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Multigenerational metabolic profiling in the Michigan PBB Registry

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Abstract

Although polychlorinated biphenyls and polybrominated biphenyls are no longer manufactured the United States, biomonitoring in human populations show that exposure to these pollutants persist in human tissues. The objective of this study was to identify metabolic variations associated with exposure to 2,2',4,4',5,5'-hexabromobiphenyl (PBB-153) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153) in two generations of participants enrolled in the Michigan PBB Registry (<http://pbbregistry.emory.edu/>). Untargeted, high-resolution metabolomic profiling of plasma collected from 156 individuals was completed using liquid chromatography with high-resolution mass spectrometry. PBB-153 and PCB-153 levels were measured in the same individuals using targeted gas chromatography-tandem mass spectrometry and tested for dose-dependent correlation with the metabolome. Biological response to these exposures were evaluated using identified endogenous metabolites and pathway enrichment. When compared to lipid-adjusted concentrations for adults

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in the National Health and Nutrition Examination Survey (NHANES) for years 2003–2004, PCB-153 levels were consistent with similarly aged individuals, whereas PBB-153 concentrations were elevated ($p < 0.0001$) in participants enrolled in the Michigan PBB Registry. Metabolic alterations were correlated with PBB-153 and PCB-153 in both generations of participants, and included changes in pathways related to catecholamine metabolism, cellular respiration, essential fatty acids, lipids and polyamine metabolism. These pathways were consistent with pathophysiological changes observed in neurodegenerative disease and included previously identified metabolomic markers of Parkinson's disease. To determine if the metabolic alterations detected in this study are replicated other cohorts, we evaluated correlation of PBB-153 and PCB-153 with plasma fatty acids measured in NHANES. Both pollutants showed similar associations with fatty acids previously linked to PCB exposure. Thus, the results from this study show metabolic alterations correlated with PBB-153 and PCB-153 exposure can be detected in human populations and are consistent with health outcomes previously reported in epidemiological and mechanistic studies.

Graphical Abstract:

Keywords

High-resolution metabolomics; Polychlorinated biphenyls; Polybrominated flame retardants; Biomonitoring; Bioeffect

1. INTRODUCTION

Due to their past widespread use and environmental persistence, exposure to polybrominated biphenyls (PBBs) and polychlorinated biphenyls (PCBs) is prevalent throughout the United States. Biomonitoring studies have shown detectable levels are still present in blood, breast milk, adipose and brain tissue, even though manufacture of these compounds in the United States was discontinued in the late 1970s [1–3]. Exposure to the developing fetus can occur through maternal transfer *in utero*, and accumulation in lipid-rich breast milk can lead to continued exposure throughout infancy and critical developmental periods [4]. While the toxic effects of these compounds have been well documented in model systems, health effects in humans from environmental exposures are uncertain.

The International Agency for Research on Cancer (IARC) lists PCBs as carcinogenic to humans (Group 1), with evidence suggesting increased risk of malignant melanoma, non-Hodgkin lymphoma and cancer of the breast following exposure [5]. PCBs are also possible obesogens [6], exhibit endocrine disrupting behavior [7], and alter thyroid function [8]. In addition, exposure to PCBs has been associated with Parkinson's disease (PD) in mechanistic and epidemiological studies [9, 10]. Less information is known about PBBs.

Bromine analogues of PCBs, PBBs have been classified as probably carcinogenic to humans (Group 2A) by IARC [5], showing association with digestive system cancer and elevated risk for lymphoma [11].

Effects of PBB exposure in humans have primarily been identified by study of the Michigan PBB Registry, which was created to address public health concerns following substantial PBB exposure in Michigan due to accidental contamination of livestock feed with a commercial PBB flame retardant mixture [12]. For the original study, the Michigan Department of Health enrolled approximately 5,000 individuals for long-term health monitoring. This was later expanded to include offspring of those exposed during the original contamination and their children, with data collection still ongoing [13]. Findings in the original cohort have included elevated risk for breast cancer [14], thyroid dysfunction [15], a suggestion of immune function alterations [16] and below median Apgar scores [17] among offspring of exposed woman. Endocrine effects of daughters born to mothers who consumed contaminated food have also been documented, including a dose related increase in miscarriages [18] and earlier age of menarche and appearance of pubic hair [19]. Male offspring with high exposure *in utero* have reported slower growth [20] and increased genitourinary problems [13]. In addition, differences in sex ratios have been associated with the father's and mother's exposure [21].

Studies of metabolic response to chemical exposures show a strong influence of environmental factors on the metabolic phenotype [22–25]. By using untargeted, high-resolution mass spectrometry to characterize biological samples (i.e. blood, urine), it is possible to provide a central measure of exposure response that includes endogenous metabolites, microbiome-related chemicals, lipids, compounds arising from diet, environmental chemicals and pharmaceuticals [26]. While originally developed as a platform for precision medicine, high-resolution metabolomics (HRM) allows the application of the metabolome wide association (MWAS) framework to identify metabolic variations associated with exposure, providing a means to assess mechanisms underlying chemical toxicity. Thus, applying these methods to the study of PCB and PBB exposure in humans can provide improved understanding of how these chemicals influence human health.

