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Transcriptomic Analysis of Gonadal Adipose Tissue in Male Mice Exposed Perinatally to 2,2',4,4'-Tetrabromodiphenyl Ether (BDE-47)

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Abstract: For the majority of lipophilic compounds adipose tissue is traditionally considered as storage depot and only rarely as a target organ. Meanwhile, abnormalities in adipose tissue physiology induced by chemical exposures may contribute to the current epidemic of obesity and metabolic diseases. Polybrominated diphenyl ethers (PBDEs) is a group of lipophilic flame retardants found in majority of human samples in North America. Their ability to alter physiology of adipose tissue is unknown. We exposed pregnant mice to 0.2 mg/kg body weight/day of BDE-47 perinatally. Transcriptomic changes in gonadal adipose tissue were analyzed in male offspring using RNA-seq approach with subsequent bioinformatic analysis. Genes of coagulation and complement cascade, de novo lipogenesis, and xenobiotic metabolism were altered in expression in response to BDE-47 exposure. The affected molecular network included the following hubs: PPAR α , HNF1A and HNF4. These findings suggest that adipose tissue should be considered a target tissue for BDE-47, in addition to its role as a storage depot. This study also builds a background for a targeted search of sensitive phenotypic endpoints of BDE-47 exposure, including lipid profile parameters and coagulation factors in circulation. Additional studies are needed to investigate the role of PBDEs as an obesogen.

Keywords: polybrominated diphenyl ether; PBDE; BDE-47; adipose; transcriptomic; genomic; obesogen; complement and coagulation cascade; de novo lipogenesis; metabolism.

1. Introduction

Epidemic of metabolic disease represents one of the biggest public health challenges. Approximately two out of every five Americans will develop Type 2 diabetes at some point during their adult lives [1]. More than 60% of children above the age of 10 either are obese or will become obese later in life. One in four overweight children suffer from impaired glucose tolerance [2]. A growing body of evidence links the current worldwide epidemics of obesity, diabetes, and related metabolic diseases with environmental exposures [3]. Many environmental xenobiotics, such as dioxins, PCBs, PBDEs and others are highly lipophilic and therefore accumulate in adipose tissue in humans, livestock and wildlife. Adipose tissue is usually considered a storage depot of lipophilic compounds and is only rarely considered a target of toxicity in toxicological research [4], although it is known to be involved in several physiological functions, including metabolic regulation, energy storage, and endocrine functions [4]. For example white adipose tissue is a major secretory cell of diverse list of proteins collectively

known as adipokines involved in regulation of metabolic functions of the organism [5]. Changes in adipose physiology induced by environmental xenobiotics may be associated with obesity, diabetes and other metabolic diseases [6,7].

Polybrominated diphenyl ethers (PBDEs), are a type of brominated flame retardants that were used in many household products such as paints, plastics, textiles, furniture padding, mattresses, and various electrical appliances until recently [8]. Although the commercial use of PBDEs has been ceased in many industrial countries, PBDEs are ubiquitously found in environment due to their resistance to degradation. The level of PBDEs in human blood, breast milk and adipose tissue has increased exponentially for three decades and continues to rise in North America [9]. Amongst the various PBDE congeners, 2,2',4,4'- tetrabromodiphenyl ether (BDE-47) is the most prevalent in human samples, including maternal milk [10-15]. PBDEs are lipophilic and bioaccumulate in fatty tissues with a half-life in human tissues estimated at 1.8-6.5 years [16]. Although PBDEs accumulate predominantly in adipose tissue, majority of studies of PBDE toxicity were focusing on neurodevelopment, endocrine and reproductive endpoints [17-19] and target organs such as the liver, thyroid gland, kidney, and reproductive organs. The ability of PBDEs to affect the physiology of adipose tissue was not thoroughly addressed in previous studies.

In this study, we analyze global gene expression in gonadal adipose tissue of mice exposed perinatally to low dose (0.2 mg/kg body weight) of BDE-47. Our data demonstrate significant changes in several functional networks including coagulation and complement cascade, lipid and lipoprotein synthesis and xenobiotic metabolism pathways.

