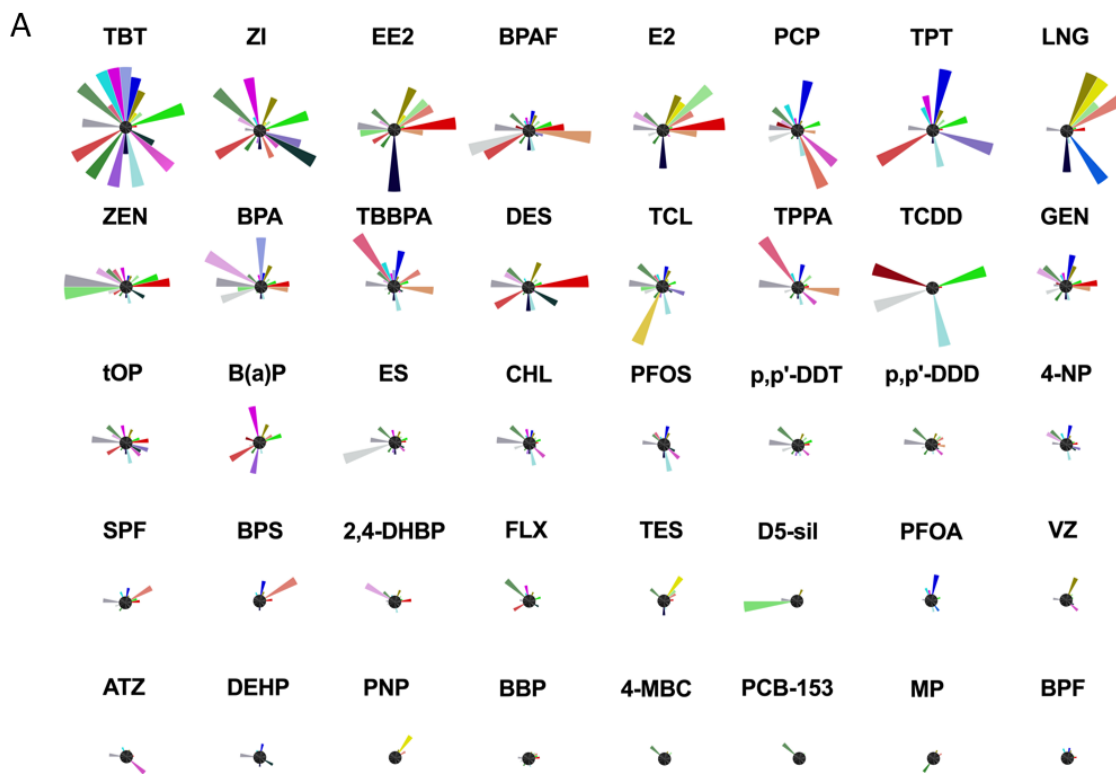
Figure 4-6 ToxPi charts of 40 EDCs (A: ToxPi score distribution of MIEs, B: Legend of MIEs)

Figure 4-6 ToxPi charts of 40 EDCs (A: ToxPi score distribution of MIEs, B: Legend of MIEs)

4.3.4. AO probability calculation

We use random work method to calculate the probability of arriving at an event in the AOP network. Fig. A and C of Fig. 4-7 show the page rank (PR) scores of all events and AOs in the six EDC-related AOP networks. A higher PR score indicates a higher probability of reaching that node (event). Since the PR score of each node decreases as the complexity of the network and the number of node increase. For example, network 6 contains the largest number of nodes, where each node has a smaller PR score than the results of other networks. In order to make the results of different networks comparable, we normalized the results of different networks. Fig. 4-7 B and D show the PR scores of all events and AOs after normalization. As shown in Table 4-4, after normalization, AO 402, 1193 and 1514 had the highest PR scores, indicating that exposure to EDC causes a greater potential for neurological impairment and breast cancer, following by impairment in liver, lungs and reproductive system. Some uncommon diseases have a low probability, such as firbrosarcoma, liposarcoma, and hemagiosarcoma. Because AOP is under development, so the terminology is not standardized. In this study, we divide AO with similar meaning: AO 459 Increased, Liver Steatosis / AO 345 N/A, Liver Steatosis and AO 1276 Lung fibrosis / AO 1458

Pulmonary fibrosis into two events based on the information collected from AOP. This may lead to some calculation bias. The future development of AOP is expected to standardize and unify the terminology of events.
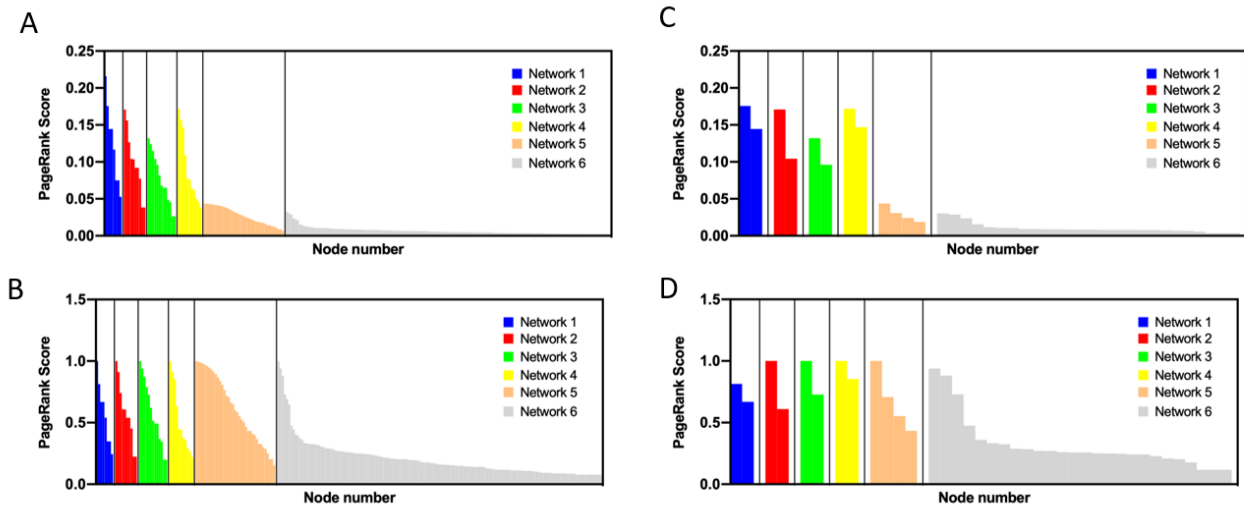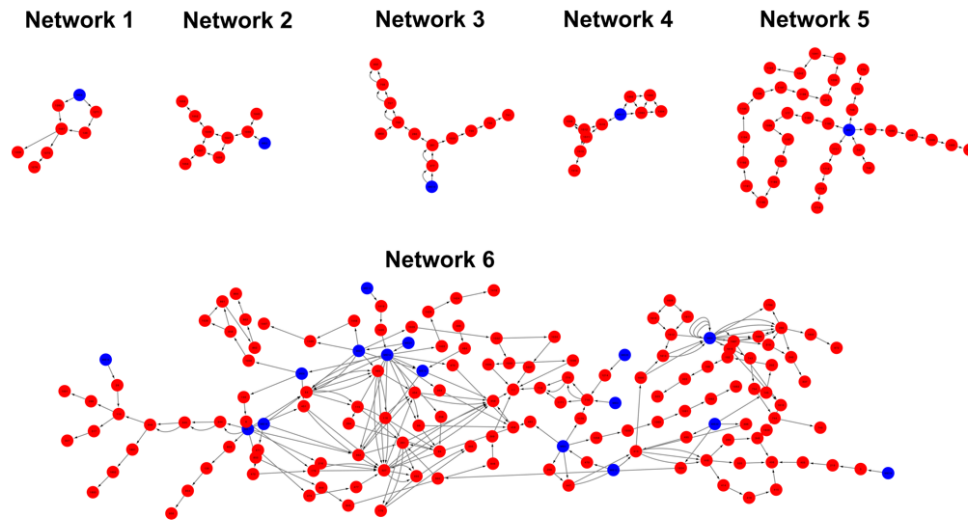
Figure 4-7 PageRank score of events in EDC-AOP networks before and after scaling (A: all events before scaling. B: all events after scaling. C: AOs before scaling. D: AOs after scaling.)

Table 4-4 PageRank score of AOs before and after scaling

AO No.	AO name	PR score before	PR score after
402	Cognitive Function, Decreased	0.132096005	1
1193	N/A, Breast Cancer	0.043608657	1
1514	Neurodegeneration	0.171897601	1
455	Accumulation, Liver lipid	0.030386358	0.939081
459	Increased, Liver Steatosis	0.028499138	0.880757
1395	Liver Cancer	0.146910729	0.854641
1276	Lung fibrosis	0.175543645	0.813477
741	Increase, Adenomas/carcinomas (follicular cell)	0.096177756	0.72809
1070	Increased, adenosquamous carcinomas of endometrium	0.030846854	0.707356
1458	Pulmonary fibrosis	0.144462936	0.669447
1053/1054	Promotion, ovarian adenomas/Promotion, ovarian granular cell	0.170912501	0.6104
1714	Exacerbation of SLE	0.024108231	0.552831
405/406	impaired, Fertility	0.023577658	0.485657
719	Increase, hepatocellular adenomas and carcinomas	0.015361978	0.474757
773	Increase, Endometrial adenocarcinomas	0.01889452	0.433275
864	Decreased, Body Weight	0.011669067	0.360629
1063	Increased, Pancreatic acinar tumors	0.010765915	0.332717
323	Increased, Disease susceptibility	0.010462088	0.323328
369	Uroporphyria	0.009310806	0.287748
1839	Testicular Cancer	0.009235273	0.285413
1688	short male AGD	0.008762	0.270799
745	Increase, Leydig cell tumors	0.008695	0.268703
337	N/A, Impairment of reproductive capacity	0.008424	0.260328
947	Increase, Early Life Stage Mortality	0.008363	0.25845
1001	Increased, Developmental Defects	0.008363	0.25845
1418	Increased, steatosis	0.008096	0.250215
856	Formation, Hepatocellular and Bile duct tumors	0.008055	0.248933
1616	Malformation, cryptorchidism	0.007966	0.246187
345	N/A, Liver Steatosis	0.007795313	0.240912
1893	increase, Preeclampsia	0.007347871	0.227084
1447	obesity	0.006764986	0.20907
1786	nipple retention (NR) male	0.006592017	0.203724
972	Decreased fertility, Reduced number of oocytes ovulated	0.0057439	0.177514
348	Malformation, Male reproductive tract	0.0054947	0.169813
1035/1036/ 1037	Increased, Firbrosarcoma/Increased, liposarcoma/Increased, hemagiosarcoma	0.003794382	0.117264

The relationship between MIEs and AOs is shown in Fig. 4-8. Liver steatosis has the highest probability with 8 MIEs having the potential to cause liver steatosis, namely PPARA Agonism, PPARG Agonism, NFE2L2 Agonism, SREBF1 Agonism, NR1I2 Agonism, NR1I3 Agonism, NR1H3 Agonism and NR3C1 Agonism. In addition to liver lesions, reproductive and developmental damage as well as tumors and cancers also account for a high probability caused by a variety of MIEs.
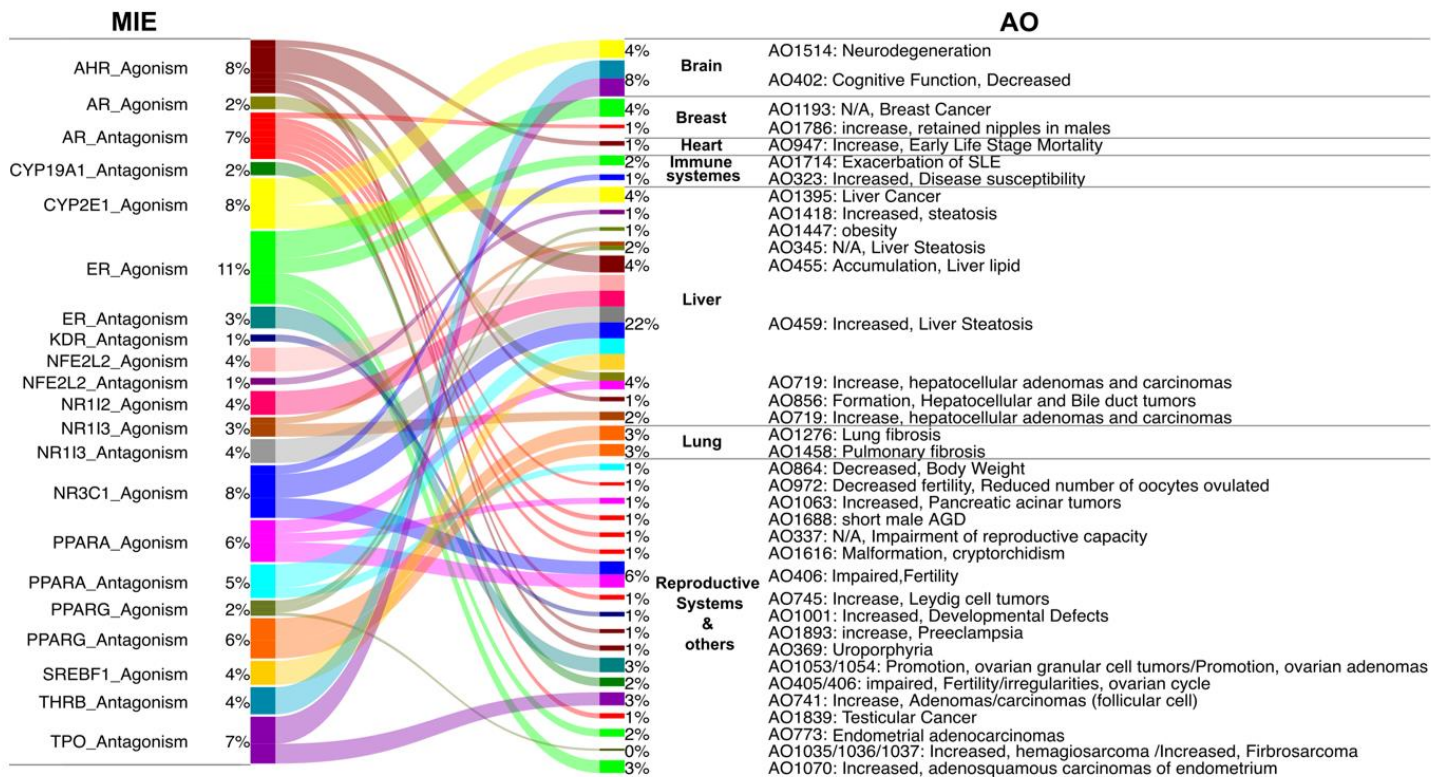
Figure 4-8 Predicted relationship between MIEs and AOs

4.3.5. Toxicity prediction of 40 EDCs

We divided the toxicity of EDC into development toxicity, reproductive toxicity, immunotoxicity, hepatotoxicity, pulmonary toxicity, neurotoxicity, cancer and tumor, and others (Fig. 4-9). TBT exhibits high toxicity levels in all toxicity. MP, BPF, ATZ, BBP, PNP and D5-sil showed relatively low levels of toxicity among all toxicity. Except for TBT, for development toxicity, ZI, EE2, E2, TPT, and LNG have higher toxicity scores; for reproductive toxicity, ZI, TPT, and PCP have higher toxicity scores; for immunotoxicity, EE2, E2 and DES have a high level of toxicity; for hepatotoxicity, BPAF, BPA, TPT, PCP and TCDD have a high level of toxicity; for pulmonary toxicity, TBBPA and TPPA exhibit high toxicity level; for neurotoxicity, ZI and LNG show high toxicity level; for cancer and tumor, a relatively large number of EDCs showed high toxicity scores, including BPAF, EE2, E2, ZEN, BPA, DES, LNG and GEN; for other toxicity, TCDD has a high level of toxicity.
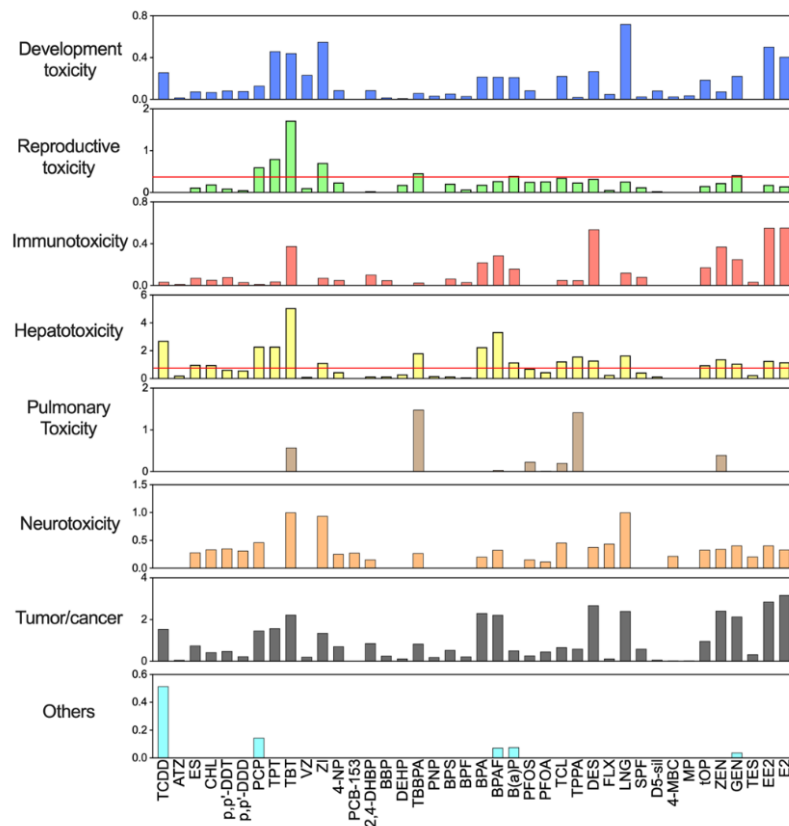
Figure 4-9 Predicted toxicity of 40 EDCs