In this study, we used HRM to identify dose-dependent changes in the plasma metabolome associated with PBB and PCB exposure. Participants were selected from the Michigan PBB Registry, and grouped based upon whether they were born before or after PBB entered the food chain. Metabolic alterations were identified by testing for correlation with plasma levels of the *ortho*-substituted PBB congener 153 (2,2',4,4',5,5'-hexabromobiphenyl) and PCB congener 153 (2,2',4,4',5,5'-hexachlorobiphenyl) in each age group. We then characterized biological response using metabolite annotation and pathway enrichment, providing new insight into how exposure to persistent organic pollutants is associated with metabolic changes.

2. METHODS

2.1 Study population

In 1976, the Michigan Department of Community Health enrolled approximately 5,000 farmers, chemical workers, and others with PBB exposure risk to participate in the Michigan PBB Registry. At enrollment, participants completed detailed questionnaires to capture demographic, health, and lifestyle information. The cohort has been followed prospectively since enrollment, with periodic updates of health status and collection of additional blood samples from original members, children and grandchildren. All original study participants and related offspring are considered members of the registry.

For this study, a subset of 174 individuals from the Michigan PBB Registry was selected to measure levels of PBB-153, PCB-153 and to perform HRM metabolic profiling. Blood collection occurred over the course of 2011–2014, and included a multi-generational cohort consisting of 1) participants over the age of 16 when the livestock feed contamination occurred, 2) participants aged 0–16 (F_0), and 3) conceived after but born to parents residing in the area (F_1). Though analyzed in the same run, a subset of participants who were older than 16 when the contamination occurred were excluded from the study due to small sample size ($n=18$) and higher risk of co-morbidities. Blood samples were collected in liquid EDTA, centrifuged, aliquoted and stored at -80°C . Participants provided informed consent and all protocols were approved by the institutional review board at Emory University and Michigan Department of Health.

2.2 Serum PBB-153 and PCB-153 levels

Serum levels of PBB and PCB congeners were measured using validated methods based on isotope-dilution gas chromatography-tandem mass spectrometry, and included quantification of PBB-77, PBB-101, PBB-153, PBB-180, PCB-118, PCB-138, PCB-153 and PCB-180, [27]. PCB congeners 118, 138 and 180 were highly correlated with PCB-153 (Pearson $r > 0.7$), while additional PBB congeners were only detected in a limited number of participants ($<7\%$) and highly correlated with PBB-153 (Pearson $r > 0.75$). Thus, we limited our MWAS to PBB-153 and PCB-153. Samples were prepared for analysis by spiking 1 mL aliquots of serum with 50 μL of 10 ng/mL internal standard solution containing ^{13}C -PBB-153 and ^{13}C -PCB-153, treated with 2 mL of a 1:1 (v/v) formic acid:water solution and extracted twice with 5 mL of hexane. The extract was then eluted through an acidified silica column, brought to dryness and reconstituted in 50 μL of isooctane.

Analysis was performed using an Agilent 7890A gas chromatograph interfaced to an Agilent 7000B triple-quad mass spectrometer with electron ionization source (Agilent Technologies, Santa Clara CA). A 2 μL aliquot of sample extract was injected into a heated, splitless inlet maintained at 325°C connected to a ZB-5HT column (15m $\times$ 0.250 ID $\times$ 0.10 μm film thickness, Phenomenex, Torrance, CA) with high-purity helium carrier gas at a constant flow of 2.25 mL/min. Analyte separation was accomplished using the following temperature program: 90°C (0.1 min), ramped to 340°C ($20^{\circ}\text{C}/\text{min}$) and held for 5 min, resulting in a total runtime of 17.6 min. The mass spectrometer was operated in multiple-reaction-monitoring (MRM) mode, and the following transitions at collision energy of 40 eV were

used for quantification: PBB-153: 467.8 → 307.9; PCB-153: 359.7 → 289.9. Concentrations were determined by relative response of the integrated peak areas using the stable isotope standards added prior to sample preparation. Calculated limits of detection (LOD) were 0.002 and 0.0016 ng/mL for PBB-153 and PCB-153, respectively. Concentrations below the LOD were replaced with an impute value of $0.5 \times \text{LOD}$ prior to data analyses.

For comparison to levels reported in the 2003–2004 National Health and Nutrition Examination Survey (NHANES), PBB-153 and PCB-153 were normalized by total serum lipids. Serum triglycerides 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 manufacturers' instructions. Total lipids were calculated from individual lipid components [28].