2. Materials and Methods

2.1. Animals and Treatment

Eight-week old male (30–35 g) and female (27–30 g) animals were obtained from Charles River Laboratories (Kingston, NY, USA) and housed in a temperature (23±2°C)- and humidity (40±10%)-controlled environments, with a 12-h light/dark cycle, and food and water available *ad libitum*. After 3 days of acclimation, the animals were bred and the day of vaginal plug detection was considered pregnancy day 1. The dams were assigned to one of two treatment groups ($n=5$ per group) based on weight match, and were exposed to tocopherol-stripped corn oil (MP Biomedicals, Solon, OH) or 0.2 mg/ml solution of BDE-47 (AccuStandard, Inc., New Haven, CT; 100% purity) in tocopherol-stripped corn oil daily from pregnancy day 8 through postpartum day 21; the females were fed 1 µl/gram BW from the tip of a pipette, resulting in exposure of 0.2 mg/kg BW/day. This method of exposure is routinely used in our laboratory as a substitution of oral gavage as the later method induces significant stress response by the endocrine system, which may interfere with analyzed health outcomes [20]. The dams were allowed to deliver naturally, and the litters were not culled to maintain consistency of nutrient distribution among the same number of fetuses/pups at pre- and postnatal periods, and to avoid catch-up growth [21]. The dams and pups were kept together until weaning on PND 21, when the male and female pups were separated. Using cervical dislocation, one randomly selected male pup per litter was euthanized on PND21. Other pups were used in different study. All animals were euthanized between 9am and 11am following 2 hours of fasting. Gonadal fat from the right side of each

animal was collected, weighed and snap frozen in liquid nitrogen and stored at -80°C. All procedures met the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and this study was approved by the Institutional Animal Care and Use Committee at University of Massachusetts, Amherst.

2.2. RNA Extraction and Sequencing

Four samples of gonadal fat per exposure group were used for RNA extraction, collected from 4 individual pups from 4 different litters per exposure group. Total RNA was isolated using TRIzol reagent (Invitrogen) and quantified using a NanoDrop 1000 instrument (Thermo Fisher Scientific, Wilmington, DE). RNA quality was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples of RNA with integrity values > 9 were used for library preparation in accordance with recommendations of library preparation and sequencing kit manuals. The NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490, New England BioLabs Inc., Ipswich, MA) was then used to isolate intact poly(A)+ RNA from 3 µg of total RNA. Libraries were constructed using NEBNext mRNA Library Prep Reagent Set for Illumina with multiplexing indexes from NEBNext Multiplex Oligos for Illumina (E6100 and E7335 respectively, New England BioLabs Inc., Ipswich, MA). The quality and purity of the libraries was assessed by Agilent 2100 Bioanalyzer, and the concentration of the libraries was measured by real-time PCR with primers for P5 and P7 flow cell oligo sequences using KAPA Library Quantification Kit (KR0405, Kapa Biosystems, Boston, MA) in a 384-well plate on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). High-throughput sequencing was performed on a NextSeq500 sequencing system (Illumina, San-Diego, CA) in the Genomic Resource Laboratory of the University of Massachusetts, Amherst. cDNA libraries were single-end sequenced in 75 cycles using NextSeq 500 High Output Kit (FC-404-1005, Illumina, San-Diego, CA) in a multiplex run. Four samples were sequenced per exposure group representing eight different litters. Sequencing was completed with minimum 20 million reads per sample. One control sample was sequenced with smaller number of reads and it was excluded from further analysis. All sequencing data were uploaded to the GEO public repository and GEO were assigned series accession number

2.3. RT-qPCR

The RNA-seq results were validated using RT-qPCR for genes selected randomly from the list of the most significantly differentially regulated genes in RNA-seq experiment (Table 1). Total RNA was purified of genomic DNA contamination using DNase (RQ1 RNase-free DNase, Cat. # M610A, Promega, Madison, WI) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Cat.# 4368814, Applied Biosystems, Vilnius, Lithuania). Using free online software Primer3Plus (primer3plus.com), forward and reverse primers were designed to anneal different exons spanning long intron (Table 1). The housekeeping gene (Polr3c) was selected from genes that were not regulated in our RNA-seq dataset, with the consideration of its ubiquitous presence in different cell types. Triplicate 5-µl real-time PCR reactions, each containing iTaq Universal SYBR Green Supermix (Cat 172-5124, BioRad), primers, and cDNA template were loaded onto a 384-well plate and run through 40 cycles on a CFX384 real time cycler (Bio-Rad Laboratories, Inc). The data were analyzed using the manufacturer's CFX

manager software, version 3.1. Relative quantification was determined using the $\Delta\Delta Cq$ method [22].

