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Assessing Metabolic Differences Associated with Exposure to Polybrominated Biphenyl and Polychlorinated Biphenyls in the Michigan PBB Registry

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Background: Polybrominated biphenyls (PBB) and polychlorinated biphenyls (PCB) are persistent organic pollutants with potential endocrine-disrupting effects linked to adverse health outcomes.

Objectives: In this study, we utilize high-resolution metabolomics (HRM) to identify internal exposure and biological responses underlying PCB and multigenerational PBB exposure for participants enrolled in the Michigan PBB Registry.

Methods: HRM profiling was conducted on plasma samples collected from 2013 to 2014 from a subset of participants enrolled in the Michigan PBB Registry, including 369 directly exposed individuals (F0) who were alive when PBB mixtures were accidentally introduced into the food chain and 129 participants exposed to PBB *in utero* or through breastfeeding, if applicable (F1). Metabolome-wide association studies were performed for PBB-153 separately for each generation and ΣPCB (PCB-118, PCB-138, PCB-153, and PCB-180) in the two generations combined, as both had direct PCB exposure. Metabolite and metabolic pathway alterations were evaluated following a well-established untargeted HRM workflow.

Results: Mean levels were 1.75 ng/mL [standard deviation (SD): 13.9] for PBB-153 and 1.04 ng/mL (SD: 0.788) for ΣPCB. Sixty-two and 26 metabolic features were significantly associated with PBB-153 in F0 and F1 [false discovery rate (FDR) $p < 0.2$], respectively. There were 2,861 features associated with ΣPCB (FDR $p < 0.2$). Metabolic pathway enrichment analysis using a bioinformatics tool revealed perturbations associated with ΣPCB in numerous oxidative stress and inflammation pathways (e.g., carnitine shuttle, glycosphingolipid, and vitamin B9 metabolism). Metabolic perturbations associated with PBB-153 in F0 were related to oxidative stress (e.g., pentose phosphate and vitamin C metabolism) and in F1 were related to energy production (e.g., pyrimidine, amino sugars, and lysine metabolism). Using authentic chemical standards, we confirmed the chemical identity of 29 metabolites associated with ΣPCB levels (level 1 evidence).

Conclusions: Our results demonstrate that serum PBB-153 is associated with alterations in inflammation and oxidative stress-related pathways, which differed when stratified by generation. We also found that ΣPCB was associated with the downregulation of important neurotransmitters, serotonin, and 4-aminobutanoate. These findings provide novel insights for future investigations of molecular mechanisms underlying PBB and PCB exposure on health. https://doi.org/10.1289/EHP12657

Background

Endocrine-disrupting chemicals (EDCs) interfere with metabolism through a number of different processes.[1] EDCs have been associated with a broad range of health outcomes, including neurological disorders,[2] obesity occurrence,[1,3] and male and female reproductive health.[1] Polybrominated biphenyls (PBBs) and polychlorinated biphenyls (PCBs) are widespread environmental contaminants linked to several different endocrine-related effects and are considered EDCs[4] (Figure S1). PBBs were widely used as flame retardants in consumer products throughout the late 1960s and 1970s until manufacturing was discontinued in 1976.[5] PCBs were also flame retardants added to consumer products, especially electronics. Compared to PBBs, their use was more prolific and long-term, with the chemical applied from the 1930s until its discontinuation in 1977.[6] In Michigan, people were exposed to high levels of PBB from the contamination of livestock feed during a 1-year period in the 1970s that resulted in highly contaminated meat and dairy products being distributed throughout the state.[7] This population is also continuously exposed to PCBs through interaction with the local environment. This exposure level is similar to other populations that live near previously operating chemical factories or routinely consume contaminated food (i.e., local fish populations).[8]

In the U.S., much of the understanding of the human health impact of PBB is from research conducted in the Michigan PBB registry. This cohort was established in 1976 after an agricultural accident caused PBB to contaminate the Michigan food supply.[9] The cohort is still active today, and ongoing follow-up is focused on understanding the impacts of PBB exposure on those who experienced the original contamination and their offspring. Although PBB production was discontinued shortly after the incident in the U.S., PBB is still detectable at very low levels in 77% of the general U.S. population as recently as 2014.[10] Participants in the Michigan PBB registry have significantly higher PBB blood levels than the general U.S. population even 40 years after the contamination event.[5] The impact of PBB exposure is important to characterize as it can serve as a model for the potential chronic and multigenerational impact that other halogenated organic compounds still in use today may have on human health. For example, PBB is structurally similar to polybrominated diphenyl ethers (PBDEs), which are still in use today.[5] Characterizing the impact of PBB exposure can provide valuable information on the potential health effects of these other halogenated organic compounds and demonstrate the need for better regulation and safer alternatives. PBB has been associated with an increased risk of breast cancer in women,[11,12] abnormal hormone levels during the menstrual cycle,[13] and changes in thyroid hormone levels.[14] Children of exposed mothers (F1 generation) were also impacted. For example, researchers found that daughters experienced earlier age at menarche,[15] whereas sons experienced a delay in puberty.[16]

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Due to their extensive use from the 1920s to the 1970s and long half-lives, PCBs are among the more widespread pollutants to which people are regularly exposed.[17] Although all people experience low-level exposure, some populations are exposed to higher levels than others due to their geography, diet, and lifestyle. The Michigan PBB participants were exposed to PCBs from different sources, such as bioaccumulation in fish in the Great Lakes region and pollution from farms, and this chemical was quantified with PBB.[18,19] The impact of PCB exposure is important to understand as it is still persistent in the environment and affects human health. PCB has been linked to increased cancer risk,[12] poor neurodevelopment in children,[17,20] and changes in thyroid hormone levels.[14]

As many adverse health outcomes have been associated with both PBB and PCB, it is important to identify the metabolic effects associated with these exposures to provide a biological framework for understanding how they impact health. These two compounds share a similar chemical structure to other halogenated hydrocarbons, consisting of carbon-X bonds, where X is a halogen (chlorine, fluorine, bromine, or iodine).[21] Characterizing the impact of PBB and PCB exposure is important as it can serve as a model for the potential chronic and multigenerational impact that other halogenated organic compounds still in use today may have on human health. To date, the molecular mechanisms underlying PBB and PCB toxicity remain largely unknown despite numerous studies associating these chemicals with adverse human health outcomes and a few initial mechanistic studies.[22–24,25] With the advent of high-throughput sequencing and analytical technology, various omics approaches have shown great promise in providing critical information detailing chemical toxicity effects across multiple molecular levels. Metabolomics, which aims to systematically measure small molecule metabolites in biological samples, is a key analytical platform that provides a unified measurement for linking complex environmental exposure to internal dose and biological responses.[25–28] Prior metabolomic-wide association studies (MWAS) have shown that environmental contaminants can impact the metabolomic phenotype.[22,26–31] Previously, a pilot metabolomics study was conducted in the Michigan PBB registry that examined metabolic changes associated with PBB and PCB in 174 participants. This study observed differences in pathways related to catecholamine metabolism, cellular respiration, essential fatty acids, lipids, and polyamine metabolism.[22] The current study continues this effort using a larger sample size and an untargeted MWAS approach to validate prior pilot findings and better characterize the detailed molecular network.

