



ENVIRONMENTAL HEALTH PERSPECTIVES

<http://www.ehponline.org>

A Systems Biology Approach Reveals Converging Molecular Mechanisms that Link Different POPs to Common Metabolic Diseases

Patricia Ruiz, Ally Perlina, Moiz Mumtaz, and Bruce A. Fowler

<http://dx.doi.org/10.1289/ehp.1510308>

Received: 5 June 2015

Accepted: 10 December 2015

Advance Publication: 18 December 2015

Note to readers with disabilities: *EHP* will provide a [508-conformant](#) version of this article upon final publication. If you require a 508-conformant version before then, please contact ehp508@niehs.nih.gov. Our staff will work with you to assess and meet your accessibility needs within 3 working days.



National Institute of
Environmental Health Sciences

A Systems Biology Approach Reveals Converging Molecular Mechanisms that Link Different POPs to Common Metabolic Diseases

Patricia Ruiz^{1†}, Ally Perlina^{2†}, Moiz Mumtaz¹, and Bruce A. Fowler³

¹Computational Toxicology and Methods Development Laboratory, Division of Toxicology and Human Health Sciences, Agency for Toxic Substances and Disease Registry, Atlanta, Georgia, USA; ²Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California, USA; ³Private Consulting Toxicologist, Emory University Rollins School of Public Health, Atlanta, Georgia, USA. [†]These two authors contributed equally to this work.

Address correspondence to Patricia Ruiz, Division of Toxicology and Human Health Sciences, Computational Toxicology and Methods Development Lab, Agency for Toxic Substances and Disease Registry, 1600 Clifton Road, MS-F57, Atlanta, 30333 USA. Telephone: 770-488-3348. Fax: 770-488-3470. E-mail: pruiz@cdc.gov

Running title: POPs linkages to common metabolic diseases

Acknowledgments: This study was presented at the 54th Society of Toxicology annual meeting (SOT2015). The authors are grateful to Dr. Linda S. Birnbaum and Dr. Dale Johnson for their valuable comments, and advice during the preparation of this manuscript. We also appreciate Mr. Donald Meadows for his editorial technical support. A.P. was employee of Sanford Burnham Prebys Medical Discovery Institute La Jolla, California, USA. B-A. F is a private consultant, in Rockville, Maryland, USA

Disclaimer: The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry. Mention of trade names is not an endorsement of any commercial product.

Competing financial interests: The authors declare they have no actual or potential financial interests.

Abstract

Background: A number of epidemiological studies have identified statistical associations between persistent organic pollutants (POPs) and metabolic diseases, but testable hypotheses regarding underlying molecular mechanisms to explain these linkages have not been published.

Objectives: To assess the underlying mechanisms of POPs that have been associated with metabolic diseases, 3 well-known POPs (2,3,7,8-Tetrachlorodibenzodioxin (TCDD), 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB 153), and *p,p'*-Dichlorodiphenyldichloroethylene (*p,p'*-DDE)) were studied. We used advanced database search tools to delineate testable hypotheses and guide laboratory-based research studies into underlying mechanisms by which this POPs mixture could produce or exacerbate metabolic diseases.

Methods: For these studies searches, a proprietary systems biology software MetaCoreTM/MetaDrugTM was used to conduct advanced search queries for the underlying interactions database, followed by directional network construction to identify common mechanisms for these POPs within 2 or less interaction steps downstream of their primary targets. These common downstream pathways belong to various cytokine and chemokine families with experimental well-documented causal associations with type 2 diabetes.

Conclusions: Our systems biology approach allowed identification of converging pathways leading to activation of common targets downstream. To our knowledge, this is the first study to propose an integrated global set of step-by-step molecular mechanisms for a combination of 3 common POPs, using a systems biology approach, which may link POPs exposure to diseases. Experimental evaluation of the proposed pathways may lead to development of predictive biomarkers of POPs' effects, which could translate into disease prevention and more effective clinical treatment strategies.

Introduction

Persistent organic pollutants (POPs) are ubiquitous environmental contaminants. They include polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and organochlorine pesticides. . TCDD, a representative of the dioxin chemical family, is unintentionally produced during chlorine bleaching processes, drinking water chlorination, and incineration processes (ATSDR 2012). *P', p'*-DDE is a metabolite of DDT that has been used as insecticide for insect vectors of malaria and typhus (ATSDR 2008). PCBs are industrial chemicals principally used as heat exchange fluids in transformers and capacitors were also banned in U S in 1977(ATSDR 2011).

Epidemiological studies have reported associations between POPs and metabolic diseases such as Type 2 diabetes (T2D), obesity and metabolic syndrome, but potential underlying mechanism(s) are not known (Langer et al.2014; Lee et al. 2006; 2010; 2011; Rylander et al. 2005; Suzuki et al. 2008). The 3 POPs evaluated here (TCDD, PCB 153, and *p,p'*-DDE) have been associated with metabolic disorders in observational studies, but potential molecular mechanisms that might underlie endocrine disruption and disease development are far from understood (Everett et al. 2007; Henriksen et al. 1997;Lee et al. 2014; Longnecker and Michalek 2000; Magliano et al. 2014; Rignell-Hydbom et al. 2007; Turyk et al. 2009;Wang et al. 2008).

Because metabolic diseases are increasing in frequency throughout the world, further investigation and understanding of the possibility that exposure to POPs contributes to the etiology of diabetes, obesity, and cardiovascular disease is critical (Taylor et al. 2013; Thayer et al. 2012). Metabolic syndrome may affect up to 1 in 5 people, and its prevalence increases with

age (Paoletti et al. 2006). It is estimated that up to 25% of the U.S. population has metabolic syndrome (Ford et al. 2004).

Researchers have hypothesized that low-level POPs exposure can cause metabolic changes through a network of pathways, including increased insulin resistance and obesity preceding the development of T2D (Barouki et al. 2012; Barrett 2013; Lee et al. 2014; Taylor et al. 2013). Within this network, different POPs might also cause the metabolic syndrome through slightly overlapping pathways to cause disturbance in glucose homeostasis. Such disturbances include inhibition of insulin action and induced down-regulation of master regulators of lipid homeostasis. The situation is further complicated by the realization that POPs –induced alterations in epigenetic regulatory mechanisms may occur during sensitive developmental periods leading to diseases such as obesity and T2D later in life (Barouki et al. 2012).

In toxicology, systems biology facilitates the identification of important pathways and molecules from large data sets. These tasks that can be extremely laborious when performed by a classical literature search. Computational systems biology offers more advantages than just providing a high-throughput literature search engine. These tools may provide the basis for establishing hypotheses on potential links between environmental chemicals and human diseases. Comprehensive databases containing information on networks of human protein–protein interactions and protein–disease associations make this possible. Experimentally determined targets data of the specific chemical of interest can be uploaded and overlaid into these networks to obtain additional information that can be used to establish hypotheses on links between the chemical and human diseases. Such information can also be applied for designing more intelligent animal and cell based laboratory experiments that can test the established hypotheses.

In this study we examine potential linkages for combined exposures to 3 specific POPs, cellular pathway alterations, and metabolic disturbances related to the development of important clinical outcomes. We used an integrated global approach that brought together 1) predictive chemical analyses based on compound structure and 2) knowledge bases of chemogenomics data associating compounds to biological and toxicological properties. We then provided an *in silico* evaluation of the possible joint effects of POPs on metabolic pathways that could lead to metabolic diseases. We sought to discover downstream activation targets in common for all 3 POPs as a mixture. Even though inhibitory targets were also analyzed, we chose to focus on the genes that could be ultimately up-regulated and lead to increased abundances on the protein level. The rationale for this was to set the stage for discovery of screening biomarkers, which, especially if present in easily accessible tissues/fluids, could be more accessible when increased in abundance, as opposed to depleted. It is hoped that these data will stimulate formation of new testable hypotheses to address some of the data gaps previously identified by Taylor et al. (2013) and Barrett 2013.