2.4. *Bioinformatic Analysis of RNA-seq Data*

To analyze the data from our mRNA sequencing experiment, read filtering, trimming and de-multiplexing were performed via the BaseSpace cloud computing service supported by Illumina (<https://basespace.illumina.com/home/index>). The preprocessed reads were mapped to the reference mouse genome (MM10) using TopHat 2 aligner [23,24]. Aligned reads were then used for assembly of novel transcripts with Cufflinks 2.1.1 and differential expression of novel and reference transcripts with Cuffdiff 2.1.1 [25]. The list of differentially expressed transcripts was filtered to select only transcripts associated with gene names and transcripts with FPKM values > 1 in at least one of conditions (control or exposed). The resulting gene list included 14289 genes.

DAVID bioinformatics resources version 6.7, Gene Set Enrichment Analysis (GSEA), and Ingenuity Pathway Analysis (IPA) were used for bioinformatics analysis of RNA-seq data. A short list of 2-fold significantly differentially expressed genes was imported to DAVID or IPA for functional analysis with default settings. GSEA analysis was conducted using a ranked list of 14289 genes against the following collections of gene-sets: hallmark genes sets (H), curated gene sets from online pathway databases, publications in PubMed, and knowledge of domain experts (C2) and gene ontology gene sets (C5) obtained from the molecular signatures database v5.2 [26] and PPAR α target genes obtained from [27]. Gene sets enriched with FDR q-value < 0.02 were considered as significant.

3. Results

We found no significant relationship between litter size and exposure to BDE-47, with the number of pups varying from 11 to 15 per litter. No weight differences were observed between the control and exposed dams and pups throughout the experiment. No changes in adipose tissue weight were found in male mice on PND21.

The resulting filtered list of differentially expressed genes included 14289 genes. 758 genes were differentially expressed 2-fold or higher (Fig. 1) and 94 genes also had FDR q-value of ≤ 0.05 (Table S1). Expression of select genes measured by RT-PCR showed good concordance with RNA-seq data (Table 1).

3.1. *DAVID analysis*

DAVID analysis revealed four enriched annotation clusters with an enrichment score > 2.0: 'secreted proteins', 'peptidase inhibitor activity', 'complement and coagulation cascades' (Fig 2), 'biosynthesis of amino acid', 'microsomal metabolism', and 'lipid metabolism' (Table 1).

3.2. *GSEA analysis*

No significantly positively enriched gene sets were found in GSEA analysis (see Table S2 for details). The following hallmark gene sets were negatively enriched with FDR q < 0.2: 'coagulation', 'xenobiotic metabolism', and 'bile acid metabolism' (Fig. 3A). Analysis of C2 collection of

gene sets revealed several negatively regulated liver specific gene sets, including genes targets of HNF1A and HNF4A (Fig. 3B). Several positively enriched (although not significant) gene sets indicated upregulation of lipid biosynthesis, including ‘SREBF targets’ (Fig. 3C), ‘obesity’ and ‘cholesterol biosynthesis’. Significantly negatively enriched gene ontology (GO) gene sets included ‘coagulation’, ‘serine type endopeptidase activity’, and ‘lipid catabolism’. Genes – targets of PPARα were also significantly downregulated (normalized enrichment score = -1.72, nominal *p*-value <0.0001, FDR *q* < 0.001).

3.3. IPA analysis

The top IPA enriched canonical pathways were ‘FXR/RXR activation’, ‘LXR/RXR activation’, ‘acute phase response signaling’, ‘coagulation system’, and ‘extrinsic prothrombin activation pathway’ (Table 3). HNF1A (*p*-value of overlap = 2.98e-37, predicted activation - inhibited), and PPARα (*p*-value of overlap = 1.41e-18, predicted activation – n/a) were identified as upstream regulators with significantly altered activity (Fig. 4).

3.4. Figures, Tables and Schemes

Table 1. RT-qPCR validation of RNA-seq data for select differentially expressed genes.