To address critical knowledge gaps in how PBB and PCB impact the human metabolome, we conducted this study in the well-established Michigan PBB registry cohort to identify biological perturbations associated with PBB and PCB exposure among 498 Michigan PBB cohort participants using targeted exposures assessment and untargeted high-resolution metabolomics (HRM). Additionally, we sought to explore how multigenerational exposure to PBB altered metabolomic processes by generation. The pathways elucidated through this study will continue to build an understanding of how these chemicals influence health.

Methods

Study Participants

All participants were part of the Michigan PBB Registry. This cohort was established in 1976 and enrolled ~4,500 chemical workers, farmers, and community members at high risk for PBB exposure.[7] All participants completed a detailed baseline questionnaire at enrollment, capturing demographic, health, and lifestyle information. Participants also provided biological samples at baseline. Since enrollment, this cohort has been followed continuously and includes periodic questionnaires collecting updated health information and additional blood samples from original cohort members, newly enrolled members, their children, and their grandchildren. Participants provided informed consent, and all protocols were approved by the institutional review board at Emory University and the Michigan Department of Health.

This analysis utilizes serum samples collected from May 2013 to April 2014 from a subset of 498 individuals. This cohort includes F0 (directly exposed to PBB; $n = 369$) and F1 (exposed *in utero* or through breastfeeding; $n = 129$) generations. The F0 generation includes individuals who worked at the Velsicol Chemical plant in St. Louis, Michigan (the source of the chemical contamination) and those who lived in the area surrounding the chemical plant. These F0 individuals were selected due to their high exposure to PBB through their occupation and residence. The F1 generation includes the offspring of the chemical plant workers and those residing near the chemical plant, selected due to their high exposure *in utero* and through breastfeeding, if applicable. Serum samples from these participants were used to analyze PBB-153 and ΣPCB levels, and plasma samples were used to conduct untargeted HRM metabolic profiling. The PBB congener, PBB-153 was selected for this analysis as it was the most prevalent congener in the fire retardant that accidentally contaminated the food supply in Michigan. Further, this congener is most commonly detected in this study population.[5] As for PCB, congeners included in ΣPCB (PCB-153, PCB-118, PCB-138, and PCB-180) were analyzed together as this more appropriately represents how individuals experience these chemicals in their local environment; these were the most common PCBs found in technical mixtures (e.g., Aroclors) and represent an estimate of total concentrations in humans experiencing background exposure.[32] This subpopulation was distinct from the population used in our previous independent analysis by Walker et al.,[22] although both sets of participants are part of the larger Michigan PBB Registry. Blood samples were collected in liquid ethylenediaminetetraacetic acid (EDTA), processed, and stored at −80°C. All participants provided informed consent. The institutional review board at Emory University oversees research on the PBB Registry. In addition, this study and its results were discussed and shared with the Michigan PBB Research team, which consists of the PBB Citizens Advisory Board, the Pine River Superfund Citizen Task Force, the Mid-Michigan District Health Department, and the academic research team. This academic-community partnership collaboratively leads the PBB Registry.

Serum PBB-153 and ΣPCB Measurements

Both PBB and PCB were measured using validated methods based on isotope-dilution gas chromatography–tandem mass spectrometry in 2014.[33] Briefly, each sample was subjected to a liquid-liquid extraction followed by a solid-phase extraction. After samples were reconstituted with isooctane, PBB and PCB levels were quantified by gas chromatography–tandem mass spectrometry in multiple reaction monitoring mode using an Agilent 7890A gas chromatograph coupled to an Agilent 7000B tandem mass spectrometer (Agilent Technologies, Santa Clara, CA).[33] Four congeners of PBB (PBB-77, PBB-101, PBB-153, and PBB-180) and four congeners of PCB were measured (PCB-118, PCB-138, PCB-153, and PCB-180). This study used PBB-153 as the most prevalent congener in the cohort and ΣPCB, which was calculated by summing the PCB congeners without molar conversion. ΣPCB was used as there was no single congener that the population was exposed to more than the others.[34]

The preparation and analysis of samples are described elsewhere.[33] Briefly, samples were extracted using liquid-liquid

extraction, cleaned over activated Florisil, and then concentrated for analysis using an Agilent 7890A gas chromatograph interfaced with an Agilent 7000B triple-quad mass spectrometer with an electron ionization source (Agilent Technologies, Santa Clara, CA). The limit of detection (LOD) for PBB-153 was 0.002 ng/mL. The LOD for ΣPCB was 0.0007 ng/mL (PCB-180: 0.0007; PCB-138: 0.0012; PCB-118: 0.0014; PCB-153: 0.0016). Samples that fell below the LOD were not included in the analysis.

High-Resolution Metabolomics Profiling

Plasma samples were analyzed using established methods in 2018 by liquid chromatography with ultrahigh-resolution mass spectrometry (LC-HRMS; Fusion, Thermo Scientific).[35] Briefly, 50 μL of plasma was treated with 100 μL acetonitrile in water containing a mixture of stable isotopic internal standards. Samples were vortex mixed, allowed to equilibrate on ice for 30 min, and centrifuged at 18,100 × *g*. The resulting supernatant was analyzed in triplicate using C18 hydrophobic reversed-phase chromatography with positive and negative electrospray ionization (ESI) to enhance the coverage of metabolic feature detection.[36] The mass spectrometer was operated using ESI mode at a resolution of 120,000 and a *m/z* range of 85–1,275, and samples were analyzed in batches of 40 study samples and 6 QA/QC pools. Raw data files were extracted and aligned using apLCMS with modifications by *xMSanalyzer*.[33,34] Uniquely detected ions consisted of *m/z*, retention time (rt), and ion abundance and are referred to as metabolite features. Prior to data analysis, metabolite features were batch-corrected using *ComBat*.[37]

Metabolic features detected in >10% of samples with a median coefficient of variation (CV) among technical replicates <30% and Pearson correlation $\rho > 0.7$ were included in further analyses to optimize data quality and filter out sample noise. For each sample with at least one nonzero intensity, we averaged the replicate samples and performed a log base 2 transformation to normalize the features before statistical analysis.

Untargeted MWAS

We used generalized linear models to examine the association between serum PBB-153 and ΣPCB levels (continuous variables) and metabolic feature intensities (continuous variable). All models were controlled for age at blood draw, sex, and serum lipid levels using the following form:

$$\log_2 Y_{ij} = \beta_0 + \beta_{1j}\text{Exposure}_i + \gamma_{1j}\text{Sex}_i + \gamma_{2j}\text{Age at blood draw}_i + \gamma_{3j}\text{Lipid levels}_i + \varepsilon_{ij},$$