Methods

For this study 3 POPs (p, p'-DDE, TCDD and PCB 153) were selected for study since they are commonly detected in the environment, and in human tissues. Based upon the data from the epidemiological and data mining literature noted above, they are also linked with metabolic diseases such as T2D (Everett et al. 2007; Henriksen et al. 1997; Lee et al. 2010; Longnecker and Michalek 2000; Turyk et al. 2009; Wang et al. 2008).

The majority of available POPs studies have focused on them on an individual basis. To our knowledge, there are no published studies on their combined potential interactive effects at the molecular level in relation to clinical disease outcomes.

The pathway analysis tools: Metacore/Metadrug

The molecular structure (.mol) files of 3 POPs: p, p'-DDE, TCDD and PCB 153 (Figure 1) were separately uploaded to MetaCore™/MetaDrug™, a proprietary systems biology software solution by Thomson Reuters (originally developed by Genego, Inc) (<http://portal.genego.com>). This software is built on proprietary database (MetaBase™) to allow functional and network analysis of primary and secondary effects of any query compound in the context of manually curated molecular interactions and pathways (Ekins et al. 2007).

MetaCore™/MetaDrug™ are analytical tools built on top of a manually curated database of literature findings that support various types of molecular interactions and ontologies, including disease relationships. These tools help analyze information from user's experimental results or use the options of mining the underlying content from the database MetaBase™ content directly.

The Advanced Search is a java application tool with in MetaCore™, which facilitate the searching of combined information, e.g. “find all compounds that inhibit EGFR with IC₅₀ lower than 1 μM”. By using Advanced Search allows us to can create such a Boolean query and retrieve the results. A detailed methodological description of the systems biology procedures and protocols for using these software are available at <http://lsresearch.thomsonreuters.com/>. However, to be available to access or reproduce the data from proprietary tools, researchers need to obtain a trial license or a paid license.

When query content from MetaBase™, Advanced Search allows us to differentiate low and high trust annotation information behind interactions. High trust means that this content was confirmed to have published small-scale experimental evidence (e.g., Co-Immunoprecipitation plus luciferase reporter assay). Low trust interactions have only been validated via high-

throughput screening / co-expression or predictive analysis studies, and lack more rigorous experimental evidence. Molecular entities can affect a target directly and indirectly. Mechanisms which are used to describe the direct physical interactions include: binding, covalent modifications, phosphorylation, etc. Indirect mechanisms include: influence on expression; co-regulation of transcription, unspecified, and others, as stated in the legend. Only high-trust direct interactions with known effects (activation or inhibition) were used for this study.

POPs Pathway Analysis

To map all the possible pathways from the 3 selected POPs to their downstream targets, first, primary targets (this is a direct chemical action of which its target will lead to a response in the cells of the mammalian organism. All others targets of the chemical are accounted for as secondary) were determined using MetaCore™ and MetaDrug™ content. Of those, only direct binding targets that have further downstream interactions were considered. To narrow down the complexity presented by primary targets that already participate in thousands of annotated molecular interactions, Advanced Search tool was used. It allowed construction of direct database queries of interactions that lead from primary/direct targets of each POP to the common targets shared by all 3 POPs in 3 steps or less and with an inferred activating effect. This meant that from the compound itself the allowed network depth / distance would be 3 steps or less. The focus on downstream activation targets was presumed to be of most utility if such targets were to be used for detection as biomarkers. All combinations of path lengths within 3 interactions were considered. For example, some targets may be 2 steps downstream of one POP, but 3 steps downstream of the other 2 POPs. For all the multi-steps paths, the assumption was made that to achieve downstream activation, not only activation interactions, but also inhibitory ones can be considered, as long as they would add up to the final effect of activation. For example, inhibition

of an inhibitor can result in subsequent activation, so all combinations of the following interaction paths were considered in our search queries:

2-step paths:

activation, activation

inhibition, inhibition

3-step paths:

activation, activation, activation

activation, inhibition, inhibition

inhibition, inhibition, activation

inhibition, activation, inhibition

Note that there were no common primary targets for all 3 POPs, hence, no 1-step paths were obtained in this analysis.

The resulting combined list of potential common activation targets was used for network construction. The “shortest paths” network building option (the maximum number of interaction steps is defined by the user from a range of 1 to 10 interaction steps, and was set to 3 as a maximum for this case; the algorithm attempts to build the shortest directed paths between selected objects using up to the maximum number of interactions defined by the user). This yielded the resulting interconnected network diagram. Interaction effects were then checked for concordance (or agreement, to make sure there was no conflicting interactions). Only direct activation or inhibition interactions were used for network construction (Direct means small-scale molecular physical interactions, as described earlier, with effect of interaction being either activating or inhibiting, which means that interactions that are indirect or have an unspecified effect would not be considered by the shortest paths algorithm). Only concordantly regulated

molecules (also referred to as network nodes) were displayed on the final network with only those interactions that lead to downstream activation. This means that the terminal nodes (those genes/proteins that only have upstream interactions) had only those sequences of interactions leading toward them that would amount to activation. Even though advanced queries resulted in target lists (queries result in a list of genes), network building was still needed for a visual representation of the interaction space that met the requirements of the queries and mechanistically tied the POPs to the targets. It was also needed to manually check and remove any signaling conflicts, which still arose between intermediate nodes along the queried paths. For example, if gene A was a logical linker downstream of a POP target and its downstream common activation target, but was regulated in an opposite direction by the primary target of another POP, then this would become a mixed message and would have to be removed from the final network. In other words, a mixed message would be one where an intermediate protein is receiving a signal from a POP through its target to behave in one way (for example to activate or induce signaling), but through a second target for another POP effect would be opposite (to inhibit or suppress signaling) this would give the intermediate protein conflicting signals.

The final network was narrowed to only those downstream targets associated with the following: diabetes/insulin resistance (IR), obesity, and metabolic syndrome X. Finally, to clarify the specific signaling paths, the final network was sub-divided into sub-networks with smaller portion of information based on one downstream target at a time for greater resolution.

Results

Interaction Queries and Network Construction

Starting with the 3 nodes that represent TCDD, PCB 153, and *p, p'*-DDE, we were able to identify primary targets by using advanced database search queries, followed by directional

network construction. Because these 3 POPs are so structurally different, they bind different primary targets: pregnane X (PXR) receptors for PCB 153, androgen receptor (AR) for *p, p'*-DDE, and aryl hydrocarbon receptor (AhR) for TCDD. This suggested different modes of action and downstream effects for these 3 chemicals. However, with the addition of only 1 or 2 more interaction steps, the literature-based pathway reconstruction/ modeling approach made it possible to determine which activation targets of 1 or 2 of these compounds can also be activated by the third compound. Such converging common activation targets were identified from cumulative results achieved by the Advanced Search queries; 349 concordance targets were identified in 3 steps or less (data not shown), and have known positive disease association. Only High-trust direct physical interactions of known effects (activation or inhibition) were used for the queries and for the network building. Network was built downstream of all 3 POPs using 3-steps or less for Dijkstra's shortest paths algorithm. All interaction effects were then checked for concordance on the final network representation.

Proposed Final Network

The connectivity of the network revealed that 6 targets (IL-6, IL-8, RelA (p65), c-Jun, FKHR, and Cyclin D1) are activated by the 3 POPs in 2.33 steps on average (2 steps from 2 of the POPs and 1 other step from the third (so 3 steps from the 3rd POP), therefore, the average of each chemical steps $(2+2+3)/3= 2.33$). The network also showed that 35 targets were activated by the 3 POPs in 2.67 steps on average (2 steps from 1 of the chemicals and 1 additional step from the 2 others, $(2+3+3)/3=2.67$), which yields 41 total targets that could be activated by the 3 POPs in less than 3 network interaction steps on average.

The complete final network (Figure 1) reveals the genes associated with diabetes/insulin resistance, or obesity, or metabolic syndrome X (union, large gray circles around gene icons).