4.3.6. Validation of toxicity of EDCs based on ToxRefDB data

We collected the results of animal experiments from the ToxRefDB database to validate the prediction results. LOAEL from *in vivo* assays and corresponding toxicity of 26 EDCs were shown in Fig. 4-10. Some results are consistent with the predicted results. For example, TCDD show high toxicity in liver, uterus and ovary. EE2 show high toxicity in reproductive effect, liver, male reproductive system and mammary gland. E2 and DES show similar properties and have greater reproductive and developmental toxicity.
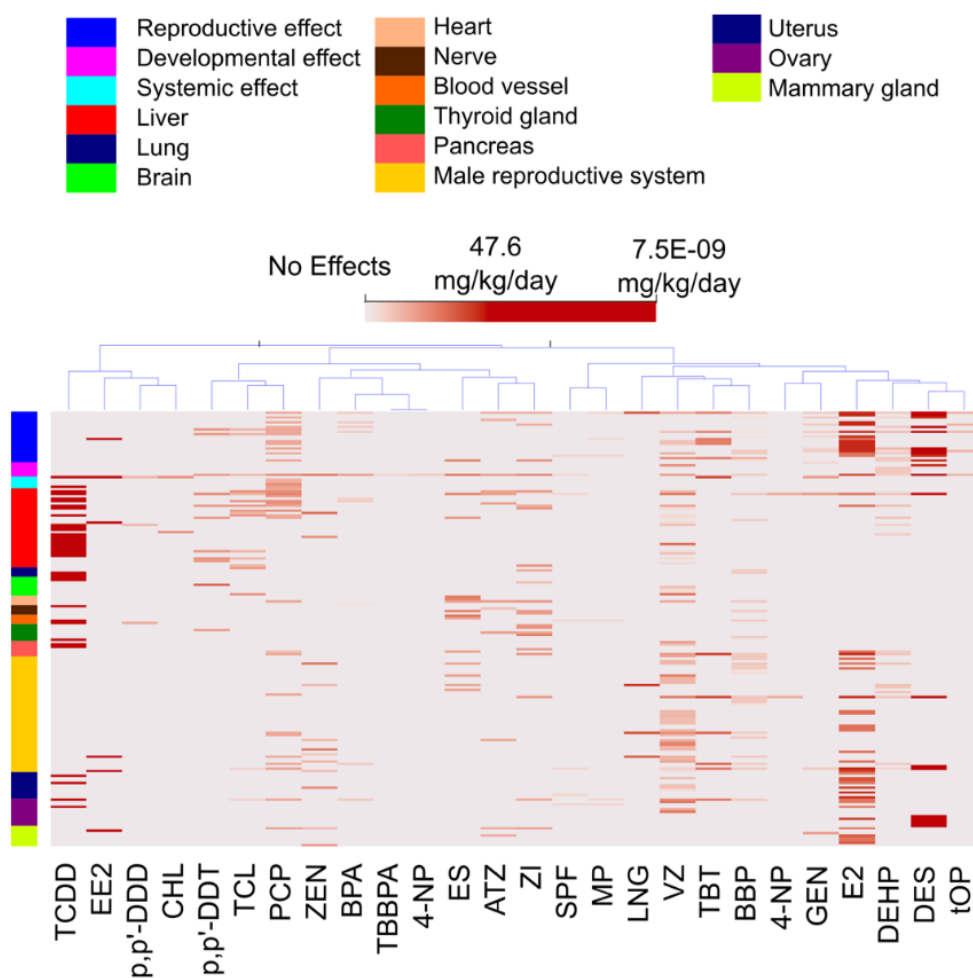
Figure 4-10 Heatmap of LOAEL from *in vivo* assays in ToxRefBD database

We predicted the toxicity of each EDC by combining the potential of the EDCs to affect the MIEs and the linking probability of the MIEs to the AOs. After PCA analysis of all

ToxAO scores (Fig. 4-11 A), ZEN, GEN, BPA, DES and LNG show the close properties as E2 and EE2. TBT possesses a higher level of toxicity than TPT. PCB-153 and p,p'-DDT show close properties with MP and 2,4-DHBP. This may be due to the limited *in vitro* assay data of PCB-153 and p,p'-DDT in ToxCast database. The distribution of 40 EDCs in PCA obtained based on ToxRefDB LOAEL (Fig. 4-11 B) was close to EDC distribution in ToxAO PCA. Due to data limitations, animal experiment data for 14 EDCs were not available and not all AO-related endpoints could be found in the animal experiment results. Table S4 listed the endpoints associated with AOs in animal experiments. Because of these data limitations, it is difficult to establish a correlation relationship between the predicted toxicity results and the results of animal studies. More data are needed to validate the prediction results in the future.
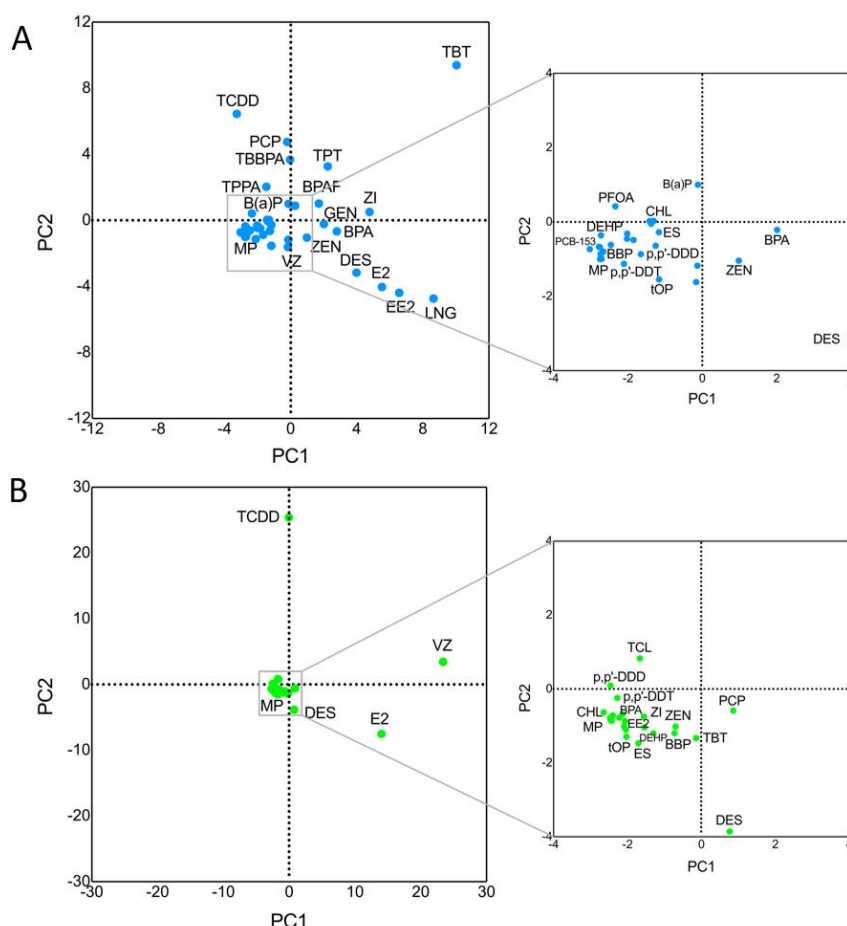
Figure 4-11 PCA of EDCs (A: ToxAO matrix, B: ToxRefDB LOAEL)

ROC illustrated that the optimal threshold of hepatotoxicity and reproductive toxicity were 0.80 and 0.36, respectively (Fig. 4-12). Therefore, half of the EDCs showed hepatotoxicity with TBT has the highest hepatotoxicity score. Seven EDCs showed reproductive toxicity. The model developed in this study can efficiently and accurately predict EDC hepatotoxicity and reproductive toxicity with AUC of 0.86 and 0.73, respectively.

Toxicity	Optimal Threshold	AUC	CA	F1	Precision	Recall	Model
Hepatotoxicity	0.80	0.86	0.83	0.89	0.86	0.92	Random Forest
Reproductive toxicity	0.36	0.73	0.60	0.72	0.76	0.68	Random Forest
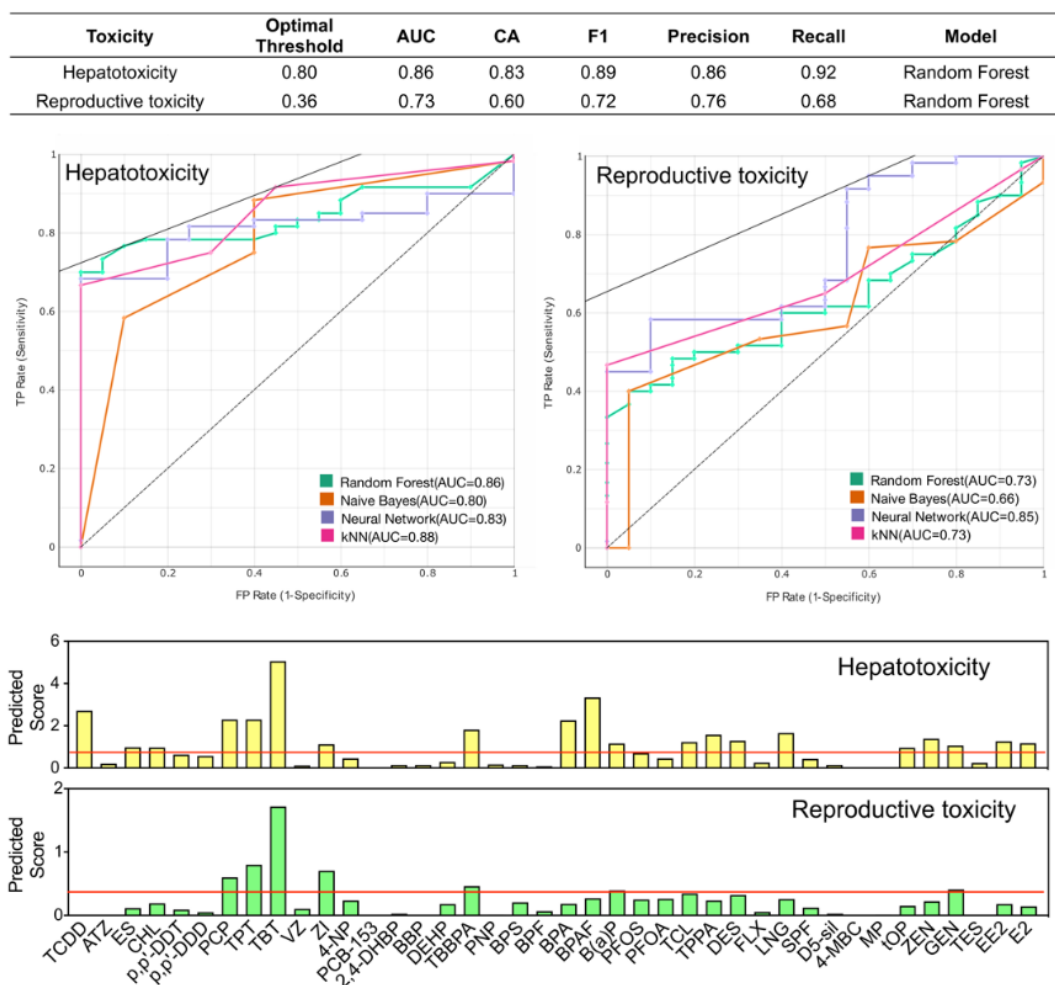
Figure 4-12 ROC for hepatotoxicity and reproductive toxicity

4.4. Conclusion

EDC is a class of chemicals of concern because of its potential health risks and complex mechanism of toxicity. In this study, we developed a *in silico* model for predicting the

toxicity of EDCs based on ToxCast data, AOP and machine learning. The 22 overlap MIEs induced by 40 selected EDCs including ER, AR, PPARA, THRB, TPO, AHR, NR1I2, etc. As indicated by ToxPi scores, TBT, ZI and BPAF have a greater potential to cause changes in endocrine-related genes. A network of 48 AOPs was established to depict the mechanism of EDC toxicity from the molecular to the individual level. AOs caused by 40 EDCs include liver lesions, reproductive system damage, immune system disorders, cancer, obesity, developmental defects, and neurodegeneration, etc. Machine learning result show that AOs in the liver and reproductive system have a higher probability of occurrence. The predicted toxicity of EDCs was validated using *in vivo* assay results. The threshold of hepatotoxicity and reproductive toxicity were 0.80 and 0.36. The AOP-based *in silico* model was a promising tool to predict hepatotoxicity and reproductive toxicity of EDC with AUC of 0.86 and 0.73.

Supplementary material

Table S4 Toxicity, AO and endpoint classification.

Figure S1 Heatmap of active in vitro assay of 40 EDCs.

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Chapter 5.   Health risk assessment of bisphenol A through adverse outcome pathway combining machine learning method and zebrafish embryo model

5.1. Introduction

Bisphenol A (BPA) is an important industrial chemical that is mainly applied to manufacture polycarbonate plastics and epoxy resins into products for most aspects of our lives [1]. Given the wide application of BPA, it is ubiquitous in the environment and human body fluids, revealing a global exposure [2]. As early as 1993, BPA has been proven to be an estrogenic compound [3]. Until now, a large amount of research has reported the negative effects of BPA exposure on reproductive system, development, nervous system, cardiovascular system, metabolic, and immune system in *in vitro* assays, laboratory animal studies, and epidemiological studies [4-6]. However, there is a lack of systemic assessment framework to analysis the linkage between the results from *in vitro* assays and animal studies and the results from human epidemiology studies to elucidate the hidden mechanism between BPA exposure and human diseases.

Due to concern about the associated health effects of BPA, the European Union, the United States, Canada and other countries have recently proposed regulations to restrict or ban the use of BPA [7]. The prohibition of BPA has stimulated the production and use of BPA-alternative chemicals. There are 16 bisphenol analogues, that have similar chemical structures to BPA being used in industrial production for manufacture of polycarbonate plastics and epoxy resins [8]. Bisphenol analogues have been detected in environmental media, foods, daily necessities, and human specimens [9-12]. Compared to the numerous BPA researches, investigations on bisphenol analogues are limited. The toxicity of bisphenol analogues which is similar to or greater than or differ from that of BPA makes "the use of bisphenol analogues is safer" become an issue worth discussing [13-14]. At the regulatory level, there are no regulatory standards for

bisphenol analogues, unlike the reference dose or tolerable daily intake value of BPA which has been determined by some government agencies [15]. More data are needed to support government management of bisphenol analogues.

The development of novel methods and IATA can provide data for supporting the regulation of emerging chemicals, which is cost effective and efficient. Previous studies have demonstrated that the combination of ToxCast data and AOP data can be used to elucidate the mechanism of EDC-induced diseases. Machine learning can be used to predict the risk of AOs. However, information about validation of the accuracy of the establish AOP network for specific problem is inadequate. One application of AOP is to identify the important KEs and their endpoints to guide further exploration of alternative test methods [16-17]. The important KEs and related endpoints identified in the AOP network may also be used to test the accuracy of the AOP network predicted results itself.

In this study, the adverse effects of BPA were predicted through AOP. The toxicological mechanisms of BPA were profiled from the molecular level to the individual level. The important KEs and associated endpoints were identified in the BPA-related AOP network through network analysis combining machine learning approaches. A zebrafish embryo model was applied to test the suitable biomarkers for MIEs and KEs. The information obtained is useful for risk assessment and chemical management of not only BPA alternatives but also other emerging chemicals.

5.2. Materials and Methods

5.2.1. Study framework
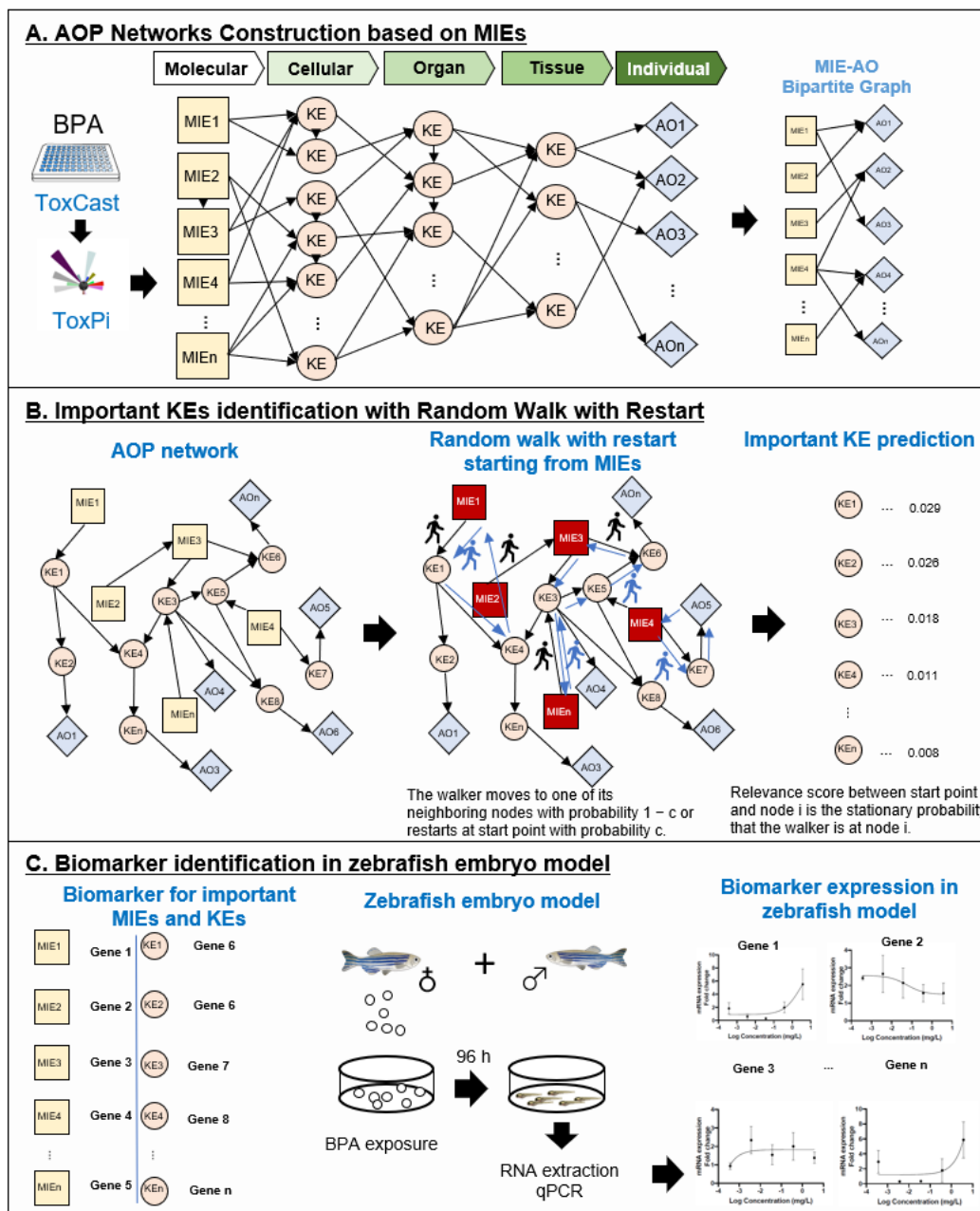
Figure 5-1 Study framework of health risk assessment of BPA

In this study, in order to establish an AOP network for predicting the adverse outcomes of BPA, firstly the MIEs induced by BPA was collected from ToxCast database, then the downstream KEs and AOs related to selected MIEs were derived from AOP-Wiki,

finally an AOP network was constructed by connecting identified MIEs, KEs and AOs. A random walk with restart method was applied to analysis the important KEs and related biomarkers in AOP network. After network analysis, the changes of gene biomarkers of important KEs and MIEs were detected in zebrafish embryo model.