where $\log_2 Y_{ij}$ refers to the log base 2 intensity of metabolic feature j for participant i, β_0 is the intercept, and Exposure_i is either PBB-153 or ΣPCB ng/mL measures for participant i. ε_{ij} represents residual random error. This analysis considered the age at blood draw (continuous in years), sex assigned at birth (male, female), and blood lipid levels (continuous) as potential confounders. Age and sex covariates were self-reported using a survey taken within 10 days of the participant's blood draw. Age at blood draw was calculated as the difference between the participant's date of birth and date of blood draw. Lipid levels were measured using an Abnova Triglyceride Quantification assay kit (Abnova Corporation), and total cholesterol content was measured by Cayman Cholesterol assay kit (Cayman Chemical Company) according to the manufacturer's instructions. Total lipids were calculated from individual lipid components using the formula proposed by Phillips et al.[38]: (Total lipids = 2.27 Total cholesterol + Triglycerides + 0.623). As both PBB and PCB are lipophilic chemicals, the lipid content of each serum sample needed to be accounted for in the analytic model to avoid biasing results.[39] When PBB-153 level was the exposure, participants were separated by generation due to the different exposure routes, as F0 ($n = 369$) was primarily exposed through the contaminated food and F1 ($n = 129$) was exposed *in utero* and while breastfeeding (born after the exposure event). When ΣPCB was the exposure variable, the population was modeled together, as people are continuously exposed through the environment, representing a different exposure pathway from PBB-153. Separate models were conducted for each ionization mode (C18 positive ESI and C18 negative ESI) for each exposure of interest. When many hypotheses are tested simultaneously, as in this study, the probability of obtaining false positive results by chance alone increases (type I error). To adjust for the increase in type I error, the significance rate for each individual test needs to be modified. Therefore, all *p*-values were corrected for multiple testing employing the Benjamini-Hochberg method for multiple comparison correction using the Benjamini-Hochberg false discovery rate (FDR).[40] All modeling was completed in R (version 4.1).

Metabolic Pathway Enrichment Analysis

Metabolic pathways enrichment analysis was conducted to predict the functional activity of the metabolomic features associated with our exposures of interest. All pathway analyses were conducted in Python (version 3.9) using *mummichog* (version 2.3).[41] This bioinformatics application is able to utilize unidentified metabolomic features to infer biological pathways based on mass spectrometry data by predicting biological activity in a network of metabolites directly from the feature table. It then provides an adjusted *p*-value for each pathway by resampling the reference input file using a gamma distribution. Two strategies were considered when selecting metabolic features for the pathway enrichment analysis: raw $p < 0.05$ and FDR-corrected $p < 0.2$. To minimize false positive discovery with the less stringent raw $p < 0.05$, pathways with less than four matching features were excluded from the results. For PBB-153, due to the limited number of significant features at FDR-corrected $p < 0.2$ cutoff, we decided to use a less stringent cutoff of raw $p < 0.05$. For ΣPCB, we used FDR-corrected $p < 0.2$. For each exposure, separate pathway analyses were conducted for the C18 positive and C18 negative columns. Separate analyses were conducted as C18 hydrophobic columns utilized both positive and negative ESI, which allows for the enhanced extraction of diverse polarity, thus increasing the number of detectable features. The different ESI modes polarity must be considered separately by the pathway analysis for accurate results, as the same metabolites may be captured in both ESI modes while others are detected in either positive ESI or negative ESI.

Metabolite Annotation and Confirmation

To minimize further false positives with metabolic features associated with PBB-153 or ΣPCB, each matched feature was screened on their retention time, isotope patterns, and spectrum peak quality (determined by a clear Gaussian peak shape and a signal-to-noise ratio >3:1) by examining the extracted ion chromatographs (EICs). Features that passed the visual examination by a primary and secondary reviewer were annotated and confirmed using established quality control guidelines.[42,43] Those features with a *m/z* (±5ppm difference) and retention time (±10 s) matched to authentic standards analyzed under identical experimental conditions were assigned a metabolic profile with level 1 evidence. Those that could not be assigned with level 1 evidence were annotated with level 2 evidence using the *xMSanalyzer* and literature values reported for authentic samples by other laboratories.[42] The

Table 1. Characteristics of participants selected for the metabolomic-wide association study ($n = 498$).

Characteristic	Total population ($n = 498$)	F0 ($n = 369$)	F1 ($n = 129$)
Sex [n (%)]			
Male	221 (44.2)	170 (46.1)	51 (39.5)
Female	277 (55.4)	199 (53.9)	78 (60.5)
Missing	0 (0.0)	0 (0.0)	0 (0.0)
Age at blood draw (y)			
Mean ± SD	51.5 ± 17.1	59.7 ± 10.6	28.2 ± 8.35
Median (minimum, maximum)	53.0 (7.27, 88.5)	60.1 (40.8, 88.5)	28.8 (7.27, 40.8)
Missing [n (%)]	0 (0.0)	0 (0.0)	0 (0.0)
Lipids (mg/dL)			
Mean ± SD	710 ± 225	717 ± 224	688 ± 230
Median (minimum, maximum)	683 (231, 1,860)	697 (231, 1,520)	656 (312, 1,860)
Missing [n (%)]	1 (0.2)	1 (0.3)	0 (0.0)

Note: F0, first generation exposed through diet and occupation; F1, second generation exposed *in utero* and through breastfeeding; PBB, polybrominated biphenyls; PCB, polychlorinated biphenyls; SD, standard deviation.

R package *xMSanalyzer* uses clustering, retention time, mass defect, and isotope/adduct patterns to assign metabolic profiles to features in the dataset. These profiles are based on multiple chemical databases, including the Kyoto Encyclopedia of Genes and Genomes (KEGG), the Human Metabolome Database (HMDB), LipidsMaps, and the Toxin and Toxin Target Database (T3DB).

Sensitivity Analyses

A sensitivity analysis was conducted for PBB-153 exposure controlling for ΣPCB and ΣPCB exposure controlling for PBB-153. We also conducted a sensitivity analysis splitting the ΣPCB models by generation. These analyses were conducted to test how robust our results were against different analytic decisions.

Results

Study Population and Exposure Serum Levels

A total of 498 participants were included in this study. This sample was divided into 369 F0 participants and 129 F1 participants (Table 1). Briefly, there were more female participants (55.4%) in the total population compared to males (44.2%), with similar proportions in both the F0 and F1. The mean age at blood draw in the population was 51.5 y old [standard deviation (SD): 17.1]. As expected, the F0 population was, on average, older (mean: 59.7; SD: 10.6) compared to the F1 generation (mean: 28.2; SD: 8.35). The average lipid level in the total population was 710 mg/dL, with similar levels in the F0 generation and slightly lower levels in the F1 generation. Of the 129 participants in F1, 55 and 24 had a mother or a father in the F0 generation, respectively.

Both PBB-153 and ΣPCB had a high detection rate (Tables 2 and 3). The median PBB-153 level was much higher in F0 [0.374 ng/mL (minimum, maximum: 0.0120, 221)] compared to F1 [0.0300 ng/mL (minimum, maximum: 0.00500, 0.425)] (Table 2). PBB-153 levels fell below the LOD in 54 samples (11%, 2 in F0 and 52 in F1) and were not included in the final analysis. The median ΣPCB was 0.890 ng/mL (minimum, maximum: 0.0420, 5.69) in the total population (Table 3). Everyone in the sample had a detectable level of ΣPCB (i.e., each individual in the study had at least one PCB congener detected). Further, PCB-153, PCB-138, and PCB-118 were at detectable levels in all participants. PCB-180 fell below the LOD in one participant (0.2% of the 498 samples). Each PCB congener (PCB-153, PCB-180, PCB-138, and PCB-118) was highly correlated with each other and with ΣPCB. PBB-153 was not correlated with either ΣPCB or any of the PCB congeners (Figure S2).