The genes that also have a small circle (tagging the gene's icon on top-right) represent the annotated associations with all 3 diseases (intersection). Based on this systems biology generated global network (Figure 1), a joint mechanism of action was proposed for the combined exposure to the 3 POPs or the additive effects that may be anticipated. Figure 1 shows some intermediate cross interactions between the downstream targets (shown in green). These common activation processes can influence or activate each other and imply even more functional and mechanistic connectivity/synergy. Although this systems biology-generated global network cannot be considered as a proof of causal linkages without further experimental validation, it provides justification for the mechanistic hypothesis and contributes to new interpretation linking available published toxicology and disease information domains.

Delineating Sub-Pathways from the Network

To clarify specific signaling pathways, the final global network was divided into sub-networks with smaller portions of information for greater resolution. For example, interleukin-8 (IL-8) can be activated by *p, p'*-DDE via AR (inhibition of an inhibitor) and activated by TCDD via AhR (activation of an activator) (Vogel et al. 2007) (Figure 2). PCB 153 is not known to activate IL-8 directly or through its primary target (PXR). However, with evaluation of one additional step, a plausible mechanism was revealed: CREB1 can be activated by PCB 153 through PXR (inhibition of an inhibitor) via documented (Kodama et al. 2007; Tabb et al. 2004) direct binding interactions. CREB1 can then lead to activation of IL-8 via well-documented promoter binding (Mayer et al. 2013) (Figure 2). The potential link between IL-8 and PCB 153 is noteworthy. It establishes a link between PCB 153 and IL-8 through CREB1 by allowing 1 immediate step.

Interleukin-6 (IL-6) is well known to promote inflammation and pro-inflammatory effects (Scheller et al. 2011). As illustrated in Figure 3, our analysis suggests that that IL-6 can be

directly activated by TCDD via AhR and ARNT complex (activation of an activator), which are close neighbors of IL-6 that often act together in many pathways. AhR can also activate RelA, which can activate IL-6. PCB 153 and *p, p'*-DDE can inhibit RelA (inhibition of an inhibitor) via PXR and AR, respectively, and then RelA activates IL-6. There is a direct path for activation of IL-6 from *p, p'*-DDE via AR (inhibition of an inhibitor). IL-6 could be activated from PCB 153 in the same pathway as described for IL-8 (Kodama et al. 2007; Tabb et al. 2004).

As illustrated in Figure 4, our analysis suggests that tumor necrosis factor-alpha (TNF- α). TNF- α can be activated by TCDD via AhR and RelA (activation of an activator) and then RelA will directly activate TNF- α , and though *p, p'*-DDE via AR and c-Jun (inhibition of an inhibitor). PCB 153 shows same pathways as described for IL-6 and IL-8. PXR (inhibition of an inhibitor) also provides a direct path for activation of TNF- α from PCB 153. CREB1 could be an important link to get to these cytokines activation pathways. RelA could represent a common step in the activation of TNF- α by all of these individual POPs. C-Jun is important in all of the inflammation pathways and, together with RelA, promotes the inflammation pathway (Ip and Davis 1998; Tak and Firestein 2001).

As illustrated in Figure 5, our analysis suggests that fetuin A can be activated by TCDD via AhR and RelA (activation of an activator) and then RelA will directly activate fetuin A. PCB 153 and *p, p'*-DDE could inhibit RelA (inhibition of an inhibitor) via PXR and AR respectively, and then RelA activates fetuin A. Again, it is a noteworthy observation that RelA could represent a common step in the activation of fetuin A, IL-6 and TNF- α by all of these individual POPs. Thus, the counterpart pro-inflammatory effects of these proteins through intracellular signaling pathways may involve the RelA systems.

Overall, the whole resulting network is populated with a combination of metabolic genes, insulin signaling, immune response signaling, and inflammation cascade of cytokines and transcription factors. Based on our analysis, we hypothesize that common pathways that converge through the cytokines may contribute to inflammatory processes that may lead to metabolic diseases via circulation and also creation of a chronic inflammatory background in adipocytes, liver, and pancreatic tissues. This can lead to impact on adipogenesis, pancreatic beta-cell dysfunction, insulin resistance, glucose intolerance, liver disease, inability to cope with increased dietary intake, which over time can amount to development of more serious metabolic disease phenotypes. Apart from inflammation, some cancer-associated targets are also present. For example, as illustrated in Figure 6, our analysis suggests that cyclin D1 and IL-8 share common pathways. Three steps from PCB 153 through PXR and CREB1, it will reach cyclin D1, as the same way of IL-8. Also, cyclin D1 is activated though AhR from TCDD, the same way as IL-8 is activated. Similar steps occur with the direct transcription methylation of AR that is then inhibited though p, p-DDE. Thus, based on our analysis, we hypothesize that could be an overlay of mechanisms between inflammatory processes and cancer development and progression that increases the potential for carcinogenicity of mixtures of these POPs.

Discussion

By integrating available information and bridging the gap between toxicology, epidemiology, and chemistry within the world of disease mechanisms, we can look further beyond the primary target of the individual POPs by 2 or 3 steps down the relevant pathway. Analysis of molecular networks and all possible downstream targets is very complex. Our approach, based on mechanistic annotated networks, allows identification of common targets that are beyond primary targets. Although molecular interactions data for individual POPs have been reported in

the literature and confirmed by published experimental studies (Goldberg 2009, Kuwatsuka et al. 2013), to our knowledge, these data have not been previously integrated as a mixture in the step-by-step continuum and sequential manner described here.

For example, *p, p'*-DDE, TCDD, and PCB 153 can act as agonists or antagonists of the AR, AhR, PXR, respectively (ATSDR 2008; 2012; 2011). These characteristics make these chemicals of specific concern for developing organisms that are highly sensitive to hormonal changes and exposure to these chemicals is critical because this could result in permanent changes throughout life. They might act over time at low exposure levels during fetal or early life periods and have a particular impact on health. The finding of potential human health effects from interactions to multiple chemicals in epidemiological studies faces many difficulties, and there is a great need for reliable biomarkers of effect and exposure. Nevertheless, recent reports support the notion that documented interactions downstream of the POPs implicate each POP in perturbation of pathways that might lead to various metabolic diseases such as obesity and T2D (Scriver et al. 2011).

A closer look at the nuclear receptor signaling pathway reveals that PCB 153, TCDD, and *p, p'*-DDE have overlapping and interconnected pathways that have potential to cause biological perturbations through the different nuclear receptor signaling pathways. AhR directly activates and transcriptionally regulates expression of IL-8 (Vogel et al. 2007), and IL-8 and TCDD was associated with diabetes in a cross-sectional analysis data from NHANES cohort (Lee et al. 2006). Our systems biology analysis suggests a link between PCB 153 and IL-8 through CREB1 by allowing 1 immediate step from its primary target. The potential link between IL-8 and PCB 153 is noteworthy. CREB1 could be an important link to get to cytokines activation pathways. Based on our analysis, we can hypothesize a joint toxic action pathway (IL-8 as well

as others cytokines) for the mixtures of these 3 specific POPs that could be experimentally tested and extended to other POPs.

Various toxic compounds may trigger abnormal inflammatory responses directly or indirectly by interfering with normal physiological functioning of cells or tissues (Medzhitov 2008). These effects could play a role in the development of insulin resistance and diabetes. In a cross-sectional study of nondiabetic individuals where most of them had cancer, Kim et al. (2014) analyzed the influence of POP concentrations on insulin resistance. A cross-sectional study of 39 Caucasians and 72 First Nations adults by Imbeault et al. 2012 reported a weak but significant association of elevated levels of POPs with cytokines. Studies on POPs and human adipose cells, showed that precursor cells and adipocytes were targets of POPs, and that these pollutants trigger mainly the inflammation pathway (Kim et al. 2012). In a Japanese study involving 40 Yusho patients and 40 controls, Kuwatsuka et al. (2013) demonstrated that serum levels of certain interleukins (IL-17, IL-1 β , and IL-23) and tumor necrosis factor- α (TNF- α) were higher in patients who were exposed to POPs, including PCBs through consumption of contaminated rice oil (Kuwatsuka et al. 2013). Circulating inflammatory biomarkers such as CRP, IL-6, TNF α , monocyte chemotactic protein 1 (MCP 1), intercellular adhesion molecule 1 (ICAM 1), vascular cell adhesion protein 1 (VCAM 1), and E-selectin have been associated with a variety of metabolic disorders and associated outcomes (Goldberg 2009). However, in a study population of 72 participants, Pal et al. 2013 reported no significant association between POPs concentrations and markers of insulin resistance when compared diabetic to non-diabetic individuals in Northern Ontario population. Similarly, in a cross-sectional study of 1016 individuals (all 70 years of age) from Sweden, Kumar et al. 2014 observed no association between levels of POPs and pro-inflammatory cytokines (IL-6, MCP-1, and TNF- α). Differences

in results between studies could be attributable to various factors, including number of individuals in the studies, presence of others diseases, gut microbiota, diet composition, early-life nutrition and non-causal associations due to confounding or other sources of bias.