Gene	qPCR Primers	Fold change	
		RNA-seq	qPCR
Grem2	GAGGAGAGGGACAGGGAGAC AGCGAGAGCTTCCAGAACAT	2.8	2.6
Cish	CGTTGTCTCTGGGACATGGT ACAGCCAGCAAAGGACAAGA	4.4	3.9
Sfrp2	AGGACAACGACCTCTGCATC TTCTTGTTTTGCAGGCTTC	4.3	4.2
Fgg	AACCCACGAGACAAGCATTC CAAGCTGGGCTACCTTCTGT	-15.7	-16.3
F2	CCCTGGTTAGCCTGGTACAC CAGCGATAGTGCTTGCTGAG	-16.2	-13.9
Apob	AAAACCTTAAGCTTCAGCTGTCCA CTTTCGGAGGTGCTTGAATC	-20.3	-17.5
B2m	CCGGCCTGTATGCTATCCAG TGTTCCGGCTTCCCATTCTCC	--	housekeeping

Table 2: DAVID functional annotation clustering for 94 2-fold differentially expressed genes (FDR *q* ≤ 0.05).

Functional Cluster	Annotation	Enrichment score	Genes	P-value	Benjamini
	Secreted proteins	11.17	38	6.7E-6 – 3.0E-18	7.0E-4 – 5.9E-16
	Peptidase inhibitor activity	5.15	10	2.7-E-2 – 1.9E-9	6.2E-1 – 9.2E-8
	Blood coagulation	3.14	22	2.7-E-2 – 1.9E-9	6.2E-1 – 9.2E-8
	Biosynthesis of amino acids	2.81	6	4.6E-2 – 1.2E-4	3.0E-1 – 5.7E-3

<i>Microsomal metabolism</i>	2.40	7	1.1E-1 – 1.9E-5	5.3E-1 – 4.5E-4
<i>Lipid metabolism</i>	2.27	5	3.8E-2 – 7.8E-4	3.9E-1 – 3.0E-2

Table 3: Ingenuity Pathway Analysis: top enriched canonical pathways.

<i>Canonical Pathway</i>	<i>P-value</i>	<i>Overlap</i>	<i>Count</i>
<i>FXR/RXR Activation</i>	2.4e-23	25.4%	32/126
<i>LXR/RXR Activation</i>	2.99e-17	21.5%	26/121
<i>Acute Phase Response Signaling</i>	3.02e-17	17.8%	30/169
<i>Coagulation System</i>	1.71e-15	42.9%	15/35
<i>Extrinsic Prothrombin Activation Pathway</i>	3.68e-11	56.2%	9/16

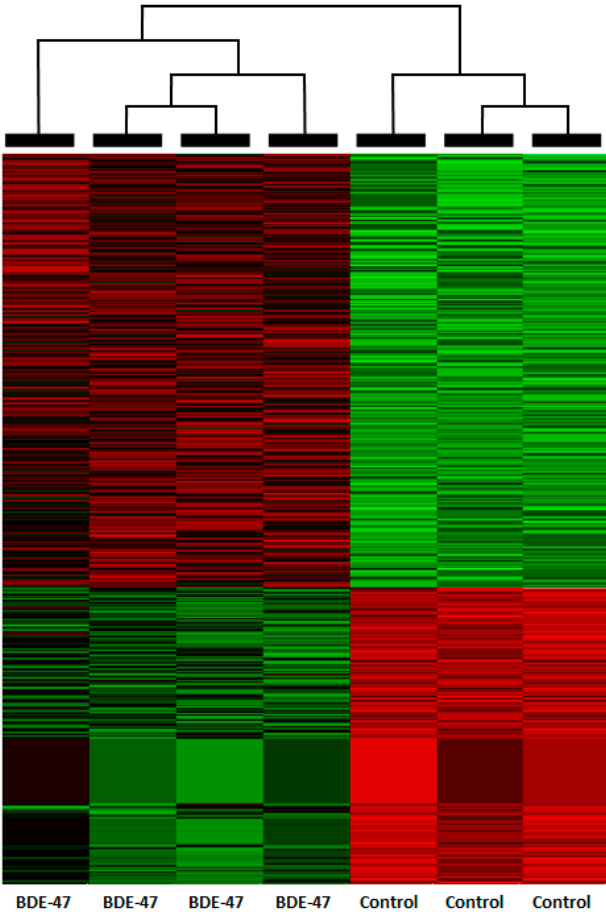


Figure 1. Heat-map for 758 2-fold differentially regulated genes in mouse gonadal adipose tissue on PND21.