Table 2. PBB-153 concentrations in participants selected for the metabolomic-wide association study ($n = 498$).

	Total population ($n = 498$)	F0 ($n = 369$)	F1 ($n = 129$)
PBB-153 (ng/mL)			
Mean ± SD	196 ± 14.7	2.37 ± 16.2	0.0467 ± 0.0611
Median (minimum, maximum)	0.295 (0.00500, 221)	0.374 (0.0120, 221)	0.0300 (0.00500, 0.425)
Geometric mean	0.270	0.429	0.030
Below LOD [n (%)]	54 (11.0)	2 (0.5)	52 (40.0)

Note: F0, first generation exposed through diet and occupation; F1, second generation exposed *in utero* and through breastfeeding; LOD, limit of detection; PBB, polybrominated biphenyls; SD, standard deviation.

High-Resolution Metabolic Profiling

After data quality filtering, 13,485 metabolic features were detected (7,493 in the C18 negative column and 5,992 in the C18 positive column, respectively). We analyzed six sets of MWAS models [one for ΣPCB, one for PBB-153 (F0), and one for PBB-153 (F1), with each analyzed using the two chromatography columns (see supplemental Excel file, "MWAS data.xls")]. Sixty-two and 26 metabolomic features were significantly associated with PBB-153 in F0 and F1 (FDR-corrected $p < 0.2$), respectively (Table 4). There were 2,861 features associated with ΣPCB (FDR-corrected $p < 0.2$) (Table 4).

Pathway Analysis

Using *mummichog* (version 2.3),[41] we examined how features significantly associated with PBB-153 ($p < 0.05$) and ΣPCB (FDR-corrected $p < 0.2$) were enriched in endogenous metabolic pathways (Figures 1 and 2; Figure S3). Thirty-eight perturbed metabolic pathways in the F0 generation and 40 within the F1 generation were associated with PBB-153 (adjusted $p < 0.05$). In

Table 3. ΣPCB and PCB congener (PCB-153, PCB-180, PCB-138, and PCB-118) concentrations in participants selected for the metabolomic-wide association study.

	Total population ($n = 498$)
ΣPCB (ng/mL)	
Mean ± SD	1.04 ± 0.788
Median (minimum, maximum)	0.890 (0.0420, 5.69)
Geometric mean	0.786
Below LOD [n (%)]	0 (0.0)
PCB-153 (ng/mL)	
Mean ± SD	0.352 ± 0.263
Median (minimum, maximum)	0.300 (0.0100, 2.22)
Geometric mean	0.264
Below LOD [n (%)]	0 (0.0)
PCB-180 (ng/mL)	
Mean ± SD	0.267 ± 0.281
Median (minimum, maximum)	0.222 (0.00300, 4.92)
Geometric mean	0.190
Below LOD [n (%)]	1 (0.2)
PCB-138 (ng/mL)	
Mean ± SD	0.267 ± 0.252
Median (minimum, maximum)	0.276 (0.0110, 1.99)
Geometric mean	0.239
Below LOD [n (%)]	0 (0.0)
PCB-118 (ng/mL)	
Mean ± SD	0.0962 ± 0.109
Median (minimum, maximum)	0.0665 (0.00700, 0.892)
Geometric mean	0.068
Below LOD [n (%)]	0 (0.0)

Note: LOD, limit of detection; PCB, polychlorinated biphenyls; SD, standard deviation.

Table 4. Number of metabolic features significantly associated with PBB-153 and ΣPCB. These features were used for the pathway analysis and metabolite annotation.

	Significance threshold	
Exposure of interest[a]	Raw p-value <0.05	FDR p-value <0.2
PBB-153		
F0 ($n=369$)	530	63
F1 ($n=129$)	475	27
ΣPCB		
Full population ($n=498$)	3,001	2,861

Note: F0, first generation exposed through diet and occupation; F1, second generation exposed *in utero* and through breastfeeding; FDR, Benjamini-Hochberg false discovery rate; PBB, polybrominated biphenyls; PCB, polychlorinated biphenyls; SD, standard deviation.
[a]Linear regression models controlled for sex, total lipids, and age at blood draw.

both generations, the identified pathways were associated with energy metabolism, fatty acid metabolism, and glycan metabolism. The pathways found in the two generations were generally consistent; however, F0 generation had 10 unique pathways and F1 generation had 12 unique pathways. Metabolic perturbations in 36 pathways were associated with ΣPCB in the whole population. Most of these pathways involved amino acid metabolism, energy metabolism, and glycan metabolism.

Chemical Identification of Associated Metabolites

After correcting for multiple testing (FDR <0.2), the results for each metabolic feature associated with either PBB-153 or ΣPCB were screened to further reduce the chance of false positive discovery. The features were screened by manually examining the spectrum peak quality and purity of EICs (Tables S4 and S5). In the F0 generation, 10 metabolites associated with PBB-153 were confirmed with level 2 evidence using *xMSanalyzer* and an external library (Table S1).[44] However, after completing the matching and quality control process, the associated peaks failed to provide high-confidence matches. Most of the rt and *m/z* were larger and are most likely lipids that do not have analytic matches in the database. Therefore, these results were not interpreted further. No statistically significant matches in the F1 generation could be confirmed with level 1 or level 2 evidence.

Among the significant features associated with ΣPCB (FDR adjusted p-values <0.2), we confirmed 29 metabolites with level 1 evidence after matching samples with authentic reference standards and verifying by tandem mass spectrometry (Table 5). The majority of the metabolites found were within pathways involved in amino acid metabolism, energy metabolism, and fatty acid metabolism. Furthermore, these confirmed metabolites were closely connected around the urea cycle network (Figure 3).

Sensitivity Analyses

The sensitivity analyses described above looking at modeling decisions (i.e., PBB-153 controlling for ΣPCB, ΣPCB controlling for PBB-153, and ΣPCB split generationally) did not drastically alter the number of detected features (Table S2, Supplemental Excel file, "MWAS data.xls"). We observed consistent and robust pathways patterns and similar statistically significant features when changing analytic decisions, including analyzing ΣPCB separated by generation (Figure S4; Supplemental Excel file, "MWAS data.xls").