Numerous studies as mentioned previously have shown a connection between cytokines and metabolic disease, cytokines levels and POPs, and POPs levels and metabolic diseases. However, few give a clear articulation of the underlying mechanisms, particularly for chemical mixtures of similar and dissimilar chemicals. In the study of disease biology and the pathogenesis of diseases, much effort is given to elucidating new pathways and validating those. It is less common to actually trace pathways all the way back to identify how toxicant exposures on an individual or mixture basis could lead to disturbances in these molecular regulatory systems.

At present, there is no clear explanation for differences in POPs exposures and T2D reported in epidemiological studies. This apparent inconsistency may be related to the idea that POPs are involved in the pathogenesis of T2D by interfering with endocrine signaling pathways. Low-dose effects have been proposed as possible biological responses to POPs as endocrine disruptors (Vandenberg et al. 2012). Endocrine function generally declines with age because hormone receptors become less sensitive and levels of most hormones change with age (Chahal and Drake 2007). Therefore, the different age distributions among study populations might have led to different results, even when comparing similar concentrations of POPs. In addition, endocrine-disturbing effects of a specific POP might differ relative to the presence and concentrations of other potential endocrine disruptors. Inconsistencies across studies may be also to the underlying risk (nutrition, polymorphism, non-chemical stressors, and diseases) in the population as well as endocrine state (sex, menopausal status). Humans are exposed to a mixture

of various POPs, and exposure patterns are unique to each study population. Although concentrations of a particular POP might be similar between 2 populations, the strength of association between that POP and diabetes can differ depending on concentrations of other POPs. These POPs are lipophilic and have similar pharmacokinetic behavior in the body, which means they have the possibility to interact and influence the overall joint toxicity, so they should be considered as mixtures instead of on an individual basis. Hence, we need highly sophisticated data analysis tools to correlate multi-chemical POP exposure and health effects associations observed in epidemiological studies.

Novel methods of analysis including machine learning, bioinformatics and systems biology tools are available and can be used to identify specific outcome pathways from complex data (Minihane et al. 2015). Such efforts if persuaded will help identify specific and sensitive biomarkers as proposed in our study. This type of cluster identification of biomarkers as signatures of chemical mixtures exposure will help advance mixtures risk assessment methods development. Epidemiological studies need to assess inflammatory markers related to metabolic diseases, therefore, the sensitivity and specificity of these available biomarkers that are influenced by a range of modifying factors (chemical mixture components, age, sex, diet, disease, gut biota, etc) can be studied using multiple sophisticated techniques. Innovative inflammation markers could be developed for their use in human population studies, disease prevention and clinical use to detect multiple chemical exposures.

The resulting global network of common downstream activation targets was significantly enriched with metabolic disease category. Interestingly, neoplasms were also over-represented among the common targets with transcription factors, receptor tyrosine kinases, and cyclin

genes. This share common pathway could guide our understanding of the potential carcinogenic mechanisms shared by the POPs.

The final network presents a novel systems biology and toxicology model of different molecular mechanisms of POPs action that point to common disease outcomes. Future experimental evaluation of this model might lead to the development of new predictive markers of POPs effects that could translate into new disease prevention and clinical use strategies. Specific avenues of laboratory research might include, but not limited to, in vitro studies of target cell populations such as liver cells and adipocytes, moreover, cell lines studies can be done with pancreatic cells, hepatocytes, and brown adipocytes. Complimentary in vivo studies in both normal and obese mouse strains dosed with POPs could be performed to determine if the observed in vitro study findings are observed after in vivo exposure. Also, transgenic mouse models with human fatty acid metabolism genes and any other potential monogenic or polygenic rodent models. Both in vitro and in vivo studies should be conducted using exposure to the 3 selected POPs on an individual or mixture basis using a factorial design approach. Specific receptors or pathway nodes of interest identified using these combined in silico laboratory model approaches could be technically evaluated by application of genomic, proteomic or metabolomic methods. Putative biomarkers identified by these combined approaches could be further developed/translated into commercial test kits for clinical applications.

In conclusion, we examined 3 representative POPs and their possible combined effects via possible protein-protein interactions. Our results, using the inflammatory biomarkers pathway, demonstrated that looking beyond individual an individual chemical's pathway reveals a complex network of pathways that could be the basis of potential mechanism of joint toxicity of mixtures. Hence, the body burden of chemical mixtures, particularly those of lipophilic

chemicals such as POPs should be considered within the larger framework of diabetes, metabolic syndrome and other chronic diseases prevention. Biomarkers identified through such pathway analyses could be studied thoroughly and used to test real life mixture exposures. Further investigations carried out to study the influence of factors such as multiple chemical exposures, nutrition, age, gender, and genetic variations will help develop personalized specific treatment protocols for these complex diseases.

References

Agency for Toxic Substances and Disease Registry (ATSDR) 2012. Addendum Toxicological profile for Chlorinated Dibenzo-*p*-Dioxins. U.S. Department of Health and Human Services, Public Health Service, Atlanta, GA

Agency for Toxic Substances and Disease Registry (ATSDR) 2008. Addendum Toxicological profile for DDT, DDE and DDD. U.S. Dept. Health Services, Public Health Service; Atlanta, GA.

Agency for Toxic Substances and Disease Registry (ATSDR) 2011. Addendum Toxicological profile for polychlorinated biphenyls (PCBs). U.S. Dept. Health Services, Public Health Service; Atlanta, GA.

Barouki R, Gluckman PD, Grandjean P, Hanson M, Heindel JJ. 2012. Developmental origins of non-communicable disease: Implications for research and public health. *Environmental health: a global access science source* 11:42.

Barrett JR. 2013. Pops vs. Fat: Persistent organic pollutant toxicity targets and is modulated by adipose tissue. *Environ Health Perspect* 121:a61.

Chahal H and Drake W. 2007. The endocrine system and ageing. *J. Pathol.*, 211: 173–180.

Ekins S, Nikolsky Y, Bugrim A, Kirillov E, Nikolskaya T. 2007. Pathway mapping tools for analysis of high content data. *Methods Mol Biol* 356:319-350.

Everett CJ, Frithsen IL, Diaz VA, Koopman RJ, Simpson WM Jr, Mainous AG III. 2007. Association of a polychlorinated dibenzo-*p*-dioxin, a polychlorinated biphenyl, and DDT with diabetes in the 1999–2002 National Health and Nutrition Examination Survey. *Environ Res* 103:413–418.

Ford ES, Giles WH, Mokdad AH. 2004. Increasing prevalence of the metabolic syndrome among U.S. adults. *Diabetes Care* 27:2444–2449.

Goldberg RB. 2009. Cytokine and cytokine-like inflammation markers, endothelial dysfunction, and imbalanced coagulation in development of diabetes and its complications. *J Clin Endocrinol Metab* 94:3171–3182.

Henriksen GL, Ketchum NS, Michalek JE, Swaby JA. 1997. Serum dioxin and diabetes mellitus in veterans of Operation Ranch Hand. *Epidemiology* 8:252–258.