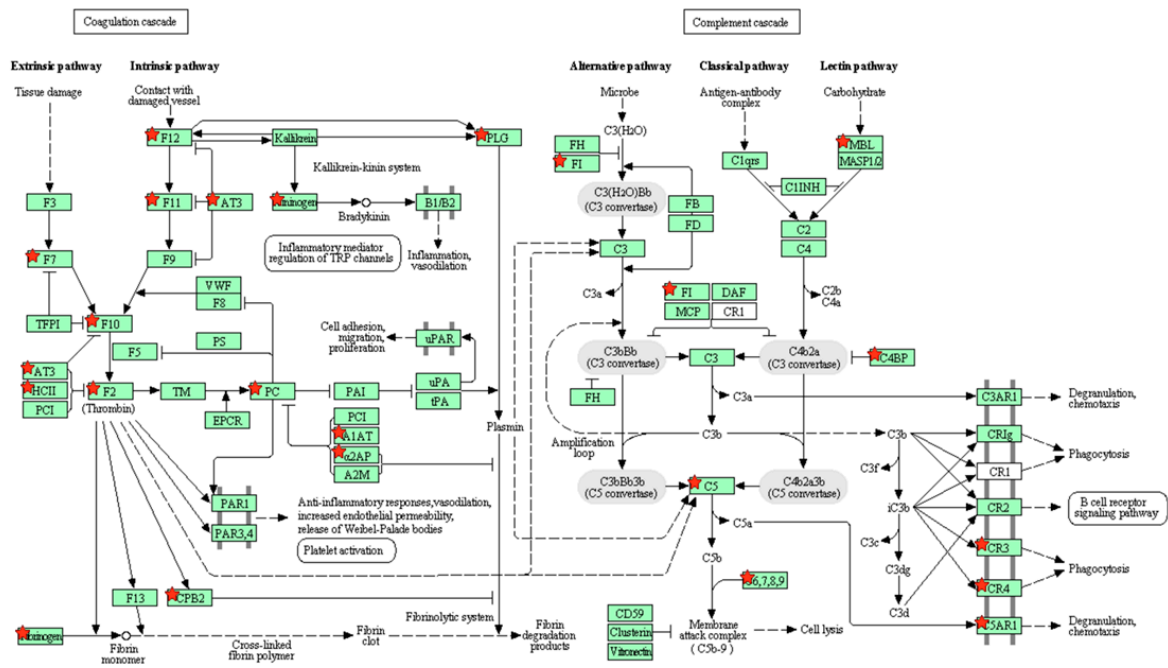


Figure 2. KEGG coagulation and complement cascades pathway. Differentially expressed genes are marked with red stars.

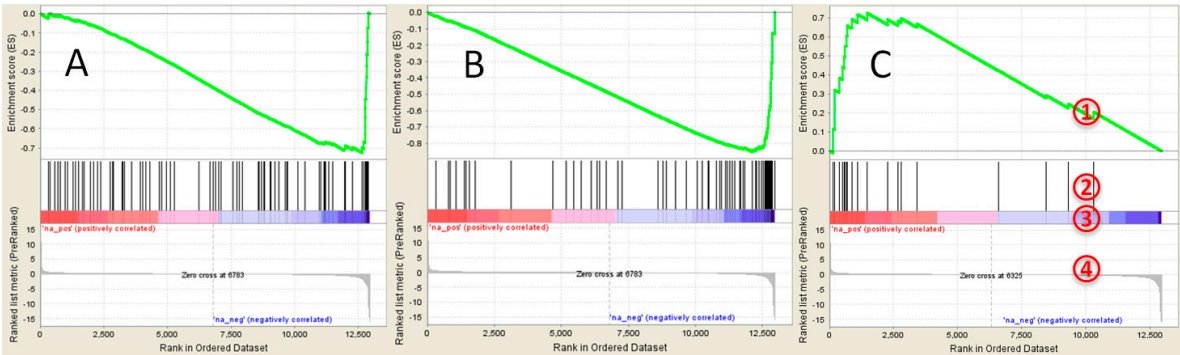


Figure 3. GSEA enrichment plots for transcriptional changes in gonadal adipose tissue of mice exposed perinatally to BDE-47: A – bile acid metabolism gene set; B - genes targets of HNF4A; and C – genes targets of SREBF. GSEA plot legend: 1 - running enrichment score for the gene set; 2 - vertical lines show where the members of the gene set appear in the ranked list of genes; 3 and 4 – ranked list of differentially expressed genes from the most up-regulated (left of each plot) to the most downregulated (right of each plot). Regulation is shown in the heat-bar (3) and in the bar plot (4).