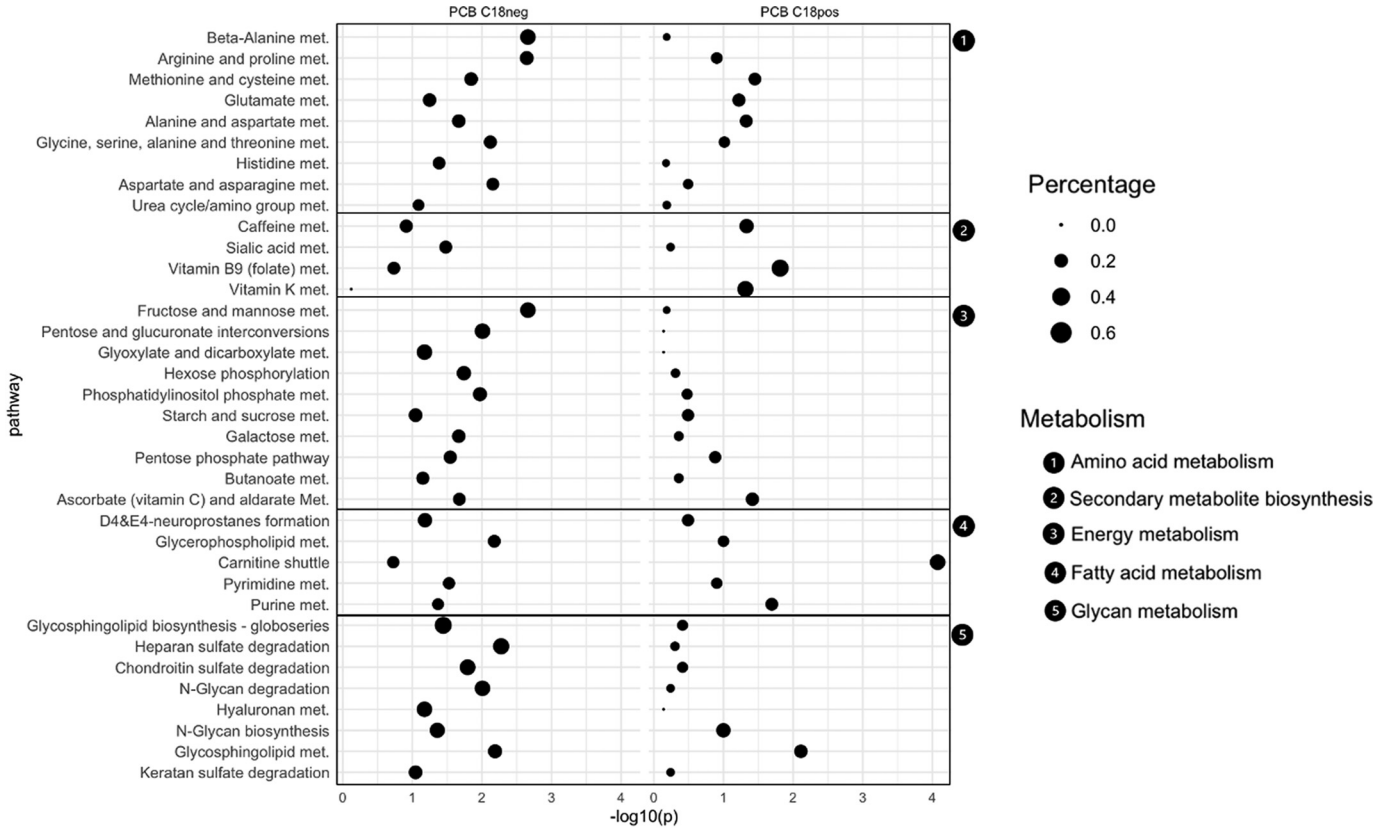

Figure 1. Biological pathways associated with ΣPCB in the total population from the pathway analysis utilizing the bioinformatics tool, mummichog ($n=498$). Pathway names are listed along the y-axis. The $-\log_{10}$ of the p-value is along the x-axis. The bubble size (percentage) relates to the number of features that overlap in the specific metabolic pathway. The number labels relate to the larger metabolism class into which the pathway falls. Note: C18neg, C18negativeion-ization; C18pos, C18positiveionization; met., metabolism; PCB, polychlorinatedbiphenyls.

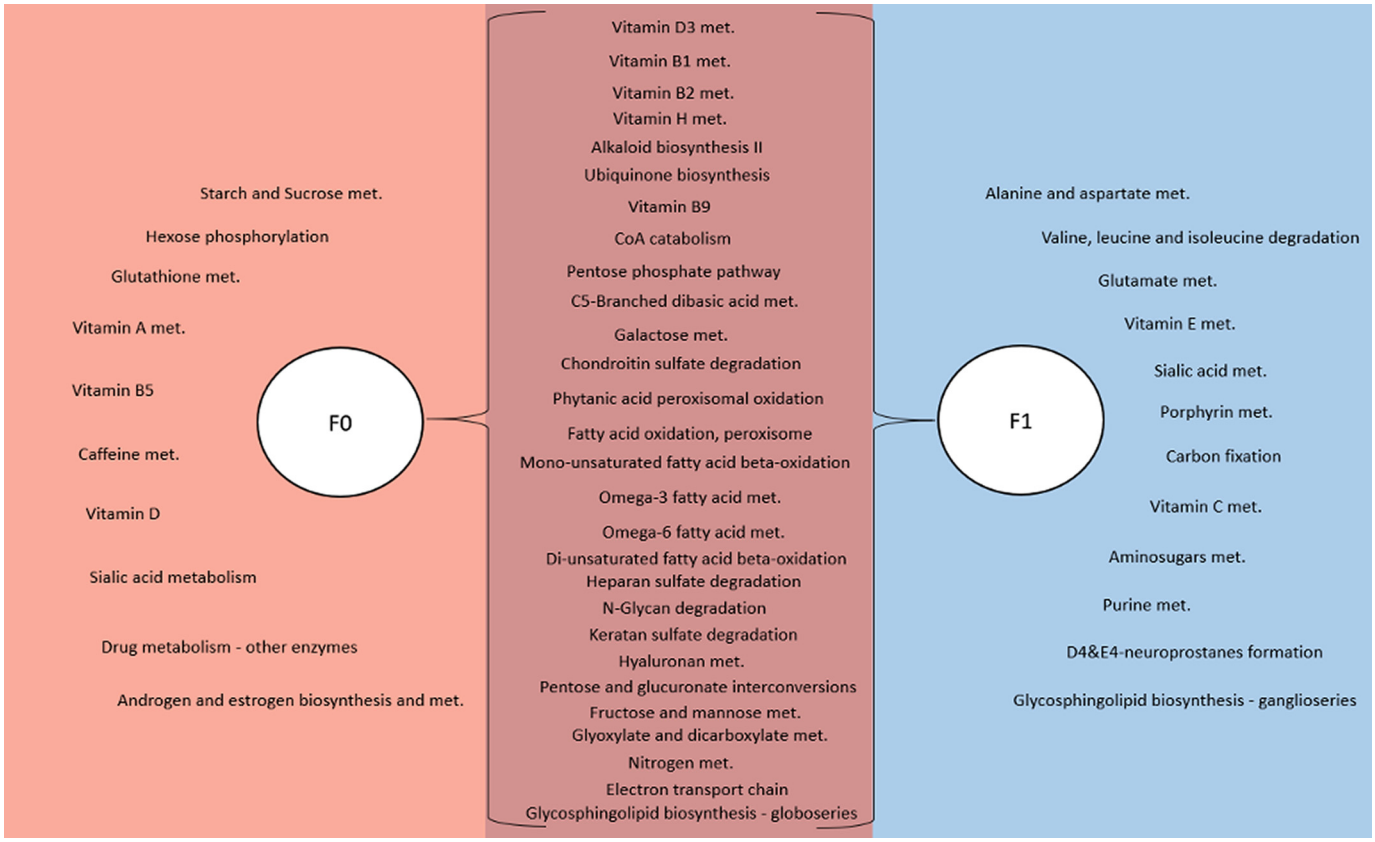

Figure 2. Biological pathways associated with PBB-153 showing the pathways unique to F0 (10) ($n = 369$) and F1 (12) ($n = 129$) and the pathways shared between the two generations (28). Pathway analyses were conducted using the bioinformatics tool *mummichog*. Note: F0, first generation exposed through diet and occupation; F1, second generation exposed *in utero* and through breastfeeding; met., metabolism; PBB, polybrominated biphenyls.