5.2.2. MIE identification and AOP network construction

Methods for MIE identification and AOP network construction were the same as the methods in Chapter 4. Briefly, active *in vitro* assay information about BPA were collected from ToxCast database for identifying MIEs. To maintain data consistency and to select MIEs for endocrine relevance, the selection criteria for MIEs were adopted from previous studies. ToxPi score was used to quantify and profile the effects of BPA on MIEs. The AOP information related to the identified MIEs and the relationship of KEs and AOs were collected from the AOP-Wiki. We chose AOPs that involve all species in order to elaborate the health and ecological risks of BPA and guide the zebrafish experiment arrangements. Network construction and analysis using Cytoscape (Institute for Systems Biology). For other details, please refer to Chapter 4 Materials and Methods.

5.2.3. Important KEs identification using random walk with restart

After constructing the AOP network, we use a machine learning method called random walk with restart to predict the important KEs. The random walk with restart can be used to calculate the affinity between a fixed node i and another node j in a network. It has been used extensively in medicine field to calculate the gene-disease associations [18]. The idea of the random walk with restart in AOP network is that a walker begins at the set of fixed nodes (MIE) and moves by a succession of random steps to KEs, traveling via the MIE-KE-AO interaction and KER edges. The walker can go back to fixed node i with the probability c or move to neighbor node j with the probability 1-c which is the difference between random walk with restart and classical random walk. In this case, we applied one of the classical random walk with restart algorithms,

personalized PageRank (PPR) algorithm to rank KEs. The resulting PPR scores demonstrate the importance of the nodes to the walker. KE (node j) with a high PPR score indicates this KE is more important in AOP network. The algorithm is shown in the following formula [19].

$$R_{t+1} = (1 - c)M^T R_t + cp$$

Where R is the vector of PageRank (refer to Chapter 4 Materials and Methods), and M^T is the transition probability matrix. c is the probability that walker can go back to fixed MIE. p is the personalized PageRank vector which reflects the importance of each node in a graph for a specific walker. The random walk with restart was performed using Python 3 (Python Software Foundation).

5.2.4. Zebrafish maintenance and embryo collection

Adult wild-type AB line zebrafish were obtained from Institute for Frontier Life and Medical Sciences, Kyoto University, Japan. All adult zebrafish were maintained in a recirculating aquarium system (Fig. 5-2).
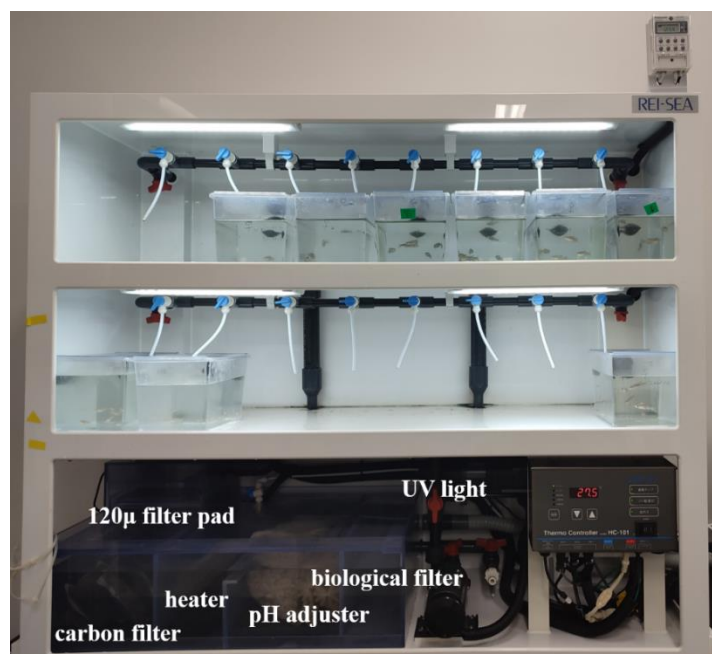
Figure 5-2 Zebrafish maintenance recirculating aquarium system

The system water was chlorine free, in temperature range between 27 ± 1 ºC and pH

between 6.0 - 8.0. In this study, tap water was used after pretreatment was conducted, by tiered filter through 120-micron filter pad, carbon filter, biological filter, pH adjustment and UV light. Water conductivity maintained between 0.5 ± 0.02 mS.cm^{-1} by adding artificial seawater powder (REI-SEA-Marine II, IWAKI Corporation, Japan). The system water flowed between 200 - 220 mL.min^{-1}. The photoperiod kept 14 hours light and 10 hours dark. The zebrafish were fed twice a day using dry flakes food and once a day using brine shrimp (Artemia sp.). To ensure optimal water quality, any remaining food was removed daily.

When the zebrafish are more than six months old, the preparation and collection of zebrafish embryos were conducted following the procedure below.

1. Fill breeding tanks with system water, then put the plastic mesh wire. Let the water flows continuously.

2. Carefully transfer adult female and male (ratio 1:2) into breeding tank and separate them by the plastic plate divider. Close the lid properly.

3. On next day, just after the artificial light turn on, remove the divider. The breeding period usually occurs during first 30 minutes.

4. After breeding time, transfer the fish to another tank, then remove the plastic mesh wire.

5. Collect all the eggs at the bottom of breeding tank using Pasteur pipette. Avoid the fish feces or other impurities.

6. Put all eggs in a plastic net, rinse the filtered eggs gently using system water 2 - 3 times.

7. After that, put all clean eggs on clean tank by turn over the net and push gently by the system water from a bottle.

8. Take the egg by Pasteur pipette into clean petri dish and select the fertilized embryo under the microscope (Shimadzu VCT-VBL 20x). Separate the fertilized embryo to another clean petri dish.
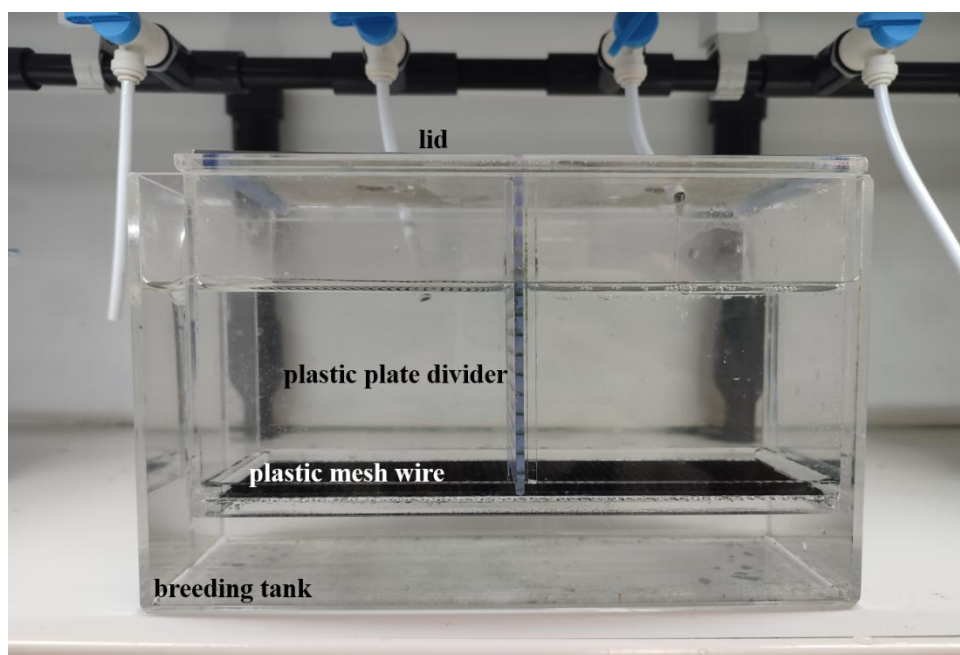
Figure 5-3 Breeding tank

Experiments were performed in accordance with Regulations on Animal Experimentation at Kyoto University.

5.2.5. Chemicals and Reagents

99.0% Bisphenol A Standard (CAS 80-05-7) were purchased from FUJIFILM Wako Pure Chemical Corporation, Japan. The stock solution of BPA used for exposure was prepared using dimethyl sulfoxide (DMSO). All other reagents utilized were of analytical grade. The standard water for exposure was prepared using deionized water and a variety of inorganic salts (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.33 mmol/L $CaCl_2$, 0.33 mmol/L $MgSO_4$).

5.2.6. Zebrafish Embryo Acute Toxicity Test

This experiment was conducted according to the OECD Test Guideline 236. Exposure solutions of BPA were prepared by dissolving the stock solution in the standard water. The exposure concentration was 2, 4, 6, 8, 10, 12 mg/L BPA. A blank control was established with 0.10% (v/v) DMSO. Embryos during the 16-cell stage (1.5−1.7 h postfertilization) were randomly transferred into exposure solutions in 24-well plates.

Each well contained 2 mL of exposure solution and one embryo. Each exposure concentration and control contained ten embryos and were performed in triplicate. The exposure lasted 96 hours. The exposure solutions were renewed every 24 hours to maintain the appropriate concentration of test compounds and water quality. All exposures were performed in a temperature-controlled incubator (26 ± 1 ºC with a light: dark period of 14:10 h). After 96 h of exposure, mortality, hatching rate, malformation rate for each exposure concentration and control and heartbeat (20 seconds) for each embryo were observed using a microscope (ZEISS Axiovert 25 CFL Inverted Microscope) equipped with a camera (Canon EOS Kiss Digital).

The dose-response curve of mortality, hatching rate, malformation rate, heartbeat rate was analyzed with the Hill model using GraphPad Prism 9 software.

Table 5-1 Water quality of exposure solution

Parameter	Dissolved Oxygen (mg/L)	pH	Conductivity (S/m)
Zebrafish Embryo Acute Toxicity Test			
Day 1	6.89	7.53	20.8
Day 2	6.78	7.40	21.1
Day 3	7.01	7.48	21.3
Day 4	7.05	7.55	20.7
Zebrafish Embryo Genotoxicity Test			
Day 1	7.04	7.03	22.8
Day 2	7.06	7.06	23.1
Day 3	7.08	7.02	23.3
Day 4	7.00	7.01	23.3

5.2.7. Zebrafish Embryo Genotoxicity Test

Fertilized embryos were collected and incubated in system water at 26 ºC for 24 h with 14 h light. Based on the Acute Toxicity Test, concentration which did not affect survival, hatchability or developmental morphology of the fish were determined and were employed to choose the highest exposure concentration for Genotoxicity Test. According to results from Acute Toxicity Test, embryos were exposed to BPA solutions at 3.7, 0.37, 0.037, 0.0037, 0.00037 mg/L in petri dishes at 24 h post fertilization (hpf).

Each petri dish contained 120 mL of exposure solution and about 60 embryos. A blank control was established with 0.10% (v/v) DMSO. Treatment and control group consisted of three replicates of 20 embryos each. After four days of exposure, at 120 hpf, hatched larvae were collected and washed twice with system water. The embryo samples were stored at -80 °C until RNA extraction. Other exposure details were the same as in the Acute Toxicity Test.

All statistical analyses were conducted using GraphPad Prism 9 software. A two-tailed Student's t test was used for comparing the means between groups. The data are expressed as mean ± SD, and p values less than 0.05 were considered statistically significant.

5.2.8. Gene Expression Analysis

Total RNA was extracted from zebrafish embryos (20 embryos for each sample) using a ReliaPrep™ RNA Tissue Miniprep System following the manufacturer's instructions (Promega Corporation, Madison, United States). The concentration and purity of RNA samples were measured by spectrophotometric absorption at 260 nm and 280 nm using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, United States). Typical 260/280 nm ratios were between 1.9 and 2.1. First-strand complementary DNA (cDNA) was synthesized from 1 µg total RNA by reverse transcription using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, United States) in a 20 µL reaction mix in accordance with the manufacturer's protocol. The reactions were stored at -25 ºC until next step analysis.

Real-time qPCR reactions were carried out using a Step-One-Plus Real-Time PCR System (Applied Biosystems, Waltham, United States) and GeneAce SYBR® qPCR Mix α (NIPPON GENE CO., LTD., Tokyo, Japan) in 10 µL reaction volume according to the manufacturer's instructions (Table 5-2). The qPCR conditions were as follows: 95 ºC/10 min; 45 cycles of 95 ºC/30 sec, 60 ºC/1 min. The fluorescence was read after the extension in each cycle. Finally, 95 ºC/15 sec; 60 ºC/1 min; 95 ºC/15 sec was used

to obtain the melting curve. After running qPCR, the values of the threshold cycle (Ct) of the biomarker transcripts were obtained from the amplification plot. Quantification of the transcripts was performed using the $2-\Delta\Delta Ct$ method.

Relative fold changes of target gene expression $= 2^{-\Delta\Delta Ct}$

$\Delta Ct = Ct$ (target gene) - Ct (housekeeping gene)

$\Delta\Delta Ct = \Delta Ct$ (treated) - ΔCt (control)

Zebrafish-specific primers were designed for the genes of interest using a primer design tool NCBI Primer-BLAST (Table 5-3). The housekeeping gene β-actin was used as an internal control. First-strand cDNA samples of 96 hpf zebrafish embryos without any exposure were used as templates to establish standard curves. Four serials of four-fold dilution were prepared from cDNA samples and were used to establish the standard curves. The amplification efficiencies of the housekeeping gene and target genes under the conditions above are given in Table 5-4.

Table 5-2 The constituents of qPCR reaction system

Constituents	Volume
GeneAce SYBR® qPCR Mix α	5 μL
Forward primer （4 μM）	0.5 μL
Reverse primer （4 μM）	0.5 μL
RNase free water	3 μL
cDNA	1 μL

Table 5-3 Primer pairs used in qPCR

Gene	Full name	Forward primer (5'->3')	Reverse primer (5'->3')
β-actin	actin beta	CGAGCTGTCTTCCCATCCA	TCACCAACCTAGCTGTCTTTCTG
ESR1	estrogen receptor 1	CAGGACCAGCCCGATTCC	TTAGGGTACATGGGTGAGAGTTTG
ESR2B	estrogen receptor 2b	CGCTCGGCATGGACAAC	CCCATGCGGTGGAGAGTAAT
ESR2A	estrogen receptor 2a	CTCACAGCACGGACCCTAAAC	GGTTGTCCATCCTCCCGAAAC
PPARA	peroxisome proliferator activated receptor alpha	TCCACATGAACAAAGCCAAA	AGCGTACTGGCAGAAAAGGA
PPARG	peroxisome proliferator activated receptor gamma	CTGCCGCATACACAAGAAGA	TCACGTCACTGGAGAACTCG
TPO	thyroid peroxidase	CCAGCCAGACCTCGTTC	CGGAGATGAGCGGAAGAAG
PXR (NR1I2)	nuclear receptor subfamily 1 group I member 2	ATGCGGCGACAAATCTACTGGC	TGTGAAGTGTGGCAGAGAGGTG
AR	androgen receptor	AGTGAAATGGGCCAAAGGAC	ATCATTGAAGACCAGGTCTGG

Gene	Description	Forward Primer	Reverse Primer
THRB	thyroid hormone receptor beta	ATCGACCAGAGCCCACACA	TAGGTGCCGATCCAATGTCTT
P53	tumor protein p53	CCTCACAATCATCACTCTGG	TTCTTGAAGTTGCTCTCCTC
GATA3	GATA binding protein 3	TACGTGTCCCGCTTAAAACC	TGAAGGGGCAATGAAGAAAG
SCD-1	stearoyl-Coenzyme A desaturase 1	GCTTTTGCGTGTTTCGTGTA	GGTTTGAGTTGTGAGGGTCG
CPT1AL	carnitine palmitoyltransferase 1a, liver	CATCCTTAGGCCTGCTCTTCAAA	ACCATGACACCCCCAACTAACAT
CPT1AM	carnitine palmitoyltransferase 1a, muscle	CCTCCATGGGCACGATTGATAA	GCAAACAGGATGGCACTCAACA
CYP17A1	cytochrome P450 family 17 subfamily A member 1	GGGAGGCCACGGACTGTTA	CCATGTGGAACTGTAGTCAGCAA
BDNF	brain derived neurotrophic factor	ATAGTAACGAACAGGATGG	GCTCAGTCATGGGAGTCC
GNRHR1	gonadotropin releasing hormone receptor 1	CTCGTGGTAAGGGCAAGGG	AGCACACCACAAATGAAGCC
DIO1	iodothyronine deiodinase 1	GTTCAAACAGCTTGTCAAGGACT	AGCAAGCCTCTCCTCCAAGTT
DGAT2	diacylglycerol O-acyltransferase 2	ACGCATAACCTGCTTCCC	TCCTGTGGCTTCTGTCCC
FOXA2	Forkhead box protein A2	CCTGGATTCACCGGACAC	AAGTCCAATCCGTCCGCAGT
VTG1	vitellogenin 1	CTGCGTGAAGTTGTCATGCT	GACCAGCATTGCCCATAACT

Table 5-4 Standard curve and amplification efficiencies of target genes

Gene	Slope	Linear Coefficient (R^2)	Amplification efficiency (%) a
β-actin	-3.2314	0.9998	103.92
ESR1	-3.4140	0.9971	96.30
ESR2B	-3.2055	0.9980	105.10
ESR2A	-3.1410	0.9905	108.15
PPARA	-3.4983	0.9997	93.13
PPARG	-3.5716	0.9888	90.54
TPO	-3.0274	0.9966	113.95
PXR	-3.2899	1.0000	101.35
AR	-3.2601	0.9916	102.65
THRB	-3.6116	0.9958	89.18
P53	-3.5642	0.9970	90.80
GATA3	-3.5243	0.9725	92.20
SCD-1	-3.1477	0.9713	107.82
CPT1A_liver	-3.4997	0.9494	93.08
CPT1A_muscle	-3.7702	0.9973	84.18
CYP17A1	-3.3488	0.9912	98.89
BDNF	-3.2655	0.9790	102.41
GNRHR1	-3.1766	0.9975	106.44
DIO1	-3.3445	0.9975	99.07
DGAT2	-3.5480	0.9838	91.36
FOXA2	-3.8515	0.9992	81.82
VTG1	-3.9141	0.9970	80.09

a Amplification efficiency (E) was calculated via the equation: $E = 10^{-1/\text{slope}} - 1$.

5.3. Results and discussion

5.3.1. MIEs and AOP network of BPA

Based on the data obtained from the ToxCast database, we determined the MIEs of BPA. Fig. 5-4 illustrates that the MIEs induced by BPA include TPO Antagonism, PPARA Antagonism, NR1I2 Agonism, NR1I3 Agonism, ER Agonism, SREBF1 Agonism, NR1I2 Antagonism, AR Antagonism, THRB Antagonism, ER Antagonism, PPARA Agonism, NFE2L2 Agonism, NR3C1 Antagonism, NR1H4 Agonism, PPARG Agonism, and AR Agonism. The largest ToxPi score of TPO Antagonism indicates that the greatest potential of BPA is to inhibit the expression of TPO. A ToxPi score of zero indicates that BPA has no effect on this gene (inactive *in vitro* assay) or there are no relevant *in vitro* assay results in the ToxCast database. The different directional effects (antagonism/agonism) of the same gene are due to the different cell lines used in the *in vitro* assay, suggesting that BPA causes different effects in different cells. For example, BPA increase the ESR1 expression in T47D cell from breast with AC50 of 0.373 μM and decrease ESR1 expression in VM7 cell from ovary with AC50 of 75.1 μM in *in vitro* assay.