Imbeault P, Findlay CS, Robidoux MA, Haman F, Blais JM, Tremblay A, et al. 2012. Dysregulation of cytokine response in Canadian first nation's communities: is there an association with persistent organic pollutant levels? *PloS One* 7:e39931.

Ip YT, Davis RJ. 1998. Signal transduction by the c-jun n-terminal kinase (jnk)--from inflammation to development. *Current opinion in cell biology* 10:205-219.

Kim KS, Lee YM, Kim SG, Lee IK, Lee HJ, Kim JH, et al. 2014. Associations of organochlorine pesticides and polychlorinated biphenyls in visceral vs. subcutaneous adipose tissue with type 2 diabetes and insulin resistance. *Chemosphere* 94:151–157.

Kim MJ, Pelloux V, Guyot E, Tordjman J, Bui LC, Chevallier A, et al. 2012. Inflammatory pathway genes belong to major targets of persistent organic pollutants in adipose cells. *Environ Health Perspect* 120:508–514.

Kodama S, Moore R, Yamamoto Y, Negishi M. 2007. Human nuclear pregnane X receptor cross-talk with CREB to repress cAMP activation of the glucose-6-phosphatase gene. *Biochem J* 407:373–381.

Kumar J, Lind PM, Salihovic S, van Bavel B, Ingelsson E, Lind L. 2014. Persistent organic pollutants and inflammatory markers in a cross-sectional study of elderly Swedish people: The pivus cohort. *Environ Health Perspect* 122:977-983.

Kuwatsuka Y, Shimizu K, Akiyama Y, Koike Y, Ogawa F, Furue M, et al. 2014. Yusho patients show increased serum IL-17, IL-23, IL-1 β , and TNF α levels more than 40 years after accidental polychlorinated biphenyl poisoning. *J Immunotoxicol* 11:246–249.

La Merrill M, Emond C, Kim MJ, Antignac JP, Le Bizec B, Clement K, et al. 2013.

Toxicological function of adipose tissue: Focus on persistent organic pollutants. *Environ Health Perspect* 121:162–169.

Langer P, Ukropec J, Kocan A, Drobna B, Radikova Z, Huckova M, et al. 2014. Obesogenic and diabetogenic impact of high organochlorine levels (HCB, *p*, *p'*-DDE, PCBs) on inhabitants in the highly polluted eastern Slovakia. *Endocrine regulations* 48:17–24.

Lee DH, Lee IK, Song K, Steffes M, Toscano W, Baker BA, et al. 2006. A strong dose-response relation between serum concentrations of persistent organic pollutants and diabetes: results from the National Health and Examination Survey 1999–2002. *Diabetes Care* 29:1638–1644.

Lee DH, Lind PM, Jacobs DR, Jr, Salihovic S, van Bavel B, Lind L. 2011. Polychlorinated biphenyls and organochlorine pesticides in plasma predict development of type 2 diabetes in the elderly: the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study. *Diabetes Care* 34:1778–1784.

Lee DH, Porta M, Jacobs DR, Jr., Vandenberg LN. 2014. Chlorinated persistent organic pollutants, obesity, and type 2 diabetes. *Endocrine reviews* 35:557-601.

Lee DH, Steffes MW, Sjödin A, Jones RS, Needham LL, Jacobs DR., Jr. 2010. Low dose of some persistent organic pollutants predicts type 2 diabetes: a nested case–control study. *Environ Health Perspect* 118:1235–1242.

Longnecker MP, Michalek JE. 2000. Serum dioxin level in relation to diabetes mellitus among Air Force veterans with background levels of exposure. *Epidemiology* 11:44–48.

Magliano DJ, Loh VH, Harding JL, Botton J, Shaw JE. 2014. Persistent organic pollutants and diabetes: A review of the epidemiological evidence. *Diabetes Metab* 40:1–14.

Mayer TZ, Simard FA, Cloutier A, Vardhan H, Dubois CM, McDonald PP. 2013.

The p38-MSK1 Signaling Cascade Influences Cytokine Production through CREB and C/EBP Factors in Human Neutrophils. *Journal of Immunology* 191:4299-4307.

Medzhitov R. 2008. Origin and physiological roles of inflammation. *Nature* 454:428–435.

Minihane AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, et al. 2015. Low-grade inflammation, diet composition and health: Current research evidence and its translation. *Br J Nutr*: 1-14.

Pal S, Blais JM, Robidoux MA, Haman F, Krummel E, Seabert TA, et al. 2013. The association of type 2 diabetes and insulin resistance/secretion with persistent organic pollutants in two first nations communities in Northern Ontario. *Diabetes & metabolism* 39:497-504.

Paoletti R, Bolego C, Poli A, Cignarella A. 2006. Metabolic Syndrome, Inflammation and Atherosclerosis. *Vascular Health and Risk Management* 2:145-152.

Rignell-Hydbom A, Rylander L, Hagmar L. 2007. Exposure to persistent organochlorine pollutants and type 2 diabetes mellitus. *Hum Exp Toxicol* 26:447-452.

Rylander L, Rignell-Hydbom A, Hagmar L. 2005. A cross-sectional study of the association between persistent organochlorine pollutants and diabetes. *Environ Health* 4:28.

Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6, *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 1813: 878-888. Scivo R, Vasile M, Bartosiewicz I, Valesini G. 2011. Inflammation as “common soil” of the multifactorial diseases. *Autoimmun Rev* 10:369-374.

Suzuki T, Hirata K, Elkind MSV, Jun Z, Rndek T, Miyake Y, et al. 2008. Metabolic syndrome, endothelial dysfunction, and risk of cardiovascular events: the Northern Manhattan Study (NOMAS). *Am Heart J* 156:405-410.

Tabb MM, Kholodovych V, Grun F, Zhou C, Welsh WJ, Blumberg B. 2004. Highly chlorinated PCBs inhibit the human xenobiotic response mediated by the steroid and xenobiotic receptor (SXR). *Environ Health Perspect* 112:163-169.

Tak PP, Firestein GS. 2001. Nf-kappab: A key role in inflammatory diseases. *The Journal of clinical investigation* 107:7-11.

Taylor KW, Novak RF, Anderson HA, Birnbaum LS, Blystone C, Devito M, et al. 2013. Evaluation of the association between persistent organic pollutants (POPs) and diabetes in epidemiological studies: a national toxicology program workshop review. *Environ Health Perspect* 121:774–783.

Thayer KA, Heindel JJ, Bucher JR, Gallo MA. 2012. Role of environmental chemicals in diabetes and obesity: a National Toxicology Program workshop report. *Environ Health Perspect* 120:779–789.

Turyk M, Anderson H, Knobeloch L, Imm P, Persky V. 2009. Organochlorine exposure and incidence of diabetes in a cohort of Great Lakes sport fish consumers. *Environ Health Perspect* 117:1076–1082.

Vandenberg LN, Colborn T, Hayes TB, et al. 2012. Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses. *Endocrine Reviews*. 33:378-455.

Vogel CF, Sciallo E, Li W, Wong P, Lazennec G, Matsumura F. 2007. Relb, a new partner of aryl hydrocarbon receptor-mediated transcription. *Molecular endocrinology (Baltimore, Md)* 21:2941-2955.

Wang SL, Tsai PC, Yang CY, Guo YL. 2008. Increased risk of diabetes and polychlorinated biphenyls and dioxins: a 24-year follow-up study of the Yucheng cohort. *Diabetes Care* 31:1574–1579.