Discussion

Using targeted exposure assessment and untargeted metabolomics, this study identified and characterized novel signals in the human metabolome linked to exposure to PBB-153 and ΣPCB among 498 participants in the Michigan PBB cohort. This study continues the work of previous metabolomic research in this cohort.[22] Both papers consistently demonstrated that PBB and PCB are associated with biological perturbations at the metabolic pathway and individual metabolite levels. Both papers found changes in metabolites and pathways linked to the urea cycle and in pathways underlying neurodegenerative diseases. Compared to our previous work, this paper included a population with a wider exposure profile and an optimized untargeted metabolic workflow, including utilizing two ESI modes instead of one and relying on level 1 evidence (authentic chemical standards) for metabolite confirmation instead of level 4 evidence (matching to external libraries). The present work provides additional insights into how EDCs influence metabolic phenotype.

Specifically, our results demonstrated that serum PBB-153 and ΣPCB levels were associated with perturbations in inflammation and oxidative stress-related pathways and that these perturbations differed when PBB-153 was stratified by generation to separate different exposure routes and the timing of the exposure window. Given the existing literature on EDCs,[1–3] we highlight findings related to steroid pathways, neurological impacts, and obesogenic effects.

In both generations, the geometric mean of PBB-153 was higher than reported in the general U.S. population [2003–2004 National Health and Nutrition Examination Survey (NHANES)[45]] on average, further demonstrating the unique exposure route of this population. Unlike PBB, ΣPCB levels in both generations were more consistent with what is observed in the general U.S. population (2003–2004 NHANES survey[45]) as the exposure route is primarily through the local environment and food chain (Table S3).

Pathways that PBB-153 impacted in the F0 and F1 generations were generally involved in energy metabolism, fatty acid metabolism, and glycan metabolism. Specifically, pentose and glucuronate interconversions have been identified as an important pathway in type 2 diabetes, and vitamin D metabolism has been linked to obesity and insulin resistence.[46] Vitamin D and B9 (folate) metabolism are also linked to several adverse reproductive health outcomes. Specifically, Vitamin D has been associated with preeclampsia, bacterial vaginosis, preterm birth, and gestational diabetes[47]; vitamin B9 has been linked to fetal neural tube defects, preeclampsia, placental abruption, preterm birth, and fetal death.[48] Vitamin B9 is also involved in oxidative stress along with the pentose phosphate pathway.[48,49] Many pathways indicated in oxidative stress, such as the pentose phosphate pathway, impact thyroid function.[50] Finally, vitamin B1 (thiamin) metabolism plays a key role in energy metabolism and has been identified as playing a role in neurological disorders, cardiovascular function, and the immune system.[51] Pathways associated with PBB-153, especially those related to oxidative stress and neurodegenerative diseases, support previous findings in this cohort.[22] Further, these results demonstrate that several pathways were associated with metabolic illnesses, supporting the role of PBB-153 in endocrine disruption.

Ten unique pathways were associated with PBB-153 in the F0 generation (Figure 2; Figure S3). Two pathways, starch and sucrose metabolism and androgen and estrogen biosynthesis, have been identified as important pathways in type 2 diabetes.[46] There

Table 5. Chemical identification of the metabolic features associated with ΣPCB in the total population of the Michigan PBB residents (FDR $p < 0.2$, $n = 498$). The chemical identity was conducted by matching peaks by accurate mass and retention time to authentic reference standards in an in-house library run under identical conditions using tandem mass spectrometry (level 1 evidence).

m/z	rt (s)	Identified metabolite	Adduct form	Association with ΣPCB exposure (β)[a]	Pathways	Biological function
104.107	27.5	Choline	M+	−0.07	Glycine, serine, and threonine metabolism	Anti-inflammation; oxidative stress; DNA damage and repair[83,84]
118.0863	29.3	Valine	M+H	0.05	Valine, leucine, and isoleucine biosynthesis	Anti-inflammation; oxidative stress; improved insulin sensitivity; adiposity reduction[85–87]
126.022	28.6	Taurine	M+H	−0.45	Primary bile acid biosynthesis	Anti-inflammation; oxidative stress; apoptosis; DNA damage and repair; lipid metabolism[88–90]
130.0499	31.1	5-Oxo-D-proline	M+H	−0.14	D-Amino acid metabolism	Anti-inflammation[91]
132.102	28.2	Isoleucine	M+H	0.09	Valine, leucine, and isoleucine biosynthesis	Anti-inflammation; antioxidant; apoptosis; lipid metabolism regulation; improved insulin sensitivity[87,92–94]
133.0975	21.2	Ornithine	M+H	−0.12	D-Amino acid metabolism	Inflammation[95]
137.0717	26.5	1-Methylnicotin-amide	M+	−0.33	Nicotinate and nicotinamide metabolism	Anti-inflammation; oxidative stress; DNA synthesis and cell proliferation[96,97]
148.0605	27.4	Glutamic acid	M+H	−0.17	Alanine, aspartate, and glutamate metabolism	Inflammation; oxidative stress[98]
160.1332	25.5	Valerobetaine	M+H	0.21	Not enough information	Inflammation[80]
176.0559	197.5	N-Acetyl-L-aspartic acid	M+H	−0.76	Alanine, aspartate, and glutamate metabolism	Antioxidant; neurotransmitter synthesis[99]
177.1022	30.4	Serotonin	M+H	−0.61	Tryptophan metabolism	Neurotransmitter; inflammation; oxidative stress; DNA repair and damage; endocrine disruption[100–102]
203.0526	26.9	Galactose	M+Na	0.43	Galactose metabolism	Inflammation; oxidative stress; DNA damage and repair; endocrine disruption[103–105]
204.1038	48.3	Ethyl 3-indoleacetate	M+H	−0.45	Not enough information	Synthetic compound; exogenous exposure
212.0429	38.4	Phosphocreatine	M+H	−0.34	Arginine and proline metabolism	Inflammation; oxidative stress; ATP production[106,107]
214.0153	83.1	Indoxyl sulfate	M+H	−0.74	Tryptophan metabolism	Inflammation; oxidative stress; endocrine disruption; angiogenesis; apoptosis[108–110]
219.0264	25.8	Fructose	M+K	0.29	Galactose metabolism	Inflammation; oxidative stress; insulin resistance; DNA damage[111,112]
282.1194	24.5	Glycolaldehyde dimer	2M+ACN+H	0.12	Glycine, serine, and threonine metabolism	Not enough information
300.2894	198.1	Sphingosine	M+H	−0.40	Sphingolipid metabolism	Inflammation; lipid; apoptosis[113,114]
302.3046	201.9	Sphinganine	M+H	−0.37	Sphingolipid metabolism	Inflammation; lipid; apoptosis[115,116]
87.0087	28.3	Pyruvate	M − H	0.22	Citrate cycle (TCA cycle)	Oxidative stress; DNA damage and repair[117,118]
102.0562	20.8	4-Aminobutanoate	M − H	−0.08	Alanine, aspartate, and glutamate metabolism	Anti-inflammation; oxidative stress; neurotransmitter; glucose homeostasis; lipid peroxidation[119–122]
115.0038	28.4	Maleic acid	M − H	−0.08	Tyrosine metabolism	Inflammation; oxidative stress[123]
128.0355	22.5	5-Oxo-D-proline	M − H	−0.35	D-Amino acid metabolism	Anti-inflammation[91]
133.0144	23.5	(S)-Malate	M − H	−0.17	Citrate cycle (TCA cycle)	Anti-inflammation; Oxidative stress[124,125]
145.0145	20.7	Alpha-ketoglutaric acid	M − H	0.15	Citrate cycle (TCA cycle)	Antioxidant; inflammation; DNA methylation[126–128]
149.0458	25.4	Arabinose	M − H	0.18	Pentose and glucuronate interconversions	Anti-inflammation; oxidative stress; insulin resistance[129,130]
171.0072	22.6	Glycerol 2-phosphate	M − H	−1.08	Not enough information	Not enough information
174.0884	20.6	Citrulline	M − H	−0.09	Arginine biosynthesis, urea cycle	Inflammation; oxidative stress; apoptosis; angiogenesis; glucose homeostasis[131–134]
197.0441	24.8	3-Methoxy-4-hydroxymandelate	M − H	0.15	Tyrosine metabolism	Prognostic biomarkers for certain diseases[135,136]
267.0731	42.8	Inosine	M − H	−0.73	Purine metabolism	Inflammation; oxidative stress; DNA damage and repair[137–139]