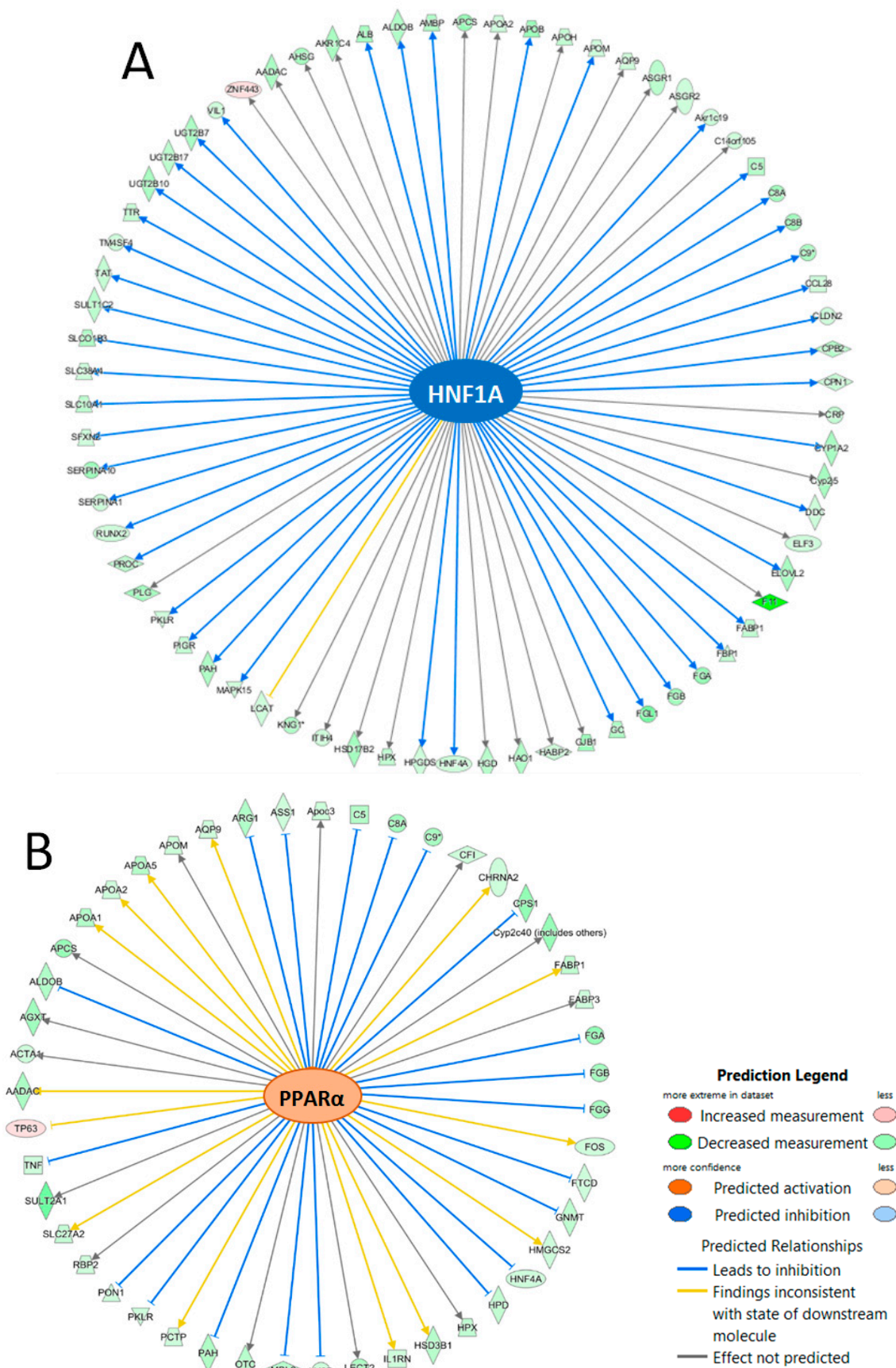


Figure 4. Molecular networks of differentially expressed genes regulated by (A) HNF1A and (B) PPARα.