Figure Legends

Figure 1. Proposed Global Network for potential converging genes associated with diabetes/insulin resistance, or obesity, or metabolic syndrome X, and the three POPs. Green arrows= activating interactions; Red arrows= inhibiting interactions. Thick lines= highlight the closest interactions. Large gray circles represent the genes that are union. Small red circles indicate the intersection genes for the 3 diseases. POPs=purple hexagon; Catalytic factors= yellow; transcription factors= red; Cytokines and lipoproteins=green; receptors and adaptor proteins=blue. Symbols as defined by MetaCore™ at <http://lsresearch.thomsonreuters.com/static/uploads/files/2014-05/MetaCoreQuickReferenceGuide.pdf>

Figure 2. Activation of IL-8. Green arrows= activating interactions; Red arrows= inhibiting interactions. Thick lines= highlight the closest interactions. Thin Lines= Intermediate and farther interactions. POPs=purple hexagon; Cytokine IL-8 in green; Transcription factors in red. Symbols as defined by MetaCore™ at <http://lsresearch.thomsonreuters.com/static/uploads/files/2014-05/MetaCoreQuickReferenceGuide.pdf>

Figure 3. Activation of IL-6. Green arrows= activating interactions; Red arrows= inhibiting interactions. Thick lines: highlight the closest interactions. Thin Lines= Intermediate and farther interactions. POPs=purple hexagon; cytokine IL-6 in green; Transcription factors in red. Symbols as defined by MetaCore™ at <http://lsresearch.thomsonreuters.com/static/uploads/files/2014-05/MetaCoreQuickReferenceGuide.pdf>

Figure 4. Activation of TNF- α . Green arrows= activating interactions; Red arrows= inhibiting interactions. Thick lines= highlight the closest interactions. Thin Lines= Intermediate and farther interactions. POPs=purple hexagon; Cytokine TNF- α in green; Transcription factors in red. Symbols as defined by MetaCore™ at <http://lsresearch.thomsonreuters.com/static/uploads/files/2014-05/MetaCoreQuickReferenceGuide.pdf>

Figure 5. Activation of fetuin A. Green arrows= activating interactions; Red arrows=inhibiting interactions. Thick lines= highlight the closest interactions. Thin Lines= Intermediate and farther interactions. POPs=purple hexagon; Generic binding protein Fetuin A in blue; Transcription factors in red. Symbols as defined by MetaCore™ at <http://lsresearch.thomsonreuters.com/static/uploads/files/2014-05/MetaCoreQuickReferenceGuide.pdf>

Figure 6. Activation of IL-8 and cyclin D1 (Proposed inflammation and cancer share the same POP's mixture pathway). Green arrows= activating interactions; Red arrows= inhibiting interactions. The POPs (TCDD, PCB 153 and p, p'-DDE are depicted in purple (hexagonal symbols), transcription factors in red (flash star symbol), Generic binding protein Cyclin D1 in blue and cytokine IL-8 in green. Thick red and green arrows emphasize primary POP binding targets with inhibiting and activating effects, respectively. Yellow highlight shows those paths that needed an additional node (CREB1) in order to further converge on the same downstream targets via PXR, whereas AR and AHR directly connect to the common downstream targets. MetaCore™ by Thomson Reuters <http://lsresearch.thomsonreuters.com/static/uploads/files/2014-05/MetaCoreQuickReferenceGuide.pdf>

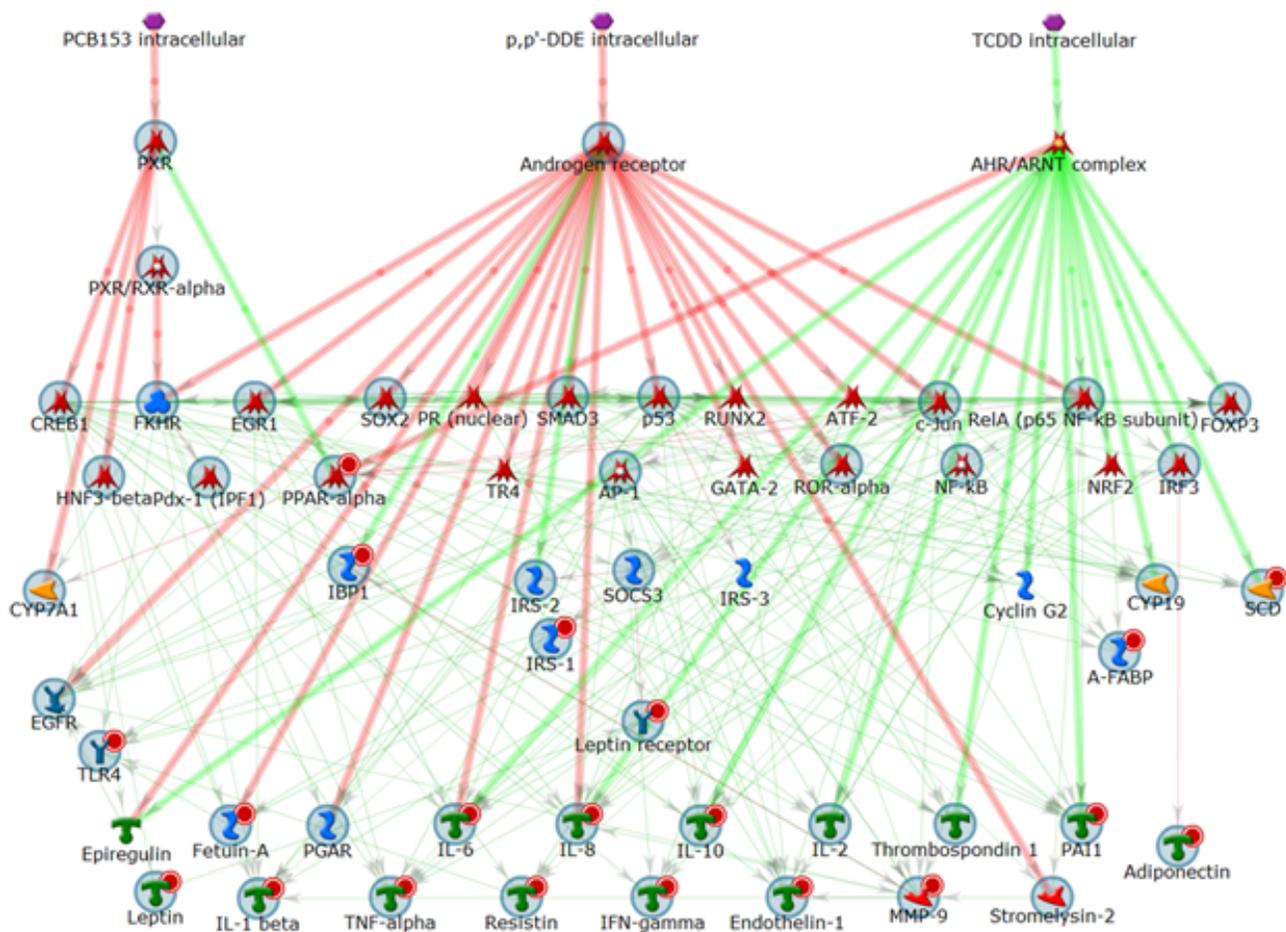


Figure 1.