Note: FDR, Benjamini-Hochberg false discovery rate; PBB, polybrominated biphenyls; PCB, polychlorinated biphenyls; rt, retention time; SD, standard deviation; TCA, tricarboxylic acid.
[a]This value represents the change in the concentration of the listed metabolite for a 1 ng/ml increase in PCB, holding all other covariates constant.

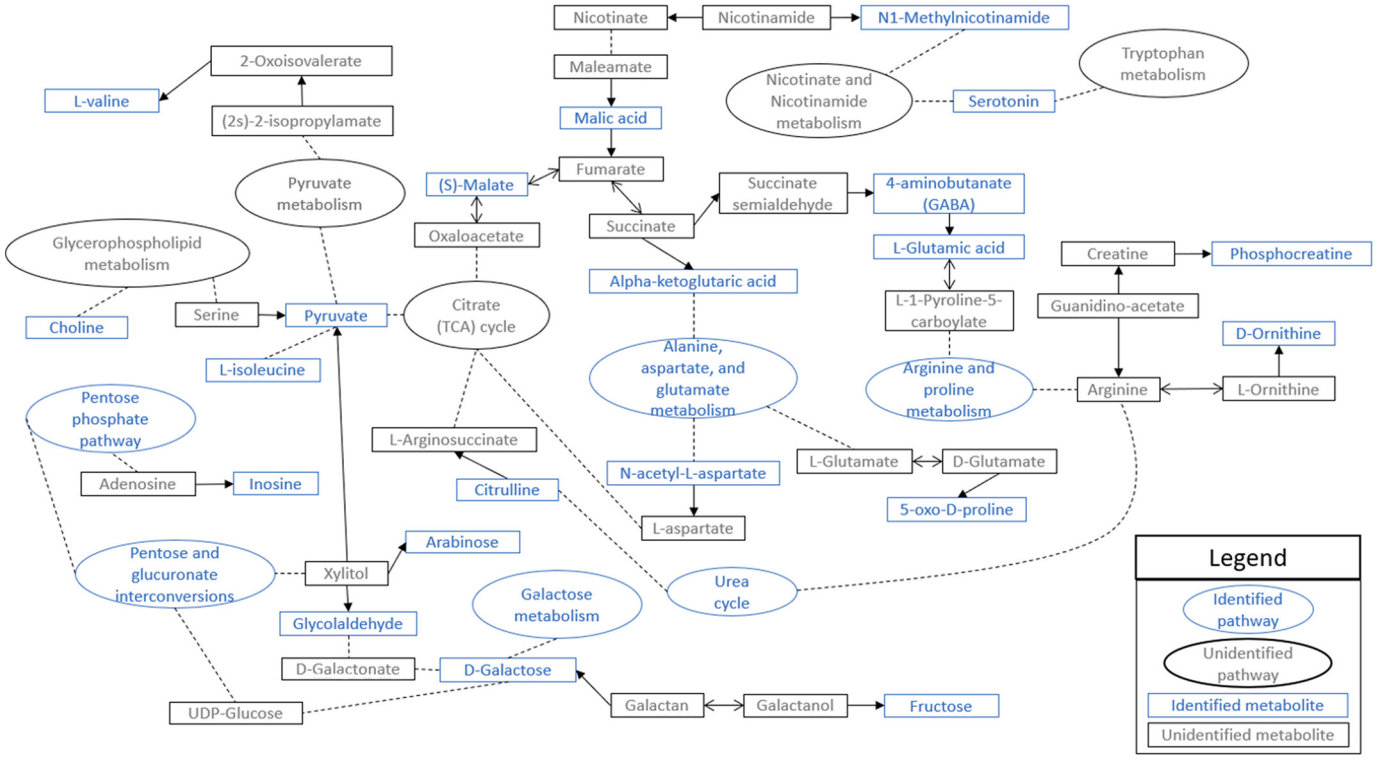

Figure 3. Potential molecular mechanisms underlying the effects of ΣPCB on individuals in the Michigan PBB registry ($n = 498$). Items in circles denote pathways and items in rectangle boxes denote metabolites. Blue pathways and metabolites were found in this research. Gray pathways and metabolites are provided for biological context but were not explicitly identified in this research. Solid arrows represent metabolites directly upstream/downstream of each other. Dashed lines represent a biochemical pathway linking metabolites not shown for clarity. Nine metabolites (taurine, valerobetaine, ethyl 3-indoleacetate, indoxyl sulfate, glycolaldehyde dimer, sphingosine, sphinganine, glycerol 2-phosphate, and 3-methyl-4-hydroxymandelte) were not included as there was no clear biological link identified. Note: PBB, polybrominated biphenyls; PCB, polychlorinated biphenyls.

were also several unique pathways related to oxidative stress, including glutathione metabolism,[52] vitamin A metabolism,[53] and vitamin B5 metabolism.[54] There were 12 unique pathways associated with PBB-153 in the F1 generation (Figure 2; Figure S3). Vitamin E and ascorbate and aldarate metabolism are both linked to oxidative stress and inflammation.[55,56] Finally, glutamate metabolism has been associated with cardiometabolic disorders, brain health, and thyroid health.[57–59] The observed differences in affected pathways could be attributable to the timing and exposure route of the PBB-153 exposure, while other differences could be attributed to the difference in participants' age. However, in this cohort, it is impossible to separate the participant's age from the exposure route due to the finite exposure experience. Future analyses in other cohorts with general exposure to PBBs or other brominated fire retardants (i.e., PBDEs) could aim to understand how the age of participants impacts the biological response to exposure.