4. Discussion

We have found that perinatal exposure to 0.2 mg/kg body weight of BDE-47 results in changes in expression of genes involved in the coagulation and complement cascades, xenobiotic metabolism and lipid metabolism in gonadal adipose tissue of mice, suggesting that adipose tissue can also be a target tissue for BDE-47, in addition to its role as a storage depo.

4.1. Relevance of the dosing paradigm

Results reported in this study were produced using environmentally-relevant exposure paradigm. In our previous study [28], exposure of pregnant rats to 0.2 mg/kg body weight of BDE-47 resulted in 234.3 ng BDE-47/g lipid in adipose tissue of dams and 1054.7 ng BDE-47/g lipid in adipose tissue of pups. These concentrations are comparable with those of the North American human population (mean concentration in adipose tissue of adult subjects = 399 ng/g lipids) [29]. Given that the rate of BDE-47 elimination is around 10 times higher in mice than in rats [30,31] the exposure used in this study is also relevant to that of the North American human population. In this study we dosed animals during pre- or neonatal period since human PBDE exposure peaks during the perinatal period of life due to the active transport of PBDE *via* cord blood and breast milk [32-34], higher rates of dust ingestion [35], and higher food intake per kilogram of body weight in toddlers [33].

4.2. Downregulation of the complement and coagulation cascades genes

Coagulation is the primary defense against blood loss at a site of injury. It is characterized by a serine protease mediated conversion of zymogens to their active form to induce blood clotting [36]. The major players in the coagulation and fibrinolytic system are circulating zymogens (fibrinogen, clotting factors, protein C and its cofactor protein S, plasminogen, kininogen), activating and converting enzymes (plasminogen and thrombin), a group of serine protease inhibitors (SERPINs), and high density lipoproteins (HDL). Our results indicate that the expression of the major players in the coagulation pathway and fibrinolytic pathway, namely fibrinogen protein chains (*FGG*, *FGA*, *FGB*), clotting factors (*F2*, *F7*, *F10*, *F11*, and *F12*), serine protease inhibitors (*SERPINC1*, *SERPIND1*, *SERPINF2*), plasminogen (*PLG*), and protein C (*PROC*) were downregulated by more than 2-fold. We also observed more than 2-fold downregulation of the complement cascade genes such as *C9*, *C8A*, *C8B*, *C4bp*, *Cfi*, *HC*, *Mbl1*, and *MBL2*. Adipose tissue has an important role in direct regulation of the coagulation and complement system by producing coagulation tissue factors and complement proteins [37,38]. Therefore, observed changes in gene expression may have significant consequence for the whole complement and coagulation system in exposed organism.

4.3. Downregulation of xenobiotic metabolism genes

A big group of heterogeneous xenobiotic metabolism genes, including but not limited to cytochromes P450 (*Cyp1a*, *Cyp2c* and *Cyp3a* subfamilies) was downregulated in exposed mice. This group of genes is usually induced in toxicological experiments, including experiments with BDE-47 [39-43], while in our experiment enrichment was negative, corresponding to significant decrease in expression of many genes. We speculate that this may be a result of overcompensation response to sub-chronic developmental exposure to low dose of BDE-47.

Similarly, changes in gene expression, opposite in direction to predicted, were reported recently for liver tissue in BDE-47 exposed mice [44].

4.4. Upregulation of *de novo* lipogenesis genes

Our bioinformatic analysis suggests that metabolic profile of adipose tissue in exposed animals is slightly shifted in a direction of activation of *de novo* lipogenesis (DNL). For example positive enrichment of GSEA '*SREBF targets*' dataset was due to upregulation of several essential DNL genes, including 1.6-fold induction of *Acaca*, encoding DNL rate-limiting enzyme acetyl-CoA carboxylase [45]. Genes of cholesterol biosynthesis were also upregulated, including 1.5-fold induction of *Hmgcs1*, encoding cytosolic 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, a rate-limiting enzyme of cholesterol biosynthesis [46]. At the same time, bile acid metabolism genes were downregulated suggesting decreased cleavage of cholesterol, concordant with previously reported increase in circulating cholesterol in rats perinatally exposed to BDE-47 [39]. However, it is though difficult to interpret the observed changes in molecular pathways responsible for cholesterol turnover in the current study, as adipose tissue is not a major player in cholesterol biosynthesis and elimination. Importantly, changes in gene expression discussed in this paragraph may affect the major function of the adipose tissue – to store energy in the form of lipids. This concern is supported by human population data reporting a positive association between PBDE concentrations in blood, milk, and placenta and BMI in nursing women [34,47-49]. In several animal studies, developmental BDE-47 exposures also resulted in increased body weights [50-52].