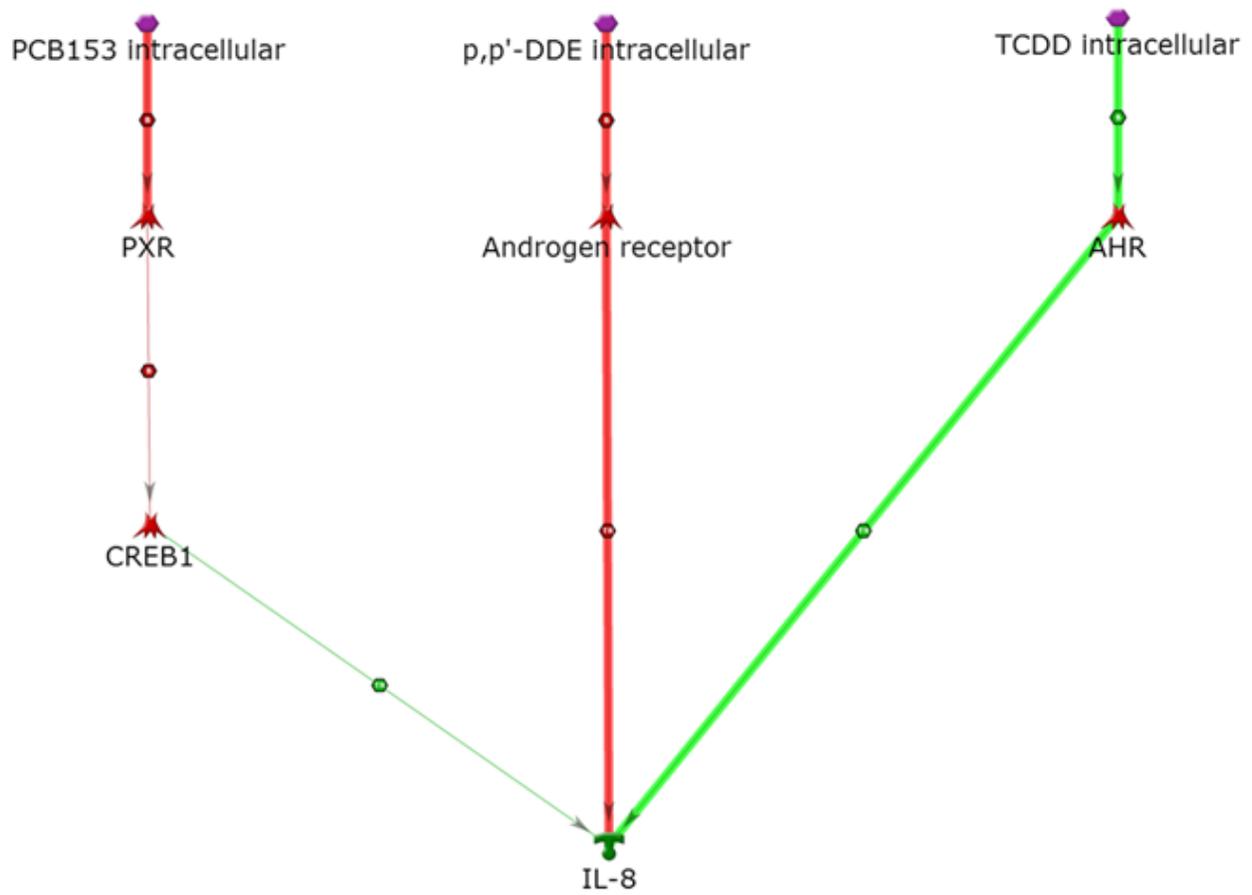


Figure 2.

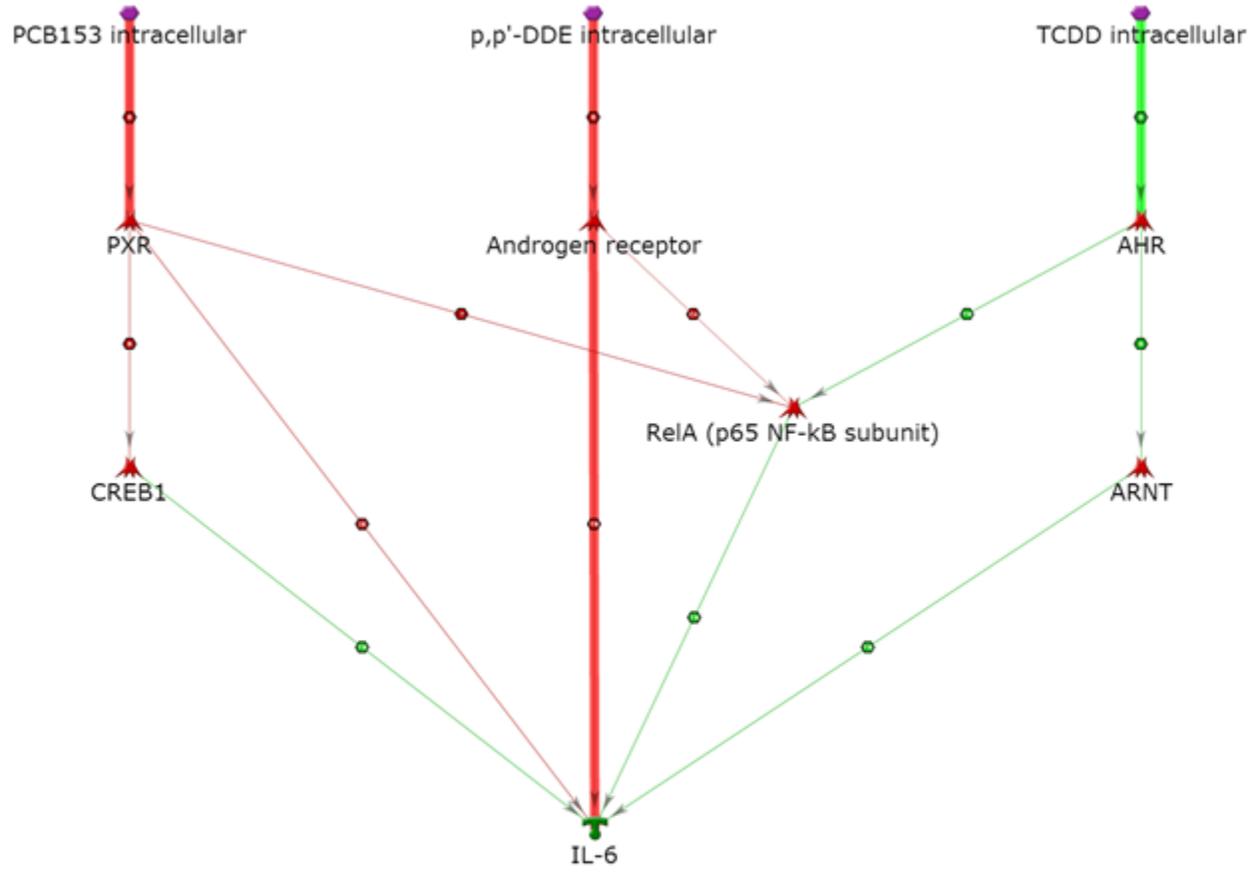


Figure 3.