The majority of pathways associated with ΣPCB were involved in amino acid metabolism, energy metabolism, and glycan metabolism. Many of these pathways are involved in pro-inflammatory responses, such as aberrant Vitamin B9 (folate), pentose phosphate pathway, and the urea cycle.[49,60,61] Glycerophospholipid metabolism and arginine and proline metabolism have been associated with inflammatory states, altered systemic immune response, and heart failure.[62] Several pathways are associated with neurological health, including glutamate metabolism,[57] histidine metabolism,[63] and Vitamin B9 (folate).[64]

Using authentic chemical standards, we confirmed the identities of 29 chemicals associated with ΣPCB (Table 5; Figure 2). Two identified chemicals are neurotransmitters, including serotonin and 4-aminobutanate (GABA). Serotonin is synthesized by the body in the central nervous system and can act as a neurotransmitter with both excitatory and inhibitory actions. It interacts with many different mammalian systems, including the cardiovascular, gastrointestinal, and endocrine systems, and behaviors like sleep, mood, cognition, and memory.[65] GABA is the primary inhibitory neurotransmitter in the adult central nervous system. It has been associated with several psychiatric disorders, including schizophrenia and major depressive disorder.[66] Both serotonin and GABA were downregulated after being exposed to ΣPCB. The findings in this research are congruent with research completed in rat models, which found that PCB mixtures inhibited the uptake of dopamine, glutamate, GABA, and serotonin.[67] Epidemiologic evidence in human populations has demonstrated that PCBs are associated with poor motor development, memory deficits, and cognitive dysfunction in children exposed *in utero*,[68–70] and motor dysfunctions in adults.[71] There is also evidence that exposure to PCB mixtures is associated with depressive symptomology.[72] Further exploration into how PCB impacts brain health and mental health is an important avenue of future research.

Several other metabolites have been associated with cognitive function in animal models. Valine is an amino acid that has a demonstrated impact on serotonin in humans by affecting the transport of serotonin precursors across the blood-brain barrier.[73] In zebrafish exposed to PCB-153, there was an association with an upregulation of valine, which impacted sensory neurons.[74] This research shows a positive association between ΣPCB exposure (including PCB-153) and valine, suggesting that there could be a similar impact to sensory neurons. This would be an area for more targeted research. An additional study found that exposure to a PCB mixture impacted creatine kinase status in the cerebellum. This system, which involves phosphocreatine, plays an important role in the energy regulation of cells with fluctuating energy requirements, like neurons.[75] Similar results

were observed in this research, with exposure to ΣPCB associated with a downregulation of the phosphocreatine metabolite. Next, galactose is able to accumulate in neurons as it is not metabolized further. This metabolite is associated with oxidative stress and is often used as a marker of brain aging in mice.[76,77] This research found a positive association between ΣPCB and galactose, which should be researched further in future work. Lastly, in rat models, inosine appeared to have a protective effect against oxidative alterations in the brain.[78] In this research, inosine was inversely associated with ΣPCB exposure, potentially downplaying any protective effect it could have for human populations.

Beyond neural impacts, two metabolites potentially involved in obesity were associated with ΣPCB: fructose and valerobetaine. Fructose is often from dietary sugar intake and is primarily metabolized through the liver. It can impact the lipid profile in the human body and contribute to increased adiposity and general inflammation.[79] Here, fructose was positively associated with ΣPCB. ΣPCB was also associated with an upregulation of valerobetaine. This metabolite is derived from the gut microbiome and has been associated with increased visceral adipose tissue in humans.[80] It has been theorized that chemical pollutants, including PCB congeners and mixtures, might impact the gut microbiota composition, impacting the host metabolism and leading to metabolic diseases.[81] Both of these metabolites could warrant further investigation.

This study had several notable strengths. This is a well-described cohort with validated exposure measurements. This study applies a comprehensive metabolomics workflow that has been successfully used for nonfasting samples.[35] Finally, this work was able to characterize potential intergenerational differences and link results to previous outcomes observed in this cohort, such as thyroid[14] and reproductive health issues.[13] While this study provided novel insights into the metabolic differences associated with exposure to either PBB-153 or ΣPCB, our research has several limitations to note. First, individuals included in this analysis were selected from those who worked at the chemical plant, their offspring, and individuals who lived in the surrounding community. This potentially limits the generalizability of the study results. Additionally, due to the highly multidimensional analysis, there is an increased risk for type I errors. This was addressed by imposing stringent criteria throughout the ΣPCB analysis, including applying the Benjamini-Hochberg FDR. Unfortunately, for the PBB-153 analysis, we could not use FDR-corrected significance thresholds as the more stringent threshold would not include a sufficient number of metabolic features to conduct a meaningful pathway analysis. Therefore, these results should be carefully interpreted and confirmed using additional studies with larger sample sizes. Next, this study focuses mainly on endogenous metabolites that can be identified using authentic chemical standards. This limits the identifiable metabolites, and due to limited sample availability, we did not complete the structural characterization of unidentified metabolites. Next, this study did not adjust for diet, education, or smoking. There was no dietary information available, and as this is a nonfasting blood draw, this could introduce bias into the results. Education and smoking were not included in the final model as these variables might introduce unknown biasing paths and cause overadjustment issues. Therefore, only basic covariates supported by the researcher's directed acyclic graph were included in the final model. Additionally, the serum samples used in this analysis were stored for 5–6 y at −80°C prior to processing. Sample processing, time to freezing, and deterioration of sample quality at −80°C can introduce analytical variabilities impacting interpretations. To minimize the effect on data quality, identical procedures were used, and frozen samples were maintained together under the same conditions. Furthermore, a review study investigating preanalytic factors found that sample quality remained stable after 30 months of storage.[81,82] Nonetheless, preanalytical collection and storage could have an unrecognized negative impact on data quality. Sample run order and batch effects are also well-recognized limitations in metabolomics research, and randomization of sample run order prior to analysis was used to minimize potential bias. To address analytical drift and other batch effects, batch effects were adjusted during data processing using *ComBat*.[37] Finally, this work is hypothesis-generating in nature, and more research is needed to validate these findings using a hypothesis-testing approach. Despite these limitations, the results of this study demonstrate metabolic perturbations and support associations observed between diseases in human populations and animal studies.

Conclusion

The detailed biological mechanisms underlying the associations between exposure to EDCs and disease remain largely uncharacterized. The results from this study demonstrate the feasibility and potential of applying HRM to elucidate biological perturbations associated with PBB-153 and ΣPCB. Specifically, these results show that PBB-153 was associated with perturbations in energy metabolism, fatty acid metabolism, and glycan metabolism and that although results were similar across the generations, there are meaningful differences when exposure route and timing (here different generationally) are considered in the analysis. ΣPCB was associated with the downregulation of important neurotransmitters, findings that are consistent with rat models and support disease associations observed in epidemiologic studies. In community outreach with members of the Michigan PBB registry, people have often expressed difficulty with weight management, and there are frequent questions regarding neurological disorders and immune function. Many of the findings in this research are relevant to these concerns and provide novel insights for future investigations of molecular mechanisms underlying PBB and PCB exposure on health.

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