Based on the active MIEs in Fig. 5-4, the downstream KEs and AOs in the AOP-Wiki database associated with these MIEs are collected. An AOP network of BPA was constructed (Fig. 5-5). In total, 43 AOPs related to 13 MIEs, 31 AOs and 147 KEs that meet data selection criteria were used for AOP network construction. Different from EDC's AOP networks, AOPs used for BPA AOP network construction are derived from humans, rodents, fish, amphibians, and birds. The AOs caused by BPA exposure involving multiple organs including immune system disorders, reproductive system cancers and other cancers, reproductive system disorders, growth and developmental disturbances, breast cancer, liver lesions, neurological developmental disturbances. And also cause fertility, larval development, sex ratio alteration and population trajectory alteration in fish (Table 5-5). The information-rich AOP network provided

details of mechanism of complex toxicity of BPA. For example, ER agonism in breast may cause breast cancer, while ER antagonism in ovary may cause ovarian adenomas. Liver Steatosis is the result of multiple MIEs acting simultaneously, including NFE2L2 Agonism, PPARA Antagonism, SREBF1 Agonism, and NR1I2 Agonism.

TPO_Antagonism	1.000
PPARA_Antagonism	0.774
NR1I2_Agonism	0.695
NR1I3_Agonism	0.632
ER_Agonism	0.398
SREBF1_Agonism	0.376
NR1I2_Antagonism	0.312
AR_Antagonism	0.301
THRB_Antagonism	0.203
ER_Antagonism	0.170
PPARA_Agonism	0.144
NFE2L2_Agonism	0.119
NR3C1_Antagonism	0.107
NR1H4_Agonism	0.025
PPARG_Agonism	0.023
AR_Agonism	0.002
PPARG_Antagonism	0.000
AHR_Agonism	0.000
NR1I3_Antagonism	0.000
NR1H3_Agonism	0.000
NR1H4_Antagonism	0.000
NR3C1_Agonism	0.000
NFE2L2_Antagonism	0.000
CYP2E1_Agonism	0.000
CYP19A1_Antagonism	0.000
KDR_Antagonism	0.000

Figure 5-4 ToxPi chart and ToxPi score of MIEs for BPA
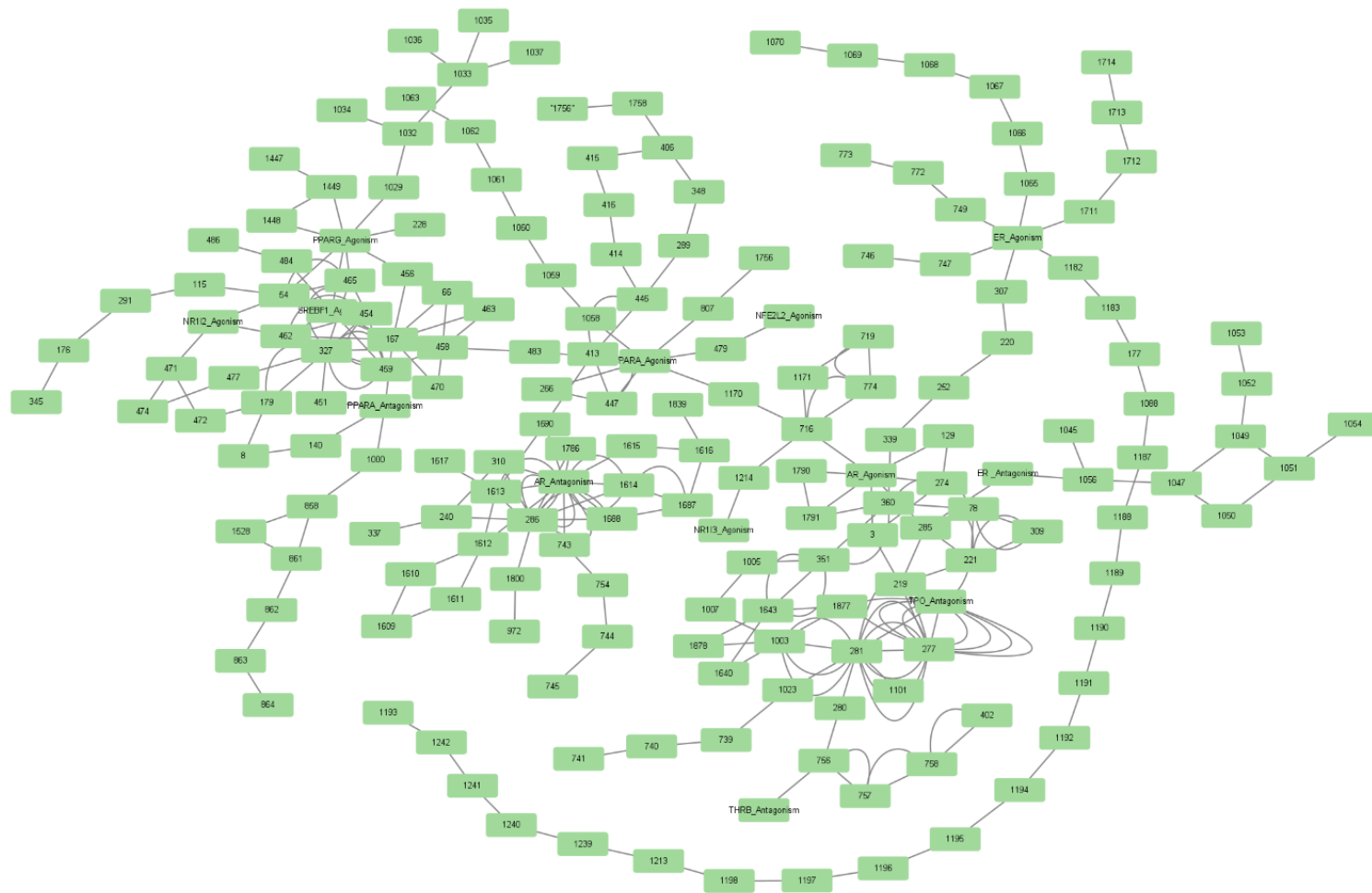
Figure 5-5 AOP network for BPA (The numbers are the KE and AO ID in the AOP-Wiki)

Table 5-5 AOs caused by BPA exposure

MIE	AOP	AO(KE)	Name
ER Agonism	314	1714	Exacerbation of SLE
	167	1070	Increased, adenosquamous carcinomas of endometrium
	200	1193	N/A, Breast Cancer
	29	360	Decrease, Population trajectory
		339	Altered, Larval development
	112	773	Increase, Endometrial adenocarcinomas
ER Antagonism	30	360	Decrease, Population trajectory
	165	1053	Promotion, ovarian adenomas
		1054	Promotion, ovarian granular cell tumors
AR Agonism	117	719	Increase, hepatocellular adenomas and carcinomas
	376	360	Decrease, Population trajectory
	23	360	Decrease, Population trajectory
AR Antagonism	345	972	Decreased fertility, Reduced number of oocytes ovulated
	344	1786	nipple retention (NR) male
	306	1688	short male AGD
	19	337	N/A, Impairment of reproductive capacity
	372	1839	Testicular Cancer
	111	745	Increase, Leydig cell tumors
	305	1688	short male AGD
	288	1616	Malformation, cryptorchidism
	307	1688	short male AGD
PPARA Agonism	166	1063	Increased, Pancreatic acinar tumors
	323	1758	Impaired, Spermatogenesis
	18	406	impaired, Fertility
	37	719	Increase, hepatocellular adenomas and carcinomas
	51	406	impaired, Fertility
NFE2L2 Agonism	61	459	Increased, Liver Steatosis
PPARA Antagonism	6	864	Decreased, Body Weight
	36	459	Increased, Liver Steatosis
	58	459	Increased, Liver Steatosis
PPARG Agonism	72	1447	obesity
	163	(1035)	Increased, Firbrosarcoma
		(1036)	Increased, liposarcoma

		(1037)	Increased, hemagiosarcoma
	34	345	N/A, Liver Steatosis
SREBF1 Agonism	62	459	Increased, Liver Steatosis
NR1I3 Agonism	107	719	Increase, hepatocellular adenomas and carcinomas
THRB Antagonism	300	402	Cognitive Function, Decreased
TPO Antagonism	271	78	Reduction, Cumulative fecundity and spawning
	175	1101	Altered, Amphibian metamorphosis
	42	402	Cognitive Function, Decreased
	159	360	Decrease, Population trajectory
	365	351	Increased Mortality
	363	351	Increased Mortality
	364	360	Decrease, Population trajectory
	119	741	Increase, Adenomas/carcinomas (follicular cell)
NR1I2 Agonism	60	459	Increased, Liver Steatosis

5.3.2. Important KEs of BPA in AOP network

After implementing random walk with restart analysis on the AOP network, the PPR scores of each event in the network were analyzed to illustrate the importance of each event. The top 30 important events in the AOP network are listed in Table 5-6 in the rank order according to PPR scores. These important KEs can be used to guide the selection of biomarkers of a specific AO. Hippocampal gene expression, fatty acid in liver, testosterone in ovary may be used as biomarkers for neurological disorders, liver steatosis, ovarian cancer, respectively. Some biomarkers of important KEs, such as vitellogenin concentration, thyroxine concentration, E2 concentration in blood are popular biomarkers in other EDC risk assessment studies [20-22]. In this study, we selected gene biomarkers of some important KEs and less important KEs for the next step of experiment (Table S5). Specific information of PPR score of all KEs is also shown in supplementary material Table S5.

Table 5-6 Top 30 important KEs in BPA-related AOP network

KE	Name	Level of Biological Organization	Biological Organization	PPR
454	Increased, Triglyceride formation	Cellular	Hepatocyte	0.02639
716	Increase, cell proliferation (hepatocytes)	Cellular	Hepatocyte	0.022351

756	Hippocampal gene expression, Altered	Organ	Brain	0.020124
327	Accumulation, Fatty acid	Organ	Liver	0.018553
462	Up Regulation, SCD-1	Molecular	Hepatocyte	0.018427
221	Reduction, Plasma vitellogenin concentrations	Cellular	Gonadotropin Releasing Neuron	0.017256
757	Hippocampal anatomy, Altered	Organ	Brain	0.017106
479	Activation, NR1H4	Molecular	Hepatocyte	0.017102
1214	Altered expression of hepatic CAR-dependent genes	Cellular	Hepatocyte	0.017102
285	Reduction, Vitellogenin synthesis in liver	(-)	Liver	0.016471
281	Thyroxine (T4) in serum, Decreased	Organ	Serum	0.014643
758	Hippocampal Physiology, Altered	Organ	Brain	0.014536
471	Inhibition, FoxA2	Molecular	Eukaryotic Cell	0.010261
774	Increase, Preneoplastic foci (hepatocytes)	Cellular	Hepatocyte	0.009497
1171	Increase, Clonal Expansion of Altered Hepatic Foci	Cellular	Hepatocyte	0.009497
1056	Decrease, E2 blood concentrations at hypothalamus	(-)	Corpus Luteum	0.008551
219	Reduction, Plasma 17beta-estradiol concentrations	(-)	Blood Plasma	0.007661
807	Decreased, cholesterol	Organ	Blood plasma	0.007363
1170	Increase, Phenotypic enzyme activity	Cellular	Hepatocyte	0.007363
1047	Increased, secretion of GnRH from hypothalamus	(-)	Epithelium Of Female Gonad	0.007268
3	Reduction, 17beta-estradiol synthesis by ovarian granulosa cells	Cellular	Granulosa Cell	0.006875
179	Decreased, Mitochondrial fatty acid beta-oxidation	Organ	Liver	0.006793
465	Increased, FA Influx	Cellular	Hepatocyte	0.006691
447	Reduction, Cholesterol transport in mitochondria	Cellular	Steroid hormone secreting cell	0.006628
451	Inhibition, Mitochondrial fatty acid beta-oxidation	Molecular	Hepatocyte	0.006413
1000	stabilization, PPAR alpha co-repressor	Molecular	Eukaryotic cell	0.006413
1756	Decreased, plasma 11-ketotestosterone level	Organ	Blood plasma	0.00626
309	Reduction, Vitellogenin accumulation into oocytes and oocyte growth/development	Cellular	Gonadotropin Releasing Neuron	0.005866
413	Reduction, Testosterone synthesis in Leydig cells	Cellular	Testosterone Secreting Cell	0.005633
286	Altered, Transcription of genes by AR	Cellular	Eukaryotic Cell	0.005558

5.3.3. BPA zebrafish embryo acute toxicity

In order to validate the predictions of MIEs and KEs using the zebrafish embryo model, we performed a BPA zebrafish embryo acute toxicity experiment to determine the exposure concentration for the genotoxicity assay. The 96 h LC50 and LC10 values of BPA for zebrafish embryo were 9.4 and 7.5 mg/L. The 96 h EC50 values of BPA for hatching rate, malformation rate, heartbeat rate of zebrafish embryo was 5.9, 5.1, 8.5 mg/L and EC10 values for malformation rate and heartbeat rate were 3.7 and 4.1 mg/L, respectively (Fig. 5-6). Because of limited data, EC10 for hatching rate was not available. An exposure concentration of 3.7 mg/L was set as the maximum exposure concentration for the genotoxicity assay. BPA also caused various defects in zebrafish embryos, including bent spine, pericardial edema, and yolk sac edema (Fig. 5-7). The present results are close to the results of other study with a 96 h-LC50 value of 8.04 mg/L [23].
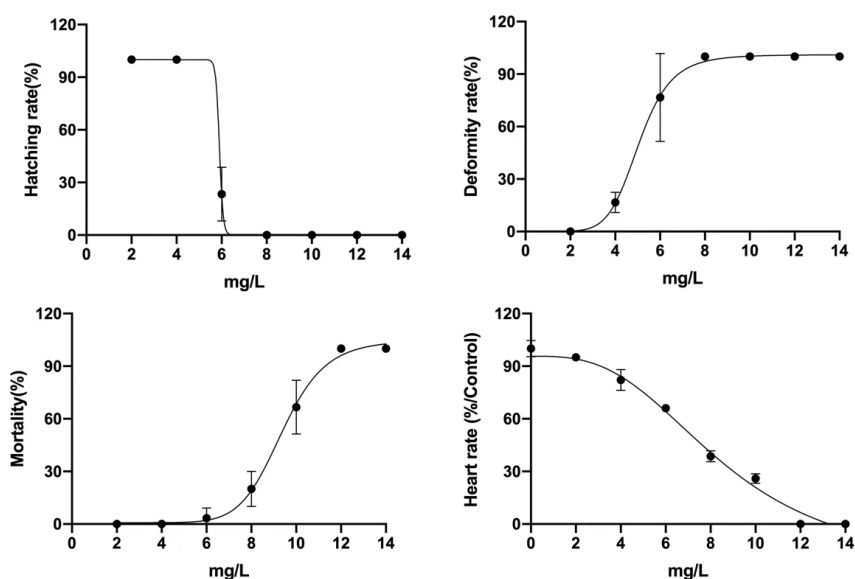
	EC50(mg/L)	Hillslope	EC10(mg/L)	EC50(mg/L) 95%CI
mortality(%)	9.381	9.701	7.5	8.992-9.789
hatching rate(%)	5.903	-72.64	-	-
deformity rate(%)	5.067	7.059	3.71	4.629-5.605
heart rate(%control, 1min)	8.527	-2.979	4.08	7.526-12.71

Figure 5-6 Dose-response curve for mortality, hatching rate, malformation rate, heartbeat rate of zebrafish embryo after 96 h BPA exposure
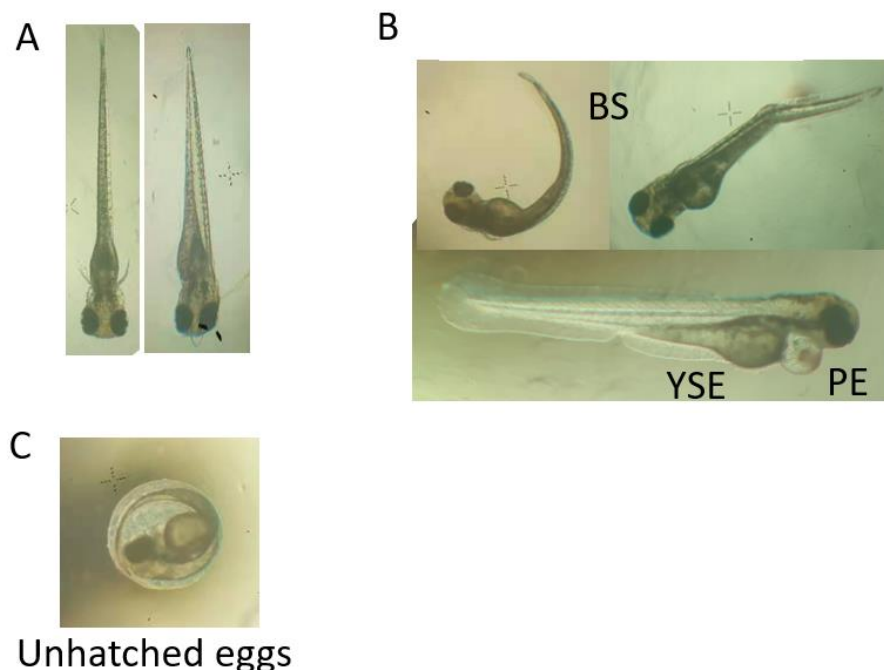
Figure 5-7 Visual images of zebrafish embryos in 96 h in control (A) and BPA exposures (B, 4 mg/L; C, 8 mg/L). PE, pericardial edema; YSE, yolk sac edema; BS, bent spine.

5.3.4. BPA zebrafish embryo genotoxicity toxicity

For MIE gene biomarkers (Fig. 5-8), we selected genetic biomarkers that are highly affected by BPA and those that are less affected to be tested. BPA led to an increase in ESR1 and ESR2B gene expression, which was consistent with the predictions of ToxPi score. The ToxPi score of ER Agonism was higher than those of ER Antagonism, suggesting that BPA has a greater potential to cause an increase in ER gene expression. The insignificant change in ESR2A gene expression indicates that ESR2A is less affected by BPA and can be considered not as a biomarker for ER. BPA down-regulated THRB expression, which was also consistent with the predicted results of ToxPi result. Therefore, ESR1, ESR2B and THRB may be appliable as MIE biomarker. The dose-response curves of AR, TPO, PPARA, PPARG, and PXR (NR1I2) show as non-monotonic dose-response (NMDR) curves. Possible mechanisms are complex including cytotoxicity, receptor down-regulation and desensitization, cell and tissue

specific receptors and cofactors, receptor selectivity, receptor competition, negative feedback loops, tissue interactions [24]. For TPO and PPARA, their expression decreased but not significant with increasing concentrations in the exposure concentration range of 0.0037-3.7 mg/L, which is consistent with the ToxPi predicted results. For AR, PPARG and PXR, in the exposure concentration range 0.00037-0.37 mg/L, BPA upregulated the expression of these genes. The results of PPARG and PXR are consistent with predicted results from ToxPi. However, the result of AR is different from the result predicted by ToxPi, which AR Antagonism has higher score than AR Agonism.