4.5. Network affected by BDE-47

The coagulation and complement system is known to be regulated among other transcription factors by HNF1, HNF4 α and PPAR α – mechanisms enriched in our bioinformatic analysis. For example the α and β promoters of fibrinogen gene contain tissue specific motif for HNF1 [53]. SERPINC1 (antithrombin) is transcriptionally activated by the binding of HNF1 α and HNF4 α to SERPINC1 gene (*α 1AT*) promoter/enhancer [54]. Additionally synthetic PPAR α agonists, fibrates, have been shown to suppress fibrinogen gene expression and impair the induction of tissue factors in endothelial cells in rodents [55,56] and downregulate the complement and coagulation pathways in cynomolgus monkey liver [57]. Both transcription factor HNF4A and PPAR α regulate expression of major protein component of the high density lipoproteins (HDL) [58,59]. In mice (contrary to humans) PPAR α upregulates expression of *APO5*, *APOA1* and *APOA2* [60-62]. In our study, the expression of several HDL genes *APOA1*, *APOA2*, *APOA5*, *APOB*, *APOC2*, *APOC3*, *APOC4*, *APOH*, *APOM* was more than 2-fold downregulated. HDL modulates protease activity by directly binding to majority of SERPINs [63]. Finally the expression of PPAR α is regulated by HNF4 in mice, and PPAR α is capable of binding to HNF4 α RE [64].

4.5. Role of PPAR α in the modulation of BDE-47 induced response

Our analysis identifies PPAR α as a hub of a molecular network affected in adipose tissue by BDE-47. No studies reported previously BDE-47 interaction with PPAR α . In fact, a theoretical structure-activity assessment predicted that PBDEs wouldn't have an interaction with PPAR

receptors [65]. However, several studies have indicated the ability of BDE-47 and/or its metabolites to activate another PPAR isoform – PPAR γ [66-68]. This evidence taken together with the fact that PPAR nuclear family is considered to have the largest and the most promiscuous binding pocket [69] suggests a possibility, that most of the observed changes in gene expression in the current study are a result of direct interaction of BDE-47 and/or its metabolites with PPAR α . An alternative hypothesis may explain changes in PPAR α activity by the ability of BDE-47 to interact with regulatory mechanisms upstream of the receptor. In our recent study we have shown that same dosing paradigm as the one used in this study results in activation of the mTOR signaling in mouse livers on PND21 [44]. PPAR α is a well-known target of mTOR complex 1 (mTORC1) [70-72]. Additional studies are needed to dissect interaction between BDE-47 and PPAR-regulated molecular network.

5. Conclusion

We have demonstrated that the low-dose perinatal exposure to BDE-47 produces significant changes in gene expression in the gonadal adipose tissue in mice, suggesting that adipose tissue should be considered a target tissue for BDE-47, in addition to its role as a storage depo. Our data implies that functional networks, including coagulation and complement cascades, de novo lipogenesis, and xenobiotic metabolism are affected by BDE-47. The affected molecular network is connected via the following hubs: PPAR α , HNF1A and HNF4. This study builds a background for a targeted search of sensitive phenotypic endpoints of BDE-47 exposure, including lipid profile parameters and coagulation factors in circulation. Additional research is needed to dissect molecular cascades affected in the adipose tissue by PBDEs, and the role of PBDEs as obesogens.

Supplementary Materials: The following are available online at ..., Table S1: Significant 2-fold differentially expressed genes in gonadal adipose tissue of mice exposed perinatally to 0.2 mg/kg body weight BDE-47; Table S2: GSEA analysis: summary of top enriched gene sets in the gonadal adipose tissue of mice exposed perinatally to 0.2 mg/kg body weight BDE-47.

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Author Contributions: A.S. conceived and designed the experiments, carried out all experimental work with animals, coordinated laboratory procedures, bioinformatic analysis and manuscript preparation. A.A. conducted bioinformatic analysis of RNA-seq data and drafted the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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