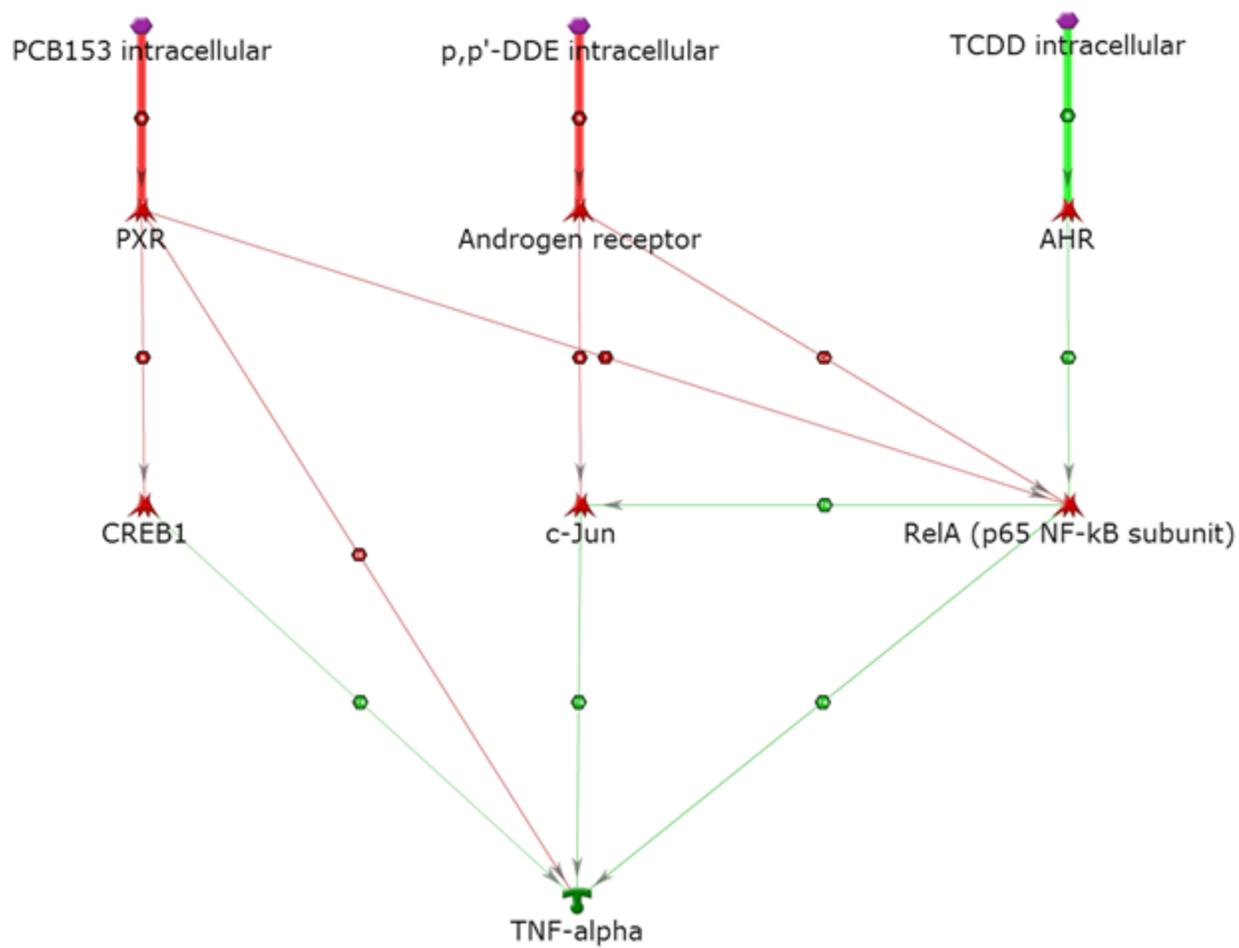


Figure 4.

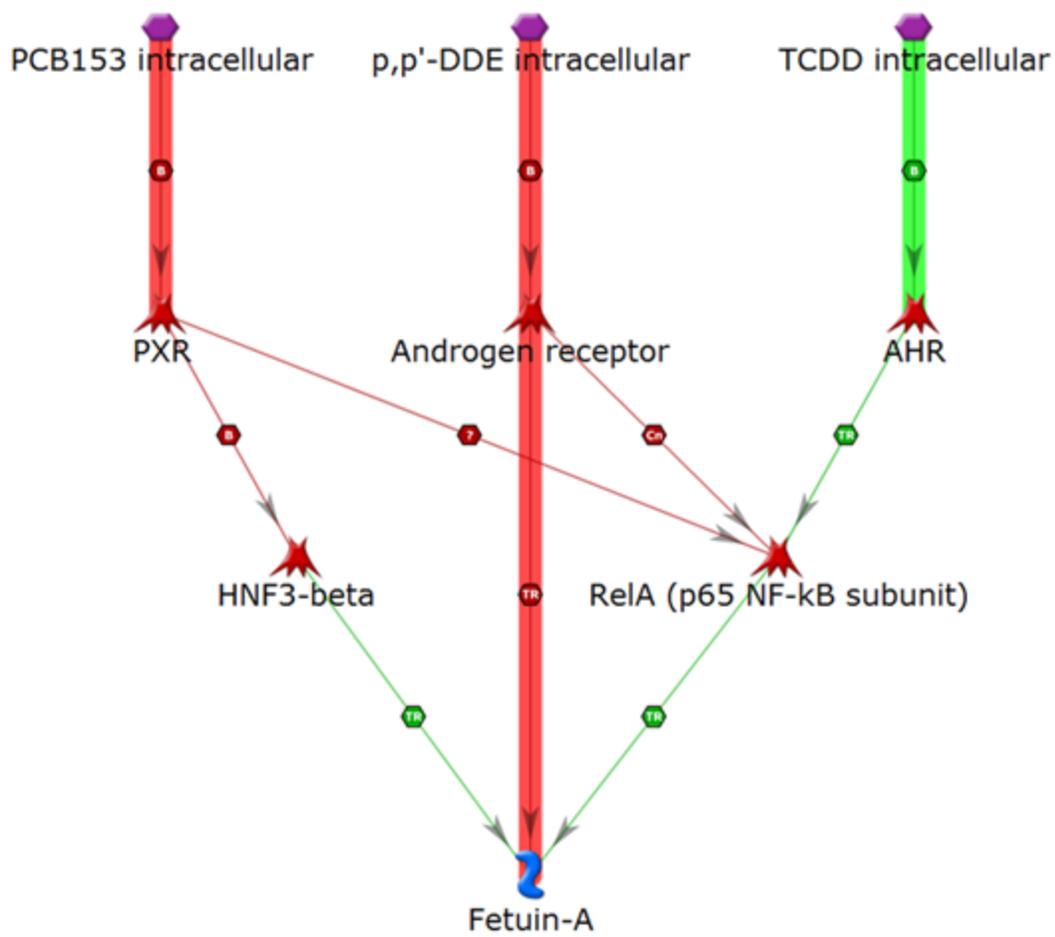


Figure 5.

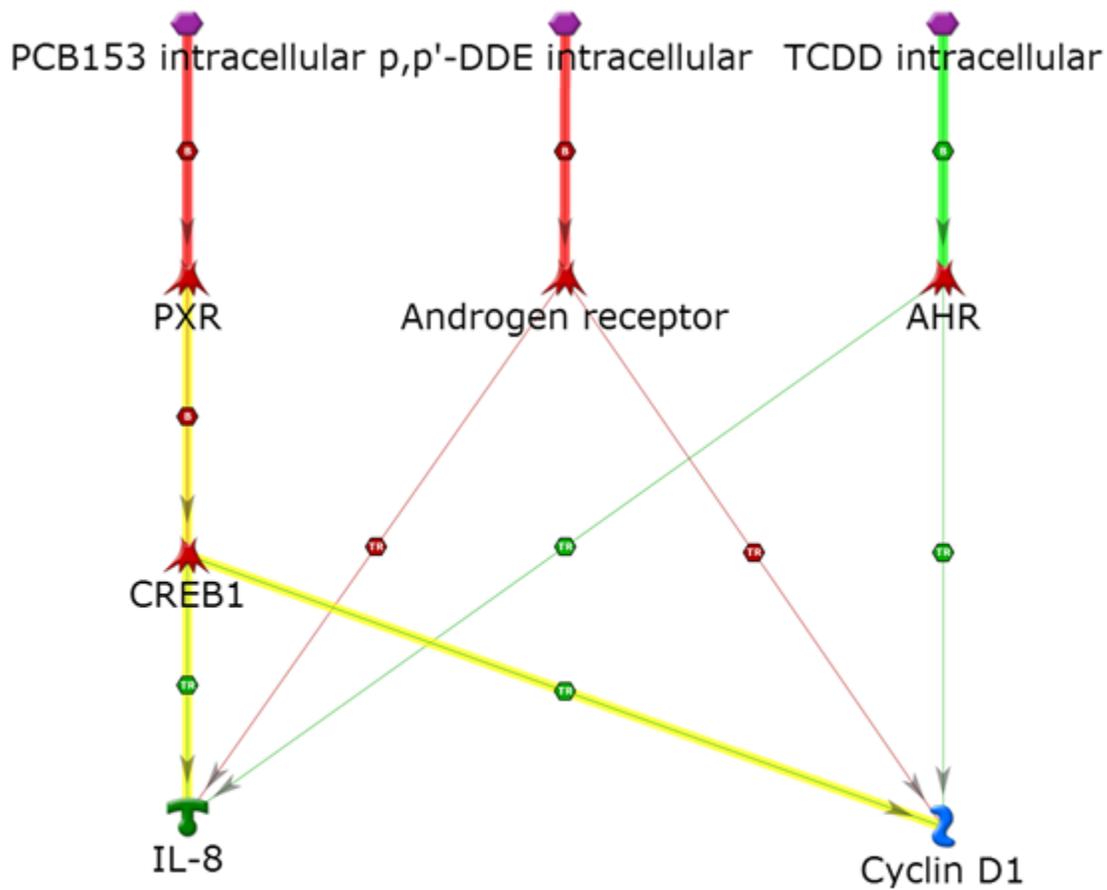


Figure 6.