Alterations in the genetic biomarkers of KEs were not significant except for VTG1, FOXA2, and DGAT2 (Fig. 5-9). P53 is an important gene associated with cancer, and a slight increase (not significant) in P53 expression suggested that BPA exposure increases cancer risk, as shown in several AOs, such as AO 1193: Breast Cancer and AO 1839: Testicular Cancer. The insignificant changes in the BDNF and GNRHR1 genes may be due to the insufficient amount of genes obtained from the brain of zebrafish embryos for analysis. The insignificant changes in the SCD1, CPT1A, GATA3, and CYP17A1 genes may be due to the low PPR values of their associated KEs, indicating that the effect of BPA on these genes was not significant. The increased VTG1 gene was consistent with the results of other studies which also showed a significant increase in VTG1 expression in zebrafish larval after BPA exposure [26]. In this study, the VTG1-related KEs showed an increasing trend in male fish and a decreasing trend in female fish. The significant decrease in DGAT2 gene at an exposure concentration of 3.7 mg/L may be attributed to cytotoxicity. A slight increase in DIO1 gene and a significant increase in FOXA2 gene were different from the predicted results. The diversity of altered gene expression affected by BPA may be due to the complexity of the endocrine system. Besides, the prediction results based on AOP network have certain limitations since AOP is under development. In addition, the AOP network established in this study included several species such as human, mouse, fish and bird.

There were also some species differences in the prediction results. A species-specific AOP network can be established in the future. In addition to species differences, gender differences also need to be taken into account. Genetic biomarkers may be not well suited for some KEs, and other models and biomarkers can be added in the future to validate the KE changes, such as adult zebrafish, *in vitro* tests, the concentration of substances in the blood and organs, etc.
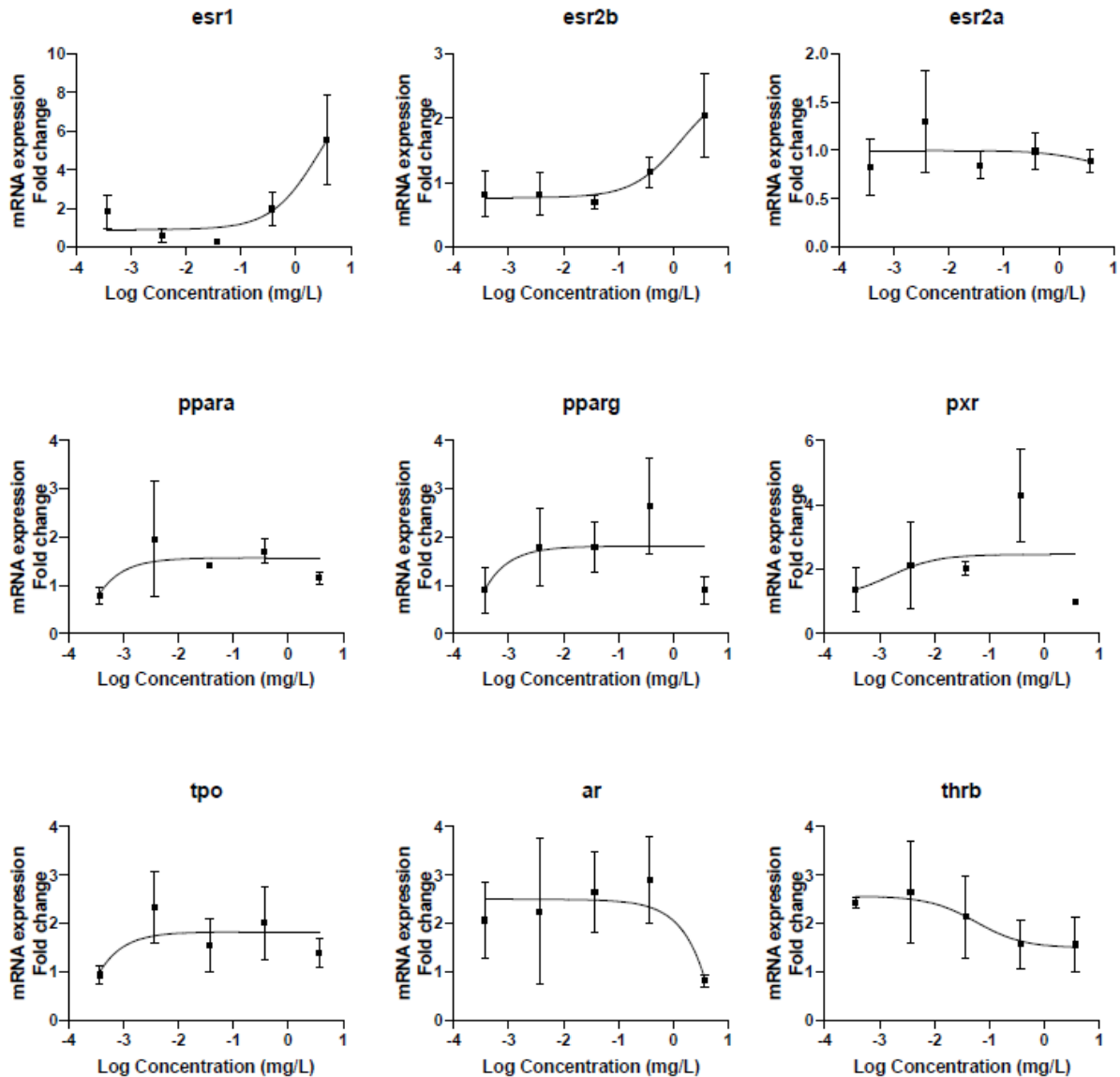
Figure 5-8 Alterations of MIE gene expression in zebrafish embryo after 96 h BPA exposure
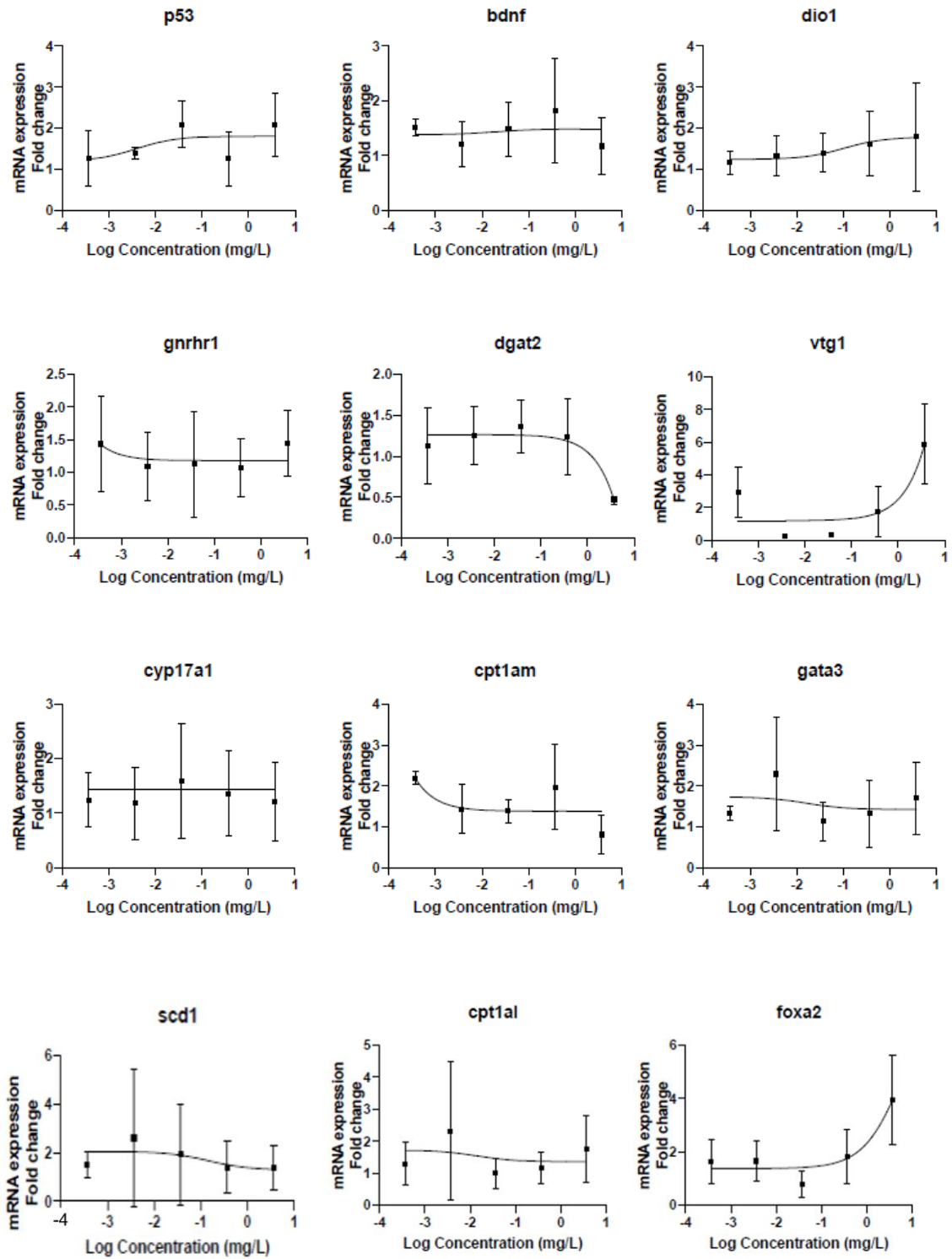
Figure 5-9 Alterations of KE gene expression in zebrafish embryo after 96 h BPA

exposure

5.4. Conclusion

BPA is an important and widely studied EDC. In order to establish a method for evaluating potential EDC risk based on AOP, a BPA-related AOP network was created. MIEs caused by BPA includes TPO Antagonism, PPARA Antagonism, NR1I2 Agonism, NR1I3 Agonism, ER Agonism, SREBF1 Agonism, NR1I2 Antagonism, AR Antagonism, THRB Antagonism, ER Antagonism, PPARA Agonism, NFE2L2 Agonism, NR3C1 Antagonism, NR1H4 Agonism, PPARG Agonism, and AR Agonism with TPO Antagonism has the greater ToxPi score. AOs caused by BPA exposure includes immune system disorders, reproductive system cancers and other cancers, reproductive system disorders, growth and developmental disturbances, breast cancer, liver lesions, neurological developmental disturbances and population trajectory alteration. The results of the machine learning analysis showed that important biomarkers in BPA-related AOP network including vitellogenin concentration, thyroxine concentration, E2 concentration in blood, hippocampal gene expression, fatty acid in liver, testosterone in ovary, etc. In zebrafish embryo model, 96 h-LC50 value of BPA was 9.4 mg/L. Because of the limitations of the AOP and zebrafish embryo models, the changes in genetic biomarkers were not significant for most MIEs and KEs. However, some genes can still be used as biomarkers, such as ESR1, ESR2B, THRB. The AOP-based risk assessment approach is a promising method to predict AOs of EDCs and identify important biomarkers of KEs.

Supplementary material

Table S5 PPR of KEs in BPA-related AOP network and selected biomarkers.

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Chapter 6.   Conclusion and Perspectives

6.1. Conclusion

EDC is a group of chemicals that are widely used in our daily lives. Because of the potentially profound and extensive adverse effects of EDCs on human health and ecosystems. Government agencies in different countries have implemented relevant policies and measures to regulate EDCs. However, the complex mechanism, transgenerational effects, low dose effects and non-monotonic dose-response of EDCs challenge the traditional risk assessment methods. The lack of toxicological data for emerging chemicals also poses an obstacle to chemical regulation. With the trend toward banning animal testing, an efficient, systematic method for evaluating the combined risks of EDCs based on computer models is needed to support EDC regulation. This study developed an *in silico* model to assess the multiple toxicity of EDC combining TaxCast data, AOP and machine learning.

In this research, there are three main conclusions are as follows:

1.   Exposure risk of BPA for the general Chinese population

Exposure risk of BPA in the general Chinese population were evaluated based BPA urine concentration data derived from the literature and a simple metabolic model. HQ values were calculated to assess the exposure risk. The results showed that the average exposure level of BPA for infants, pregnant women, children, and adults were 30.92 ± 22.70, 24.85 ± 9.40, 34.13 ± 20.65, and 22.48 ± 16.21 ng/kg bw/day, respectively. Although the average HQ is less than 1 indicating that the BPA exposure risk of Chinese population is not high. However, the high exposure levels of children and pregnant women suggest the need to focus on the exposure of sensitive populations.

2.   Development of a risk assessment method for EDC integrating TaxCast data, AOP and machine learning

In this study, MIEs of 40 EDCs were collected and determined based on data collected from the ToxCast database. The MIEs identified included ER, AR, PPARA, THRB, TPO, AHR, NR1I2, etc. The MIEs were quantified and ranked by ToxPi score, and the results indicated that TBT, ZI and BPAF have a greater potential to interact with MIEs. AOP networks covering 48 AOPs, 22 MIEs, 39 AOs and 164 KEs were established based on the MIE-related AOP information collected from the AOP-Wiki. The relationship between AOs and MIEs and the possibility of AOs were analyzed by a random walk method, and the results showed that 40 EDCs had a higher possibility of causing liver and reproductive system damage. Combining the results of ToxPi score and PageRank score, the toxicity profiles of 40 EDCs were depicted and validated by comparing *in vivo* assay data from ToxRefDB, including reproductive toxicity, developmental toxicity, hepatotoxicity, neurotoxicity and cancer, etc. The threshold of hepatotoxicity and reproductive toxicity were 0.80 and 0.36. The AOP-based *in silico* model was applicable to predict hepatotoxicity and reproductive toxicity of EDC with predictivity of 0.86 and 0.73.

3. Development of a risk assessment method for BPA combining AOP, machine learning and zebrafish embryo model

A BPA-related AOP network covering 43 AOPs, 13 MIEs, 31 AOs and 147 KEs was established based on MIE information collected from ToxCast database and AOP information collected from AOP-Wiki database. Through the network analysis, BPA-induced MIEs include TPO, PPARA, NR1I2, NR1I3, ER, AR, THRB, ect. BPA-induced AOs include immune system disorders, reproductive system cancers and other cancers, reproductive system disorders, growth and developmental disturbances, breast cancer, liver lesions, neurological developmental disturbances and population trajectory alteration. A random walk with restart approach was used to analyze the important KEs in the AOP network. Important KE biomarkers in the BPA-AOP network include vitellogenin concentration, thyroxine concentration, E2 concentration in blood; hippocampal gene expression, fatty acid in liver, and testosterone in ovary, etc. Some

KE genetic biomarkers in the AOP network were selected to test in zebrafish embryo model. The non-significant effects of BPA on the genetic markers of KEs may be related to the complexity of the AOP and the limitations of the zebrafish embryo model. Some biomarkers were identified for MIEs including ESR1, ESR2B, THRB.

Overall, combining ToxCast data, AOP and machine learning, a method to evaluate the risk of EDC was developed to rapidly and systematically predict the possible AOs and their toxicity of EDC as well as to predict the important pathways and their biomarkers. The model was validated by the results of *in vivo* assays and zebrafish embryo models. This study provides a useful reference for applying novel approaches to support government management of EDC.

6.2. Perspectives

Some chemicals have not been tested *in vitro* assays, resulting in a lack of data in ToxCast database. Therefore, in the future, other databases are needed to support the determination of MIE.

The AOP database is still developing and there are many AOP pathways waiting to be explored. The imperfection of AOP may bring uncertainty to the analysis results. In addition, the terminology of AOP is not standardized, which causes the duplication of events and also affects the construction of AOP network. Therefore, the future development of AOP should have a unified standard and be more comprehensive.

The random walk method used in this study has some limitations, for example, when analyzing networks with different sizes, the results need to be normalized for comparison. Therefore, in the future, more advanced *in silico* models such as deep learning can be used to explore the intrinsic connections between events in AOP networks.

The BPA-related AOP network integrates multiple species, and species differences may lead to bias in the results. In the future, hierarchical AOP networks can be established

to assess health risk and ecological risk separately. A multilayer machine learning approach is also needed to analyze multilayer AOP networks.

Although zebrafish share 70% of human genes, there are still genes that cannot be detected by the zebrafish model, such as NR1I3, and the zebrafish embryo model only reflects the gene level of the whole body and cannot distinguish the gene level of each organ. In addition, gene expression is affected by many factors, such as negative feedback loops. Therefore, in the future, other models with various endpoints can be chosen as KE biomarker, such as adult zebrafish model and protein level, blood hormone level, organ changes, etc.

Supplementary Material

Table S1 Urinary concentration data of BPA in China

Region	Sampling time	Characteristics of study subjects	Age range /average (years)	Urine sample size	Sample type	Urinary total BPA concentration (ng/ml)		Detection rate (%)	Analysis equipment b	LOD(LOQ) (ng/ml)	Ref.
						Average	Range/(25th-75th percentile)/[5th-95th percentile]				
Infant											
Zhoushan	March 2012 to December 2014	infants	<6 months	48	disposable diaper	0.13(median)	<LOD-5.04	93	HPLC-MS/MS	0.048	1
Xiamen	March to April 2011	infants	<3	40	disposable diaper	1.55(median)	0.58-76.3	-	HPLC-MS/MS	0.27	2
Pregnant woman											
Laizhou Wan	September 2010 to December 2013	pregnant women	28.1	506	spot	0.48(median)	<LOD-216.56	86.6	HPLC-MS/MS	0.1	3
Suzhou and Kunshan	August 2008 to November 2011	pregnant women	28.04	162	morning spot	0.58(median)	-	82.1	LC-MS/MS	0.20	4
Wuhan	2014 to 2015	pregnant women	28.6	2823	spot	1.18(median)	(0.14-3.45)	72.0	UPLC-MS/MS	0.04	5
Wuhan	November 2012 to April 2014	pregnant women	28.9	412	spot	2.45(median)	<LOD-201.05	90	UPLC-MS/MS	0.2	6
Wuhan	July 2011 to June 2012	pregnant women	28.7	322	first morning spot	0.96(median)	(0.37-2.12)	92.2	HPLC-MS	0.04	7
Wuhan	October 2013 to April 2015	pregnant women	28.58	1841	spot	1.11(median)	(0.27-2.66)	79.25	UHPLC-MS	0.2	8

Location	Period	Population	Age	N	Sample	Median	Range	%	Method	LOD	Ref
Sheyang	June 2009 to January 2010	pregnant women	26.4	386	spot	1.75(median)	0.16-224	100	GC-MS/MS	0.01	9
Qingyuan	April to August 2017	pregnant women	18-40	15	spot	0.9(median)	(0.5-1.3)	100	LC-MS/MS	(0.01-0.2) a	10
Shanghai	April to December 2012	pregnant women	28	982	spot	1.09(median)	(0.40-2.1)	77.9	HPLC	0.31	11
Shanghai	2012 to 2013	pregnant women	30	620	spot	1.24(median)	<LOD-154.60	98.9	HPLC-MS/MS	0.1	12
Guiyu and Haojiang	September 2010 to September 2011	pregnant women	27.50	137	spot	2.6(median)	<LOD-16.4	78.8	GC-MS	0.12	13
Nanjing	September 2010 to April 2012	pregnant women	27.8	567	spot	0.67(median)	<LOD-355.33	60	UPLC-MS/MS	0.36	14
Tianjin	October 2017 to January 2018	pregnant women	29.5	390	spot	0.51(median)	0.11-14.58	100	GC-MS/MS	0.014	15
Child											
Guangzhou	July 2014	kindergarten children	3-6	100	spot	1.44(median)	<LOD-37.1	87.0	HPLC-MS/MS	0.20	16
Guangzhou	-	students	3-6	56	morning spot	1.38(median)	(1.02-2.66)	100	GC-MS	(0.0018)	17
Guangzhou	-	students	7-12	95	morning spot	3.69(median)	(3.05-4.90)	100	GC-MS	(0.0018)	17
Guangzhou	-	students	13-17	72	morning spot	3.17(median)	(2.08-4.66)	100	GC-MS	(0.0018)	17
Guangzhou	September 2015	school children	3-7	70	first morning spot	1.60(median)	<LOQ-31.1	100	HPLC-MS/MS	(0.10)	18
Guangzhou	May 2014 to September 2017	children	6-12	465	morning spot	2.97(median)	<LOD-58.9	90.8	HPLC-MS/MS	0.25	19
Yangtze River Delta	March to May 2012	school children	9–12	666	first morning spot	1.00(median)	<LOD-326.00	98.9	UPLC-MS/MS	0.06	20

Location	Period	Population	Age	N	Sample	Concentration	Range	Detection %	Method	LOD	Ref
Shanghai	July 2011 to September 2012	girls with idiopathic central precocious puberty	8.13	136	morning spot	6.88(median)	<LOD-40.73	83.8	HPLC	0.5	21
Shanghai	July 2011 to September 2012	girls, healthy volunteers	8.14	136	morning spot	1.02(median)	<LOD-15.73	58.8	HPLC	0.5	21
Shanghai	2011 to 2012	school children	9-18	754	spot	1.6(GM)	-	-	HPLC	0.31	22
Shanghai	May 2011 to June 2011	school boys	9–18	671	spot	2.06(median)	(<LOD-7.94)	62.7	HPLC	0.31	23
Shanghai	January 2012	school children	8-15	259	first morning spot	0.6(median)	0.05-16.3	84.9	UPLC-MS/MS	0.07	24
Shenzhen	September 2015	school children	8-11	213	first morning spot	0.25(median)	<LOQ-3.05	91	HPLC-MS/MS	(0.10)	18
East China	2012 to 2014	school children	7-11	818	first morning spot	1.69(median)	<LOD-94.1	97.9	UHPLC-MS/MS	0.13	25
Nanjing	November 2016	preschool-aged children	3-5	80	morning spot	0.369(median)	<LOD-3.0358	97.5	HPLC-MS/MS	(0.01)	26
Tianjin	April to May 2014	school children	8-10	256	spot	1.58(GM)	<LOD-24.9	99.2	LC-MS/MS	(0.05)	27
Hongkong	2016	kindergarten children	4-6	31	spot	1.69(median)	<LOD-7.15	77	HPLC-MS/MS	0.06	28
Sheyang	2012	preschool-aged children	3	229	spot	1.04(median)	0.19-22.9	100	GC-MS/MS	0.01	29
Sheyang	2016	school children	7	412	spot	1.41(median)	<LOD-770	99.3	GC-MS/MS	0.01	29
Sheyang	August 2019	children	9.89	386	spot	1.29(median)	<LOD-440	95.1	GC-MS/MS	0.01	9
Adult											
Shanghai	June to August 2009	residents, age>40	59	3423	morning spot	0.81(median)	(0.47-1.43)	-	HPLC-MS/MS	0.30	30

Location	Time period	Population	Age	N	Sample type	Concentration	Range	Detection %	Method	LOD/LOQ	Ref
Shanghai	1998 to 1999	residents, women	40-72	50	spot	0.622(GM)	[< LOD-2.30]	65.3	HPLC-MS/MS	0.2	31
Shanghai	2006	residents, women	40-72	50	spot	0.661(GM)	[< LOD-2.10]	65.3	HPLC-MS/MS	0.2	31
Shanghai	2006	residents, women	40-72	50	spot	0.888(GM)	[< LOD-3.80]	80.0	HPLC-MS/MS	0.2	31
Shanghai	2003 to 2004	residents, men	40-72	50	spot	0.683(GM)	[< LOD-2.20]	75.0	HPLC-MS/MS	0.2	31
Shanghai	2006	residents, men	40-72	50	spot	0.774(GM)	[< LOD-3.40]	74.0	HPLC-MS/MS	0.2	31
Shanghai	2006	residents, men	40-72	50	spot	0.794(GM)	[< LOD-1.90]	86.0	HPLC-MS/MS	0.2	31
Shanghai	-	general women	20-41	123	spot	<LOD(median)	0.01-161	39.8	GC-MS/MS	0.08	32
Shanghai	March to May 2013	residents, age>40	>40	1326	morning spot	1.10(median)	(0.64-1.89)	89.1	LC-MS	0.3	33
Shanghai	2013 to 2015	women	29	700	spot	1.29(median)	<LOD-81.42	98.3	HPLC-MS/MS	0.1	34
Shanghai	-	women	20-55	246	morning spot	2.27(mean)	-	100	LC-MS/MS	0.3	35
Tianjin	May 2010 to June 2010	adults	22-62	50	morning spot	1.63(median)	<LOQ-8.70	84.0	HPLC-MS/MS	(0.10)	36
Qingyuan	July to August 2014	residents in an e-waste recycling region	-	116	morning spot	3.00(median)	0.233-27.6	100.0	UPLC-MS/MS	(0.05)	37
Qingyuan	July to August 2014	residents in rural area	-	22	morning spot	0.648(median)	<LOQ-4.12	91.0	UPLC-MS/MS	(0.05)	37

Location	Time	Population	Age	N	Sample type	Concentration	Range	%	Method	LOD	Ref
Entire country	September 2010	young adults	18-22	109	first morning spot	2.00(median)	0.19-23.9	100.0	HPLC-MS/MS	(0.03)	38
Suzhou and Kunshan	August 2008 to November 2011	women with recurrent miscarriage	28.36	102	morning spot	1.66(median)	-	85.29	LC-MS/MS	0.20	4
Guangzhou	July to August 2014	residents in urban area	18-60	20	morning spot	1.42(median)	<LOQ-4.07	80.0	UPLC-MS/MS	(0.05)	37
Guangzhou/Shanghai/Harbin	May to July 2010	general population	31	116	spot	1.10(median)	<LOQ-29.4	-	HPLC-MS/MS	(0.1)	39
Guangzhou	-	students	18-24	64	morning spot	2.41(median)	(1.21-4.14)	100	GC-MS	(0.0018)	17
Guangzhou	January 2016	university students	19-34	169	first morning spot	0.33(median)	<LOD-12.7	59	LC-MS/MS	0.2-0.5 a	40
Guangzhou	March 2018	university students	19-25	160	spot	3.57(median)	<LOQ-90.4	99	LC-MS/MS	(0.02)	41
Wuhan	October 2016 and August 2018	healthy volunteers	59.2	615	spot	1.03(median)	(0.55-2.14)	98.4	UHPLC-MS	0.031	42
Wuhan	October 2016 and August 2018	adults with non-small cell lung cancer	58.0	615	spot	1.31(median)	(0.51-4.11)	97.2	UHPLC-MS	0.031	42
Wuhan	May 2016 to May 2018	general population	56	1437	morning spot	0.598(median)	(<LOD-0.970)	61.59	HPLC-MS	(0.1)	43
Jinan	February 2013 to September 2013	53 papillary thyroid carcinoma patients and 60 nodular goiter patients and 65 healthy volunteers	-	178	morning spot	4.18(median)	0.05-34.46	83.0	HPLC-MS/MS	(0.1)	44

Location	Date	Population	Age	N	Sample	Concentration	Range	%	Method	LOD/LOQ	Ref
Jinan	2015 to 2016	general women	20-40	111	first morning spot	0.95(median)	<LOD-9.93	99.1	UPLC-MS/MS	0.1	45
Jinan	June to October 2014	infertile women with Polycystic Ovarian Syndrome	27	268	spot	2.35(median)	(1.47-3.95)	100	HPLC-MS/MS	0.1	46
Sandu	July to August 2012	adult men	19-54	560	spot	0.38(median)	[0.08–6.96]	70.4	HPLC	0.12	47
East and middle China	-	factory workers and their family members (without occupational exposure)	33.9	922	spot	0.87(GM)	(<LOD-5.26)	50	HPLC	0.31	48
Shenzhen	September 2016 to June 2017	residents	>18	183	first morning spot	4.32(median)	<LOD-35.2	98.4	LC-MS/MS	(0.1)	49
Nanjing	-	women with unexplained recurrent spontaneous abortion	20-40	30	spot	4.14(median)	<LOQ-31.3	96.7	UPLC-MS/MS	(1.8)	50
Nanjing	-	women, healthy volunteers	20-40	30	spot	3.42(median)	<LOQ-9.4	96.7	UPLC-MS/MS	(1.8)	50
Nanjing	2013 to 2014	men	18-43	364	spot	0.45(median)	<LOD-39.99	58	UPLC-MS/MS	0.36	51
Nanjing	-	men whose spouse with unexplained spontaneous abortion	30.20	80	morning spot	0.53(median)	-	65.7	UPLC-MS/MS	0.36	52

Location	Time period	Population	Age	N	Sample	Concentration	Range	Detection %	Method	LOD	Ref
Nanjing	-	women with unexplained spontaneous abortion	28.15	80	morning spot	0.64(median)	-	67.1	UPLC-MS/MS	0.36	52
Nanjing	-	men, healthy volunteers	30.03	170	morning spot	0.65(median)	-	63.3	UPLC-MS/MS	0.36	52
Nanjing	-	women, healthy volunteers	27.22	170	morning spot	1.04(median)	-	63.9	UPLC-MS/MS	0.36	52
Nanjing	March 2005 to April 2010	men, healthy volunteers	29.83	713	morning spot	0.522(median)	-	62.4	UPLC-MS/MS	0.36	53
Nanjing	March 2005 to April 2010	men with idiopathic infertility	28.50	877	morning spot	0.492(median)	-	62.1	UPLC-MS/MS	0.36	53
Xuzhou	April 2017 to February 2018	women with thyroid nodules and healthy volunteers	44.46	1416	first morning spot	1.35(median)	(0.83-2.34)	99.7	HPLC-MS/MS	0.1	54
Hangzhou	January 2015 to September 2018	women with premature ovarian insufficiency	34.48	159	spot	0.57(median)	<LOD-19.169	78.62	UPLC-MS	0.014	55
Hangzhou	January 2015 to September 2018	healthy volunteers, women	33.58	186	spot	0.616(median)	<LOD-28.437	74.19	UPLC-MS	0.014	55
Hangzhou	September 2013 to October 2016	women	31.0	351	spot	0.378(median)	<LOD-12.862	83.47	HPLC-MS/MS	0.1	56
South China	June 2013	residents residing near a BPAF	26-84	94	spot	0.900(median)	<LOD-4.380	>80	UPLC-MS/MS	0.090	57

	manufacturing
	plant

GM: Geometric Mean. LOD: Limit of Detection. LOQ: Limit of Quantification.

a The LOD/LOQ of several compounds including BPA.

b U(ultra) H(high) P(performance) L(liquid) C(chromatography) G(gas) M(mass) S(spectrometry)

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Table S2 Daily urinary excretion volume and body weight used in calculation

Reference values for daily urinary excretion a, d		
Age	Excretion (ml/day)	
	male	female
New born	300	300
1 year	400	400
5 years	500	500
10 years	700	700
15 years	1200	1200
Adult	1600	1200

Reference values for body weight b, d					
Age (years)	Body weight (kg)		Age (years)	Body weight (kg)	
	male	female		male	female
3	16.6	15.9	17	63.3	53.0
4	18.3	17.5	18	63.5	52.6
5	20.6	19.6	19	63.5	52.4
6	23.0	21.6	20~24	67.2	53.8
7	26.6	24.7	25~29	70.4	55.3
8	29.9	27.6	30~34	71.4	56.8
9	33.6	31.3	35~39	71.5	57.8
10	37.2	35.5	40~44	71.2	59.0
11	41.9	40.6	45~49	71.2	59.7
12	46.6	44.5	50~54	70.6	60.4
13	52.0	48.0	55~59	69.1	59.6
14	56.2	50.4	60~64	67.6	59.7
15	59.5	51.6	65~69	66.6	59.2
16	61.5	52.7			

Reference values for infant c	
Age (years)	Excretion (ml/day)/Body weight (kg)
0~1	79.3
0~5	35.9

a   Valentin J. Basic anatomical and physiological data for use in radiological protection: reference values: ICRP Publication 89[J]. Annals of the ICRP, 2002, 32(3-4): 1-277.

b   National Bureau of Statistics (China). 2014 National Physique Monitoring Bulletin. 2015. Available from the website of General Administration of Sports of China: http://www.sport.gov.cn/n315/n329/c216784/content.html.

c   Heffernan A L, Aylward L L, Toms L M L, et al. Age-related trends in urinary excretion of bisphenol A in Australian children and adults: evidence from a pooled sample study using samples of convenience[J]. Journal of Toxicology and Environmental Health, Part A, 2013, 76(18): 1039-1055.

d   If a study included both men and women as study subjects, daily urinary excretion and body weight were averaged between men and women.

Table S3 Urinary concentration data of BPA in different countries

Country	Sampling time	Characteristics of study subjects	Urine sample size	Urinary BPA concentration (ng/ml)	Ref.
Infant					
United States	2012-2013	3-27 days	78	0.27(median); 0.79(mean) a	1
Canada	2009–2011	0-3 months	100	0.2(median); <LOD(GM)	2
Germany	2008	1-5 months	91	<LOQ(median)	3
Switzerland	2019	6-36 months	109	2.40(mean)	4
Korea	2012-2013	9-15 months	187	0.9(median); 1.3(GM)	5
Pregnant woman					
Netherlands	2002-2006	30.5 years	1267	1.61(median)	6
Sweden	2007-2010	30.9 years	1996	1.51(median)	7
Denmark	2011-2012	30.7 years	565	1.52(median); 1.17(GM)	8
Canada	2008–2011	>18 years	1936	0.82(median); 0.80(GM)	9
Spain	2004-2006	≥16 years	479	2.0(median); 2.1(GM)	10
France	2003-2006	29.7 years	520	2.5(median)	11
Korea	2007-2010	-	757	1.08(median); 1.29(GM)	12
Mexico	1994-2004	boys' mother	49	0.6(median); 0.7(GM)	13
Mexico	1994-2004	girls' mother	50	0.7(median); 0.8(GM)	13
United States	2009–2010	16-44 years	506	1.3(median); 1.4(mean)	14
Child					
United States	2013-2016	6-19 years	1831	1.3(median)	15
Japan	2012-2017	7 years	396	0.89(median)	16
Slovenia	2011-2012	6-11 years	145	2.39(median); 1.81(GM)	17
Brazil	2012-2013	6-14 years	300	1.66(median); 1.74(GM)	18
Korea	-	8-11 years	1008	1.23(median)	19
Australia	2012-2013	2-4 years	100	2.74(median); 2.72(GM)	20
Denmark	2011	6-11 years	143	1.7(median)	21
Germany	2003-2006	3-14 years	599	2.74(median); 2.66(GM)	22
Spain	2011–2012	9-10 years	296	4.76(median); 4.58(GM)	23
Belgium	2008–2009	14-15 years	193	2.21(median); 2.22(GM)	24
Greece	2007-2008	4.24 years	500	1.2(median); 1.1(GM)	25
India	2012-2013	2-14 years	76	7.43(mean); 5.08(GM)	26
Egypt	2009	10-13 years	57	0.70(median); 0.84(GM)	27
Canada	2016-2017	3-5 years	547	0.99(median); 0.94(GM)	28
Canada	2016-2017	6-11 years	516	0.94(median); 0.97(GM)	28
Canada	2016-2017	12-19 years	524	0.96(median); 0.96(GM)	28
Mexico	2010	8-13 years, boys	53	1.2(median); 1.1(GM)	13
Mexico	2010	8-13 years, girls	55	1.1(median); 1.2(GM)	13
Adult					
Canada	2016-2017	20-39 years	362	1.0(median); 0.84(GM)	28
Canada	2016-2017	40-59 years	348	0.79(median); 0.73(GM)	28

Canada	2016-2017	60-79 years	350	0.79(median); 0.77(GM)	28
United States	2013-2014	20-39 years	601	1.5(median)	29
United States	2013-2014	40-59 years	609	1.2(median)	29
United States	2013-2014	60-79 years	490	1.1(median)	29
Korea	2012-2014	20-39 years	1547	1.29(median)	29
Korea	2012-2014	40-59 years	2665	1.13(median)	29
Korea	2012-2014	60-79 years	2139	0.85(median)	29
Japan	2000-2001	24-43 years, women	140	1.57(median)	30
Denmark	2009	20.0 years, men	100	2.27(median)	31
Denmark	2013	20.0 years, men	100	1.44(median)	31
Denmark	2017	20.0 years, men	100	1.33(median)	31
Germany	1995-2009	20-30 years	600	1.49(median); 1.55(GM)	32
India	2010	45 years	21	1.97(mean); 1.59(GM)	33
Kuwait	2010	23 years	32	4.10(mean); 1.24(GM)	33
Malaysia	2010	30 years	29	1.89(mean); 1.00(GM)	33
Vietnam	2010	49 years	30	3.32(mean); 1.42(GM)	33
Italy	1998-2000	20-74 years	720	3.5(median); 3.59(GM)	34
Israel	2011	20-74 years	246	2.99(median); 2.39(GM)	35
Sweden	-	70 years	1016	3.76(mean)	36
Finland	2011	22-67 years	121	1.8(median); 1.9(GM)	37
France	2013-2014	21-59 years	195	3.54(median); 3.52(GM)	38
United Kingdom	1993-1998	63.8 years, healthy volunteers	861	1.24(median); 1.23(GM)	39
United Kingdom	1993-1998	64.1 years, Coronary Artery Disease patients	758	1.35(median); 1.39(GM)	39
Australia	2011	45.9 years	420	2.61(GM)	40

a: urinary BPA-glucuronide concentration.

GM: Geometric Mean. LOD: Limit of Detection. LOQ: Limit of Quantification.

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Table S4 Toxicity, AO and endpoint classification

Toxicity	AO No.	AO name	Endpoints in ToxRefDB	
Developmental toxicity	AO1786	Increase, retained nipple in male	development	nipple development
	AO947	Increase, Early Life Stage Mortality	reproductive	viability
	AO1688	short male AGD	development	anogenital distance (agd)
	AO1616	Malformation, cryptorchidism	testes	epididymis small
			testes	testes small
			testes	testes degeneration
	AO864	Decreased, Body Weight	systemic	body weight
	AO1447	obesity	systemic	body weight gain
	AO1001	Increased, Developmental Defects	development	developmental malformation
Reproductive toxicity	AO406	impaired, Fertility	reproductive	fertility
	AO405	irregularities, ovarian cycle/impaired, Fertility	reproductive	reproductive performance
	AO972	Decreased fertility, Reduced number of oocytes ovulated	ovary	follicle count
	AO337	N/A, Impairment of reproductive capacity	reproductive	reproductive performance
Immunotoxicity	AO1714	Exacerbation of SLE	(-)	(-)
	AO323	Increased, Disease susceptibility	(-)	(-)
Hepatotoxicity	AO1395	Liver Cancer	Liver	hepatocellular carcinoma
	AO1418	Increased, Steatosis	Liver	hypertrophy
	AO345	N/A, Liver Steatosis	Liver	hypertrophy
	AO459	Increased, Liver Steatosis	Liver	hypertrophy
	AO455	Accumulation, Liver lipid	Liver	fatty change
	AO719	Increase, hepatocellular adenomas and carcinomas	Liver	hepatocellular carcinoma
	AO856	Formation, Hepatocellular and Bile duct tumors	Liver	cholangio carcinoma

				hepatocellular carcinoma
Pulmonary toxicity	AO1276	Lung fibrosis	(-)	(-)
	AO1458	Pulmonary fibrosis	(-)	(-)
Neurotoxicity	AO1514	Neurodegeneration	nerve	degeneration
	AO402	Cognitive Function, Decreased	(-)	(-)
Tumor/Cancer	AO1193	N/A, Breast Cancer	mammary gland	adenoma/carcinoma combined
				adenocarcinoma
	AO1395	Liver Cancer	Liver	hepatocellular carcinoma
	AO719	Increase, hepatocellular adenomas and carcinomas	Liver	hepatocellular carcinoma
	AO856	Formation, Hepatocellular and Bile duct tumors	Liver	cholangio carcinoma
	AO1053/1054	Promotion, ovarian adenomas/Promotion, ovarian granular cell tumors	ovary	cyst
	AO741	Increase, Adenomas/carcinomas (follicular cell)	reproductive	adenoma
			thyroid gland	adenoma/carcinoma combined
	AO773	Increase, Endometrial adenocarcinomas	uterus	adenocarcinoma
	AO1070	Increased, adenosquamous carcinomas of endometrium	uterus	squamous cell carcinoma
	AO1063	Increased, Pancreatic acinar tumors	(-)	(-)
	AO1839	Testicular Cancer	testes	testes cyst
	AO745	Increase, Leydig cell tumors	testes	interstitial cell tumor benign
	AO1035/1036/1037	Increased, Firbrosarcoma/liposarcoma/hemagiosarcoma	systemic	hemangiosarcoma
Others	AO1893	increase, Preeclampsia	(-)	(-)
	AO369	Uroporphyria	(-)	(-)

Table S5 PPR of KEs in BPA-related AOP network and selected biomarkers

KE	Name	Biological Organization	PPR	Biomarker
454	Increased, Triglyceride formation	Hepatocyte	0.02639	
716	Increase, cell proliferation (hepatocytes)	Hepatocyte	0.022351	
756	Hippocampal gene expression, Altered	Brain	0.020124	BDNF, GNRHR1
327	Accumulation, Fatty acid	Liver	0.018553	DGAT2
462	Up Regulation, SCD-1	Hepatocyte	0.018427	SCD-1
221	Reduction, Plasma vitellogenin concentrations	Gonadotropin Releasing Neuron	0.017256	
757	Hippocampal anatomy, Altered	Brain	0.017106	
479	Activation, NR1H4	Hepatocyte	0.017102	
1214	Altered expression of hepatic CAR-dependent genes	Hepatocyte	0.017102	
285	Reduction, Vitellogenin synthesis in liver	(-)	0.016471	VTG1
281	Thyroxine (T4) in serum, Decreased	Serum	0.014643	DIO1
758	Hippocampal Physiology, Altered	Brain	0.014536	
471	Inhibition, FoxA2	Eukaryotic Cell	0.010261	FOXA2
774	Increase, Preneoplastic foci (hepatocytes)	Hepatocyte	0.009497	
1171	Increase, Clonal Expansion of Altered Hepatic Foci	Hepatocyte	0.009497	
1056	Decrease, E2 blood concentrations at hypothalamus	Corpus Luteum	0.008551	
219	Reduction, Plasma 17beta-estradiol concentrations	Blood Plasma	0.007661	
807	Decreased, cholesterol	Blood plasma	0.007363	
1170	Increase, Phenotypic enzyme activity	Hepatocyte	0.007363	
1047	Increased, secretion of GnRH from hypothalamus	Epithelium Of Female Gonad	0.007268	GNRHR1
3	Reduction, 17beta-estradiol synthesis by ovarian granulosa cells	Granulosa Cell	0.006875	
179	Decreased, Mitochondrial fatty acid beta-oxidation	Liver	0.006793	
465	Increased, FA Influx	Hepatocyte	0.006691	

447	Reduction, Cholesterol transport in mitochondria	Steroid hormone Secreting cell	0.006628	
451	Inhibition, Mitochondrial fatty acid beta-oxidation	Hepatocyte	0.006413	
1000	stabilization, PPAR alpha co-repressor	Eukaryotic cell	0.006413	
1756	Decreased, plasma 11-ketotestosterone level	Blood plasma	0.00626	
309	Reduction, Vitellogenin accumulation into oocytes and oocyte growth/development	Gonadotropin Releasing Neuron	0.005866	
413	Reduction, Testosterone synthesis in Leydig cells	Testosterone Secreting Cell	0.005633	
286	Altered, Transcription of genes by AR	Eukaryotic Cell	0.005558	
858	Decreased, PPARalpha transactivation of gene expression	Eukaryotic cell	0.005451	
54	Up Regulation, CD36	Hepatocyte	0.00513	
307	Increase, Vitellogenin synthesis in liver	Liver	0.00513	VTG1
1029	Increased, adipogenesis	Eukaryotic cell	0.00513	
1065	Activation, estrogen receptor alpha	(-)	0.00513	
266	Decrease, Steroidogenic acute regulatory protein (STAR)	Steroid hormone secreting cell	0.004909	
1058	Decreased, bile flow	Liver	0.004909	
274	Reduction, Testosterone synthesis by ovarian theca cells	Theca Cell	0.004431	
220	Increase, Plasma vitellogenin concentrations	Blood Plasma	0.004361	
472	Down Regulation, CPT1A	Eukaryotic Cell	0.004361	CPT1AL, CPT1AM
474	Down Regulation, HMGCS2	Eukaryotic Cell	0.004361	
1032	Increased, secretion of local growth factors	Eukaryotic cell	0.004361	
1066	Promotion, SIX-1 postive basal-type progenitor cells	Basal Cell	0.004361	
140	Decreased, HSD17B10 expression	Hepatocyte	0.004275	
277	Thyroid hormone synthesis, Decreased	Thyroid follicular cell	0.004275	
861	Decreased, Ketogenesis (production of ketone bodies)	Eukaryotic cell	0.004216	

1059	Increased, cholestasis	Bile duct	0.004173	
1051	Hyperplasia, ovarian stromal cells	Blood	0.003939	
252	Increase, Renal pathology due to VTG deposition	Kidney	0.003706	
477	Decreased, Ketogenesis	Eukaryotic Cell	0.003706	
1067	Proliferation/Clonal Expansion, aberrant basal cells	Basal Cell	0.003706	
8	Decreased, 3-hydroxyacyl-CoA dehydrogenase type-2 activity	Hepatocyte	0.003634	
862	Not Increased, Circulating Ketone Bodies	Blood	0.003585	
280	Thyroxine (T4) in neuronal tissue, Decreased	Brain	0.003556	
1060	Alteration, lipid metabolism	Eukaryotic cell	0.003547	
1711	Induction of GATA3 expression	Immune System	0.00342	GATA3
1449	increased adipogenesis	Liver	0.003164	
1068	squamous metaplasia, aberrant basal cells	(-)	0.003151	
1615	Impaired inguinoscrotal phase	(-)	0.003117	
1049	Increased, secrection of FSH from anterior pituitary	(-)	0.003088	
1050	Increased, secretion of LH from anterior pituitary	Dopaminergic Neuron	0.003088	
863	Increased, Catabolism of Muscle Protein	Musculoskeletal system	0.003047	
1061	prolonged, elevation of serun CCK	Serum	0.003015	
1712	Increase of Th2 cells producing IL-4	T-Helper 2 Cell	0.002907	
1791	Increased, Male Biased Sex Ratio	(-)	0.002876	
1528	Fatty Acid Beta Oxidation, Decreased	Eukaryotic cell	0.002779	
1069	Increased, Hyperplasia (glandular epithelial cells of endometrium)	Glandular Epithelial Cell	0.002679	
1062	Increased, Cellular proliferation / hyperplasia of acinar cells	Acinar cell	0.002563	
1713	Increase of autoantibody production	B Cell	0.00247	
483	Activation, LXR alpha	Hepatocyte	0.002454	
240	Feminisation or incomplete development, Primary and accessory male sex organs	Male Reproductive System	0.002417	
446	Reduction, testosterone level	Blood	0.002394	

1690	reduction, testosterone levels	(-)	0.002394	
310	Alteration, Wnt pathway	Eukaryotic Cell	0.002149	
458	Increased, De Novo FA synthesis	Hepatocyte	0.002087	
1033	Increased, proliferation of mesenchymal cells	Mesenchymal cell	0.001853	
1034	Increased, IGF-1 (mouse)	Eukaryotic cell	0.001853	
1003	Decreased, Triiodothyronine (T3) in serum	Serum	0.001778	
1023	Increased, Thyroid-stimulating hormone (TSH)	Serum	0.001778	
749	Decreased, Progesterone from corpus luteum	Oocyte	0.00171	
1182	Increase, Cell Proliferation (Epithelial Cells)	Epithelial Cell	0.00171	
1448	activation of CCAAT/enhancer-binding protein alpha	Hepatocyte	0.00171	
743	Decreased, Testosterone binding to androgen receptor (hypothalamus)	Hypothalamus Native Cell	0.001558	
1614	Decrease, AR activation	(-)	0.001558	
129	Reduction, Gonadotropins, circulating concentrations	Blood Plasma	0.001555	
1790	Increased, Differentiation to Testis	Testis	0.001555	
739	Increase, Hypertrophy and proliferation (follicular cell)	Thyroid follicular cell	0.001511	
772	Increase, Hyperplasia (glandular epithelial cells of endometrium)	(-)	0.001454	
1183	Decreased, Apoptosis (Epithelial Cells)	Epithelial Cell	0.001454	
414	Increase, Luteinizing hormone (LH)	Leydig cell	0.001357	
754	Increased, Luteinizing hormone (LH)	Blood	0.001325	
1052	Hyperplasia, ovarian epithelium	(-)	0.001313	
740	Increase, Hyperplasia (follicular cells)	Thyroid follicular cell	0.001285	
177	N/A, Mitochondrial dysfunction 1	Eukaryotic Cell	0.001235	
1800	Reduced granulosa cell proliferation	Eukaryotic Cell	0.001181	
416	Increase proliferation, Leydig cell	Leydig cell	0.001153	
744	Increase, Hyperplasia (Leydig cells)	Leydig Cell	0.001126	
115	Increase, FA Influx	Hepatocyte	0.00109	
1088	Increased, Oxidative Stress	Eukaryotic Cell	0.00105	

1613	Decrease, DHT level	(-)	0.001018	
415	Hyperplasia, Leydig cell	Leydig cell	0.00098	
291	Accumulation, Triglyceride	Hepatocyte	0.000926	
1187	Increased, ER binding to DNA (classical pathway)	Eukaryotic Cell	0.000893	
176	Damaging, Mitochondria	Eukaryotic cell	0.000788	
1643	Altered, Visual function	(-)	0.000771	
1188	Increased, ER binding to T.F. to DNA (non-classical pathway)	Eukaryotic Cell	0.000759	
289	Decrease, Translocator protein (TSPO)	Steroid hormone secreting cell	0.000678	
1189	Increased, Proliferation (Endothelial cells)	Endothelial Cell	0.000645	
1007	Reduced, Anterior swim bladder inflation	Swim bladder	0.000604	
348	Malformation, Male reproductive tract	Male reproductive system	0.000577	
1190	Increased, Migration (Endothelial Cells)	Endothelial Cell	0.000548	
1035	Increased, Firbrosarcoma	Fibrous connective tissue	0.000525	
1036	Increased, liposarcoma	Adipose tissue	0.000525	
1037	Increased, hemagiosarcoma	Blood vessel endothelium	0.000525	
1005	Reduced, Swimming performance	(-)	0.000514	
1191	Increased, Non-genomic signaling	Epithelial Cell	0.000466	
1687	decrease, transcription of genes by AR	(-)	0.000442	
1192	Increased, Ductal Hyperplasia	Mammary Duct	0.000396	
1194	Increase, DNA Damage	Eukaryotic Cell	0.000336	
1640	Altered, Visual function	Retina	0.000302	
1877	Altered, retinal layer structure	Eye	0.000302	
1878	Decreased, Eye size	Eye	0.000302	
1195	modulation, Extracellular Matrix Composition	Eukaryotic Cell	0.000286	
1196	Increased, Invasion	Mammary Duct	0.000242	
1197	Activation, Fibroblasts	Fibroblast	0.000206	
1198	Activation, Macrophages	Macrophage	0.000175	
1213	Increased, Angiogenesis	Eukaryotic Cell	0.000149	

1239	Altered, Gene Expression	Eukaryotic Cell	0.000127	
1240	Altered, Protein Production	Eukaryotic Cell	1.06E-04	
1241	Increased, Motility	Eukaryotic Cell	9.09E-05	
1242	Increased, Second Messenger Production	Eukaryotic Cell	7.36E-05	
66	Activation, ChREBP	Hepatocyte	0	
167	Activation, LXR	Hepatocyte	0	
228	peroxisome proliferator activated receptor promoter demethylation	Hepatocyte	0	
456	Suppression, Constitutive androstane receptor, NR1l3	Hepatocyte	0	
463	Up Regulation, FAS	Hepatocyte	0	
470	Up Regulation, Acetyl-CoA carboxylase-1 (ACC-1)	Eukaryotic Cell	0	
484	Activation, AKT2	Hepatocyte	0	
486	inflammation	(-)	0	
746	Increase, Dopaminergic activity	Hepatocyte	0	
747	Decreased, Prolactin	Liver	0	
1045	Decreased, Ovarian E2	(-)	0	
1609	Inhibition of Cyp17A1 activity	(-)	0	CYP17A1
1610	Reduction, DHEA	(-)	0	
1611	Reduction, androstenedione	(-)	0	
1612	Decrease, testosterone level	(-)	0	
1617	5α-reductase, inhibition	(-)	0	

Figure S1 Heatmap of active in vitro assay of 40 EDCs

gene \ EDC	ATZ	DEHP	B(a)P	VZ	TES	TCDD	PFOS	DES	ZEN	p,p'-DDT	PNP	ES	TBT	TCL	ZI	p,p'-DDD	CHL	BPAF	tOP	TBBPA	TPT	PCP	4-NP	4-MBC	FLX	TPPA	BPA	E2	EE2	2,4-DHBP	SPF	BBP	GEN	PFOA	LNG	BPS	BPF	MP	D5-sil	PCB-153	Frequency of actived assay for 40 EDCs
AR	1	1	3	11	4	0	4	11	6	5	3	7	10	10	8	7	7	8	7	6	3	4	4	2	2	3	10	12	12	6	2	2	5	0	13	5	2	1	1	0	92.5
ESR1	0	1	4	2	3	2	4	16	15	8	8	7	8	4	6	6	6	16	16	7	2	5	0	1	1	6	15	15	14	13	12	10	14	3	10	13	6	3	1	0	92.5
ESR2	1	2	1	1	1	0	2	8	7	5	5	2	1	2	2	5	3	5	5	1	1	1	0	0	1	5	5	7	6	5	5	5	7	1	1	6	2	3	2	0	90
PGR	0	0	2	4	2	0	3	2	4	5	2	1	3	2	1	5	1	5	2	4	1	1	1	2	1	4	5	10	7	3	3	3	4	0	5	2	1	3	0	1	87.5
NR1I2	2	3	0	2	0	0	3	3	4	2	1	1	2	4	1	2	2	2	3	2	2	2	0	0	1	2	3	2	3	2	2	1	1	1	1	1	1	0	3	0	82.5
NR1I3	0	0	2	0	0	1	1	1	2	2	1	3	2	2	1	1	3	1	1	1	2	0	0	1	1	3	1	3	1	2	1	2	1	0	0	1	0	0	0	0	72.5
ESRRA	0	0	2	1	1	1	0	2	2	2	1	2	3	2	2	2	2	1	1	2	2	0	0	2	2	2	2	1	0	2	0	2	1	1	0	0	0	0	0	0	70
NFE2L2	0	0	1	0	1	1	3	3	1	1	2	1	2	2	1	2	2	2	2	3	1	3	0	0	0	3	2	0	0	2	1	1	1	3	1	0	2	0	0	0	70
PPARG	1	1	1	1	0	0	2	1	2	0	1	0	5	3	2	0	1	2	0	3	1	2	0	0	1	5	1	1	0	1	2	1	2	3	0	2	1	0	0	0	67.5
NR3C1	0	1	1	2	1	0	1	6	5	2	0	2	4	1	3	2	3	3	2	3	1	1	0	0	0	1	2	6	6	0	0	0	3	0	4	3	0	0	0	0	65
THRB	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	2	1	0	0	1	0	0	0	0	0	0	1	62.5
NR1H4	0	0	2	1	0	0	0	2	1	3	0	3	5	4	1	3	4	4	1	4	1	2	0	0	0	3	4	3	4	0	2	2	4	0	0	0	0	1	0	0	60
PPARA	0	1	0	1	0	0	2	1	0	1	0	2	1	0	0	1	3	0	3	1	2	0	0	0	1	2	0	0	1	1	1	2	2	0	1	1	0	0	0	0	52.5
CYP2E1	1	0	0	1	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	50
NR1H3	0	0	0	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	0	2	1	0	0	0	0	0	1	0	0	0	50
CYP19A1	0	1	0	1	0	0	1	2	1	1	0	1	1	1	1	1	1	2	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	45
PPARD	0	0	1	0	0	0	0	0	1	0	1	1	2	1	2	0	1	1	1	1	2	0	0	1	0	1	0	0	0	0	0	1	2	0	0	0	0	0	0	0	42.5
TPO	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	0	1	1	1	0	1	0	0	0	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	37.5
KDR	0	0	0	0	0	0	1	0	0	1	1	1	0	1	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25
SREBF1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	25
AHR	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	2	0	0	1	2	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	22.5
PTGS2	1	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	12.5
HTR2C	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	10
ADRB2	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.5
HNF4A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.5
GRIN1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
SLC6A4	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	5
ADRA2A	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CYP2C19	2	1	0	2	0	0	2	1	0	1	0	3	1	1	1	2	2	3	0	1	1	1	0	0	2	2	2	2	0	1	1	0	1	2	0	0	0	0	0	0	62.5
TP53	0	0	5	1	0	0	5	8	5	4	0	5	7	7	7	7	8	6	7	8	1	7	0	1	0	3	3	8	8	0	0	1	2	0	0	0	0	0	0	0	60
CYP2B6	4	3	0	4	0	0	3	2	0	1	0	6	1	0	1	1	2	3	2	1	0	1	1	0	0	1	3	1	1	0	1	0	0	2	1	0	0	0	0	0	57.5
CCL2	0	1	0	0	0	0	0	6	0	6	6	6	6	6	6	6	5	5	6	6	6	3	0	0	0	4	0	2	4	1	0	1	5	0	0	0	1	0	0	0	55
CSF1	1	1	0	0	0	0	0	1	0	3	3	3	3	3	3	3	2	2	3	3	3	2	0	0	0	2	0	1	1	0	0	1	1	0	1	0	0	0	0	0	55
HLA-DRA	0	0	0	1	0	0	1	2	0	2	3	3	3	3	3	3	2	3	3	3	2	0	0	0	0	2	0	1	1	0	0	0	2	0	1	0	0	0	0	0	55
RARA	0	0	0	1	1	0	1	0	1	1	0	1	2	1	1	1	1	1	1	2	0	0	0	1	0	1	1	1	1	0	1	0	1	0	1	0	0	0	0	0	55
CXCL8	0	1	0	0	0	0	1	4	0	4	5	5	5	5	5	4	4	5	5	4	5	0	0	0	0	3	0	1	2	0	0	1	4	0	1	0	0	0	0	0	52.5
CYP3A4	3	1	0	1	0	0	1	2	0	1	1	5	1	1	1	1	1	2	0	0	1	1	0	0	0	1	1	1	2	1	0	0	0	0	0	0	0	0	0	0	52.5
PLAUR	1	0	0	0	0	0	0	3	0	3	3	1	4	4	4	3	2	2	4	4	4	3	0	0	0	2	0	2	2	0	0	0	2	1	0	0	1	0	0	0	52.5
VCAM1	0	1	0	0	0	0	1	5	0	4	5	5	5	5	5	4	5	5	5	5	4	0	0	0	0	3	2	0	1	0	0	0	2	3	0	0	0	0	0	0	52.5
CD40	0	2	0	0	0	0	1	2	0	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0	0	2	1	1	0	0	0	0	2	0	0	0	0	0	0	0	50
CYP1A1	3	1	0	2	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	0	1	2	1	0	0	0	1	0	1	0	0	0	0	0	0	50
HIF1A	0	0	1	0	0	0	0	2	0	1	1	1	2	2	1	1	2	2	2	3	2	2	0	0	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	0	50
SELE	1	0	0	0	0	0	0	3	0	3	3	3	3	3	3	3	3	3	3	3	2	0	0	0	0	1	0	1	1	0	0	2	1	0	0	0	0	0	0	0	50
TNF	1	1	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	50
VDR	0	1	0	0	0	0	0	1	1	0	0	0	2	1	0	1	1	1	1	2	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0	1	1	1	0	0	50
CD38	0	1	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	47.5

Gene concluded in AOP database

Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	Score
COL3A1	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	47.5
CXCL10	1	2	0	0	0	0	3	3	0	2	3	3	3	3	3	3	3	3	3	3	3	3	0	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	47.5
F3	0	0	0	0	0	0	0	3	0	3	3	2	3	3	2	3	2	1	3	3	4	0	0	0	0	1	0	1	1	1	1	0	1	0	0	0	0	0	0	0	47.5
IL1A	1	0	0	0	0	0	1	3	0	2	3	3	3	3	3	2	2	3	3	3	3	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	47.5
MMP1	0	0	0	0	0	0	0	2	0	1	2	2	2	2	2	2	4	2	2	2	0	1	0	0	0	1	0	0	0	0	0	2	1	1	0	1	0	0	0	0	47.5
MTF1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	0	1	1	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	47.5
UGT1A1	3	1	0	1	0	0	1	1	0	1	1	1	1	0	1	1	1	2	0	1	0	1	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	47.5
CCL26	0	1	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	45
CD69	0	1	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	45
MMP9	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	0	0	2	0	0	1	0	0	0	0	0	0	0	45
TGFB1	0	0	0	0	0	0	3	3	0	1	2	2	2	2	3	2	3	3	2	3	1	2	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	45
CXCL9	0	0	0	0	0	0	0	4	0	3	5	5	5	5	5	4	4	3	5	5	5	2	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	42.5
CYP1A2	5	1	0	4	0	0	1	1	0	0	0	1	1	1	0	1	0	1	0	0	1	0	0	0	2	2	1	0	0	1	2	0	0	0	0	0	0	0	0	0	42.5
CYP3A7	1	0	0	1	0	0	0	1	0	1	1	1	1	0	1	0	1	0	0	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	42.5
CYP4A11	1	0	0	0	0	0	2	1	0	1	1	1	1	0	1	1	1	0	0	1	0	0	0	0	1	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	42.5
FABP1	0	0	0	0	0	0	1	1	0	1	1	1	1	0	1	1	1	1	0	0	1	0	0	0	1	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	42.5
HSF1	0	0	1	0	0	0	0	2	2	0	0	1	2	1	1	2	2	2	1	2	1	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	42.5
RORC	0	0	0	1	0	0	0	1	1	1	1	1	2	1	1	1	2	1	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	42.5
RXRB	0	0	0	0	0	0	0	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1	1	0		42.5
SELP	0	1	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	42.5
SERPINE1	0	0	0	0	0	0	1	2	0	1	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	42.5
PLAU	0	0	0	0	0	0	1	2	0	1	2	1	2	2	2	1	1	2	2	2	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	40
SLC5A5	0	0	0	0	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	40
TIMP1	0	0	0	0	0	0	2	1	0	1	1	1	1	1	2	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	40
ABCB1	1	0	0	1	0	0	1	1	0	1	0	1	1	0	1	1	1	0	2	0	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	37.5
CYP2C9	0	1	0	0	0	0	2	1	0	1	0	1	1	1	1	0	0	1	0	1	0	1	0	0	0	0	1	0	2	0	1	0	0	0	0	0	1	0	0	0	37.5
CYP4A22	1	0	0	0	0	0	2	0	0	1	1	1	1	1	0	1	1	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	37.5
EGR1	0	0	0	0	0	0	1	2	1	0	0	0	2	1	2	1	1	2	0	2	1	2	0	0	0	2	1	0	1	0	0	0	0	0	0	0	0	0	0	0	37.5
FOS	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	0	1	1	0	0	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	37.5
HMGCS2	0	0	0	0	0	0	1	0	0	0	1	1	2	1	1	0	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	0	2	0	0	0	0	0	0	37.5
IL6	0	1	0	0	0	0	0	1	0	0	1	0	2	1	2	1	0	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	37.5
POU2F1	0	0	0	0	0	0	0	1	1	1	0	0	1	1	0	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	0	0	1	0	0	0	1	0	0	0	37.5
XBP1	0	0	0	0	0	0	0	2	0	0	0	0	1	1	1	1	1	2	1	1	2	0	0	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	37.5
JUN	0	0	0	0	0	0	0	1	0	1	0	1	2	1	1	1	1	2	1	2	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	35
CYP2C8	0	0	0	1	0	0	2	0	0	1	0	1	1	1	0	1	1	0	0	0	1	0	0	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	35
DDIT3	0	0	0	0	0	0	1	1	0	1	0	1	1	0	1	1	1	1	0	0	0	1	0	0	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	35
H2AFX	0	0	0	1	1	0	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	35
ICAM1	0	0	0	0	0	0	0	3	0	2	2	2	3	2	2	2	1	0	2	3	2	3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	35
IRF1	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	1	1	1	0	1	1	1	0	0	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	35
PEG10	1	0	0	1	0	0	0	1	0	0	1	0	1	1	1	1	0	1	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	35
PTGER2	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	1	0	1	0	0	1	0	0	0	0	1	0	0	1	1	1	0	1	0	1	0	0	1	0	0	35
SAA1	0	0	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	35
SLC6A3	0	0	0	0	0	0	0	1	0	0	2	2	2	0	0	0	2	2	0	1	0	0	0	0	2	2	2	2	0	1	0	0	0	0	0	0	2	1	0	0	35
TIMP2	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	35
TSPO	0	2	0	1	0	0	0	2	2	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	2	1	0	2	1	2	1	1	0	0	1	0	0	0	0	0	35
ABCC2	1	0	0	0	0	0	1	1	0	1	0	1	1	0	1	1	1	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	32.5
ABCG2	0	0	0	1	0	0	1	1	0	1	0	1	1	0	1	1	1	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	32.5
FOXO1	0	0	0	0	0	0	1	1	1	0	0	0	1	0	1	1	0	1	1	1	1	2	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	32.5
PAX6	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	1	1	1	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	32.5
PLAT	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	32.5
TCF7	0	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	1	1	0	1	0	1	0	0	0	1	1	0	1	1	0	1	0	0	0	0	0	1	0	0	32.5

Gene																																									Score
THBD	0	0	0	0	0	0	0	0	0	1	2	1	2	0	2	1	1	2	1	2	2	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	32.5
ABCB11	0	0	0	1	0	0	1	0	0	1	0	1	1	1	0	1	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	30
FASN	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	30
IGF1	1	0	0	0	0	0	1	1	0	1	0	1	1	0	0	0	1	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	30
LDLR	0	0	0	0	0	0	0	1	0	1	0	1	1	1	1	0	1	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	30
LPL	0	0	0	0	0	0	1	0	0	1	1	1	1	0	1	1	0	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	30
MYC	0	0	0	1	0	0	1	0	0	1	0	1	0	0	1	1	1	1	0	2	1	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	30
PDK4	0	0	0	1	0	0	1	1	0	0	1	1	0	1	1	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	30
RARB	0	0	0	0	0	0	0	0	1	0	0	1	2	0	0	1	1	0	1	2	2	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	30
RXRA	0	0	1	0	0	0	0	0	0	2	1	0	2	2	0	2	2	0	2	2	2	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	30
SLC22A1	0	0	0	0	0	0	1	1	0	1	0	1	1	1	0	1	1	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	30
SP1	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	1	1	1	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	30
SULT2A1	0	0	0	1	0	0	0	1	0	1	0	1	1	0	1	0	0	1	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	30
AFP	0	0	0	0	0	0	1	0	0	1	0	1	1	0	1	1	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	27.5
APOA5	0	0	0	0	0	0	1	0	0	1	0	1	1	1	0	1	1	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	27.5
CYP7A1	0	0	0	0	0	0	1	1	0	0	0	1	1	0	1	1	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	27.5
FMO3	0	0	0	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	27.5
GSTA2	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	1	0	1	0	1	0	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0	0	0	27.5
HSPA1A	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1	1	1	1	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	27.5
KRT19	1	0	0	0	0	0	1	1	0	1	0	0	1	1	1	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	27.5
NFKB1	0	0	0	0	0	0	0	1	0	0	1	3	1	1	0	1	0	0	1	1	2	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	27.5
USF1	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	1	1	1	1	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	27.5
ACOX1	0	0	0	0	0	0	1	0	0	1	0	1	1	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	25
ALPP	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	25
CYP24A1	0	0	1	0	0	0	0	1	0	0	0	2	1	1	1	0	2	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25
IGFBP1	0	0	0	0	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25
MMP3	1	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	1	1	0	0	2	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	25
OPRM1	0	0	0	0	0	0	0	2	0	0	1	0	2	1	0	0	0	2	1	0	1	0	0	0	0	0	3	0	2	0	1	0	0	0	0	0	0	0	0	0	25
SLC6A2	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1	0	0	1	1	0	1	0	0	0	0	0	1	0	2	0	1	0	0	0	0	0	1	0	0	0	25
TGFA	0	1	0	0	0	0	1	1	0	0	0	1	2	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	25
THRSP	0	0	0	0	0	0	1	1	0	1	0	1	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	25
ACLY	0	0	0	0	0	0	1	1	0	0	1	0	1	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	22.5
ADORA2A	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	22.5
ATF6	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22.5
CAT	0	0	0	0	0	0	1	1	0	0	0	1	1	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	22.5
CREB3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	22.5
CYP3A5	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1	1	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	22.5
GADD45G	0	0	0	0	0	0	0	1	0	1	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	22.5
SMAD1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	22.5
STAT3	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0	0	1	2	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	22.5
TRHR	0	0	0	0	0	0	0	0	0	0	0	2	3	2	2	0	0	2	1	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22.5
UGT1A6	0	0	0	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22.5
BCL2	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20
CCND1	0	0	0	0	0	0	1	1	0	0	0	1	0	1	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20
CDKN1A	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	2	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	20
IL6R	0	0	0	0	0	0	1	1	0	0	1	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	20
LIPC	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20
NFIA	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	20
PPP2R4	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	20
TFAP2A	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	20
ADORA1	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	2	0	0	0	0	0	0	0	17.5

Gene																																													Score
ADRB1	0	0	0	0	0	0	1	1	0	0	0	0	2	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	17.5
CFLAR	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	■	17.5
FOXO3	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	■	17.5
GCLC	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	17.5
MAOA	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	■	17.5
MIR122	0	0	0	0	0	0	1	0	0	1	0	1	2	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	17.5
MMP10	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	17.5
PTEN	0	0	0	0	0	0	1	2	0	0	0	0	1	0	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	17.5
RORA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	■	17.5
SLCO1B1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	■	17.5
SOX1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	2	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	17.5
TBXA2R	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	■	17.5
TNFRSF1A	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	17.5
ABCC3	0	0	0	0	0	0	0	0	0	1	0	1	1	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	■	15
ADK	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	15
BCL2L11	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	15
CASP3	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	15
CASP8	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	15
CEBPB	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	■	15
EGF	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	15
FAS	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	15
GLI1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	■	15
HTR7	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	15
NRF1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	■	15
OPRD1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	15
SDHB	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	■	15
ADRA2C	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	12.5
BAX	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	12.5
CYP2A2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	■	12.5
CYP2C11	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	■	12.5
DIO1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	■	12.5
DRD1	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	12.5
GADD45B	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	■	12.5
HTR4	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	12.5
HTR6	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	12.5
LTB4R	0	0	0	0	0	0	2	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	■	12.5
NR4A2	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	12.5
SLC18A2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	12.5
THRA	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	■	12.5
BACE1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	10
CCKAR	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	10
CYP2C13	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	10
CYP2C18	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	10
CYP3A2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	■	10
DRD2	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	10
DUSP3	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	10
EGFR	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	■	10
ETS1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	10
EZR	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	10
GADD45A	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	10
HGF	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	■	10

Gene	Values	Score
NQO1	0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	10
NR3C2	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 1 0 0 0 0 0 0	10
ONECUT1	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0	10
OPRK1	0 0 0 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0	10
PIK3CA	0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0 0	10
RARG	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	10
SCN1A	0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0	10
SIRT1	0 0 0 0 0 0 0 0 0 1 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0	10
SLC29A1	0 0 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	10
TACR2	0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	10
ADRA1A	0 0 0 0 0 0 0 0 0 0 0 0 2 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0	7.5
AVPR1A	0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
BID	0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
CACNA1A	0 0 0 0 0 0 0 1 0 0 0 0 2 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
CCKBR	0 0 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
CYP2D6	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0	7.5
CYP2J2	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0	7.5
CYP3A23/3A1	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0	7.5
CYP4F12	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
CYSLTR1	0 0 0 0 0 0 1 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
DRD4	0 0 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
GABRA1	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 1 0 0 0 0 0 0 0 0	7.5
GATA1	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0	7.5
GRIA1	0 0 0 0 0 0 0 1 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
HTR5A	0 0 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
MAOB	0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 2 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0	7.5
MMP13	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0	7.5
MMP7	0 0 0 0 0 0 1 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
NTRK1	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
OPRL1	0 0 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
PTPN1	0 0 0 0 0 0 1 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	7.5
TEK	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0	7.5
ACP1	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0	5
ADRA2A	0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
ADRA1B	0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
ADRB3	0 0 0 0 0 0 0 1 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
AKT2	0 0 0 0 0 0 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
ATAD5	0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0	5
BAD	0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
CHRM1	0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
CHRM2	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
CHRM3	0 0 0 0 0 0 0 0 0 0 0 0 2 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
CHRM4	0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
CSNK1D	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
CYP2A1	0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
CYP2B1	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0	5
EPHA2	0 0 0 0 0 0 1 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
FGFR1	0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
GSTM3	0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
HRH1	1 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5
HTR2A	0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	5

Gene	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25	C26	C27	C28	C29	C30	C31	C32	C33	C34	C35	C36	C37	C38	C39	C40	C41	C42	C43	C44	C45	C46	Score
INSR	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
KCNK1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
LCK	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
MYB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	5
NPY2R	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
OXTR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
PDE4A	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
PDE5A	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
PPP1CA	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	5
PPP2CA	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	5
PRKAA1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
PTPN2	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
PTPN6	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
PTPRB	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	5
PTPRF	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
RORB	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
SIRT2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
SIRT3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
SSTR1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
ADCY5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
ADRA2B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
AGTR1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
AGTR2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
AKT1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
AURKA	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
BDKRB2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
BTK	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CASP5	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CDK6	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CHRM5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CHUK	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CRHR1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CSF1R	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CSNK1A1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CYP2C12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CYP2C6V1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CYP2D1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
CYP2D2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
DYRK1A	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
E2F1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
EDNRA	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
EDNRB	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
FGFR3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
FLT1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
FLT4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
FOXA2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
FYN	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
GALR1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
GRM1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
GRM5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5
GSK3B	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5

Category	Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40		Value
	HDAC3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	HRH2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	HRH3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	HTR1A	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	KCNH2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	LYN	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	MAPK1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	MAPK3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	MAPKAPK5	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	MARK1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	MET	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	NEK2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	NPY	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	NPY1R	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	P2RY1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	PAK4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	PRKACA	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	PTAFR	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	PTPN11	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	PTPN12	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	PTPN13	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	PTPN9	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	PTPRC	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	PTPRM	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	RAF1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	ROCK1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	RPS6KA5	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	SGK1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	SIGMAR1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0		2.5
	SLC10A1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	SRC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0		2.5
	TACR3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	VIPR1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
	ZAP70	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		2.5
Others	Mitochondria Dysfunction	0	0	3	2	0	0	4	9	3	5	3	3	6	8	8	4	5	5	5	7	0	6	3	1	1	3	8	4	5	2	5	0	5	2	0	2	3	0	0	0		75
	Oxidative Stress	0	0	0	0	0	0	2	3	0	3	0	1	2	4	4	3	4	0	1	2	0	0	0	0	0	2	2	2	3	0	0	0	1	0	0	0	0	0	0	0		40