2.3 High-resolution metabolomics

Untargeted metabolic profiling was completed using previously described methods [24, 29]. Briefly, plasma aliquots were removed from storage at -80°C , and thawed on ice, upon which 65 μL of plasma was added to 130 μL of acetonitrile containing a mixture of stable isotope standards, vortexed, and allowed to equilibrate for 30 minutes. Following protein precipitation, triplicate 10 μL aliquots were analyzed by reverse-phase C_{18} liquid chromatography (Targa 100 mm $\times$ 2.1 mm $\times$ 2.6 μm , Higgins Analytical Inc) with detection by high-resolution mass spectrometry (Q-Exactive, Thermo Scientific, San Jose, CA). Three replicates of each sample extract were injected, and analyte separation was accomplished using water, acetonitrile and solution A (2% [v/v] formic acid in water) mobile phases operating under the following gradient: initial 2 min period of 5% water, 15% acetonitrile, 80% solution A, followed by linear increase to 5% water, 95% acetonitrile, 0% solution A at 6 min held for an additional 4 min. Mobile phase flow rate was held at 0.35 mL/min for 6 min, and then increased to 0.5 mL/min. The high-resolution mass spectrometer was equipped with an electrospray ionization source operated in positive ion mode with spray voltage of 4.5 kV, probe, capillary temperature 275°C , sheath gas flow 45 (arbitrary units), auxiliary gas flow 5 (arbitrary units) and S-lens RF level of 69. Resolution was set at 70,000 and mass-to-charge (m/z) scan range 85–1275. Samples were analyzed in batches of 20 study samples and two quality control (QC) pooled reference sample included at the beginning and end of each batch.

Upon injection of all study and quality control samples, mass spectral features with replicate coefficient of variation (CV) $\leq 100\%$ were extracted and aligned using *apLCMS* [30] with modifications by *xMSanalyzer* [31] and batch effect correction by *ComBat* [32]. Detected chemical signals were defined by accurate mass-to-charge ratio (m/z), retention time and intensity, referred to as m/z features. Prior to statistical analysis, replicate injections were averaged, and m/z features not detected in $\geq 25\%$ of the participants were removed

2.4 Statistical analyses

Summary statistics were calculated using GraphPad Prism version 6.0 for MacOSX (GraphPad Software, La Jolla CA); metabolomic data processing and analyses were

completed in R, version 3.1.2 [33]. To evaluate the relationship between PBB-153, PCB-153 and metabolic phenotype, a network-based MWAS approach was employed [34, 35]. Relationship between serum levels of PBB-153, PCB-153 and detected m/z features was assessed using Spearman rank correlation coefficient, which was calculated separately for each generation with *MetabNet* [35]. Since the aim of our study was to assess biological response to PBB-153 and PCB-153 exposure, we only evaluated correlations between serum levels of the two pollutants and m/z features. Correlations between HRM detected m/z features were not calculated with *MetabNet*. Correlations exhibiting a Spearman $|r| \geq 0.3$ and $p < 0.01$ were selected for further characterization. An unadjusted p threshold of 0.01 was utilized to balance Type I and Type II errors. The use of unadjusted p with effect size measures and pathway enrichment has been shown to protect against discarding metabolites with weak but important biological interactions [34]. For these m/z features, effect size variability was evaluated by estimating the 95% confidence intervals using a bootstrap approach (1,000 iterations) and R package *RVAideMemoire*. The resulting correlation networks were visualized using Cytoscape [36].

2.5 Annotation and metabolic pathway enrichment

High-resolution mass spectrometry provides accurate mass measures of ion m/z , which is related to chemical monoisotopic mass, an intrinsic molecular property. The m/z features correlated with PBB-153 and PCB-153 exposure were first matched to a reference database of 120 metabolites previously confirmed with MS² and co-elution studies [29]. Additional m/z features not matching these metabolites were annotated based upon positive electrospray ionization adducts using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [37] and the Human Metabolome Database (HMDB) [38]. Identities were assigned using ± 5 parts-per-million (ppm) mass tolerance ($m_{\text{error}} / m_{\text{theoretical}} \times 10^6$) and xMSannotator, which combines intensity- and time-based modularity clustering to improve annotation confidence [39]. Annotation of mass spectral signals is a major bottleneck of untargeted HRM profiling, with typically less than 50% of signals matching known metabolites listed in metabolomics databases [40]. The combination of correlation and retention time group can improve characterization of unidentifiable mass spectral signals by providing insight into relationship with identified metabolites from known biological processes and annotation of additional adducts, isotopes and in-source fragments [35, 41].

Enriched metabolic pathways were selected using a Mummichog $P \leq 0.05$ [42]. Pathway enrichment significance (P) is based upon a permutation framework that accounts for the many-to-many mappings of m/z features and metabolites. In this approach, pathway p -values are calculated using Fisher's exact test for the list of significant m/z features, and compared to the distribution of permuted pathway p -values generated by randomly sampling the full feature table. This accounts for the additional complexity of untargeted mass spectral data while isolating biological effects and reducing Type I error. The algorithm has been validated in Mummichog [42–44], and has been applied to range of studies examining metabolic effects of disease, drug and environmental exposures [45–47].

2.6 Comparison to the National Health and Nutrition Examination Survey

To evaluate the exposure levels and metabolic associations observed in this study relative to a representative US population, plasma fatty acid and lipid adjusted PBB-153 and PCB-153 concentrations were obtained from the 2003–2004 NHANES survey and stratified into two age groups (20–39 and 40–59) for comparison [48] (accessed January 21, 2017). For the 20–39 age group, 483 and 436 individuals were available with PBB-153 and PCB-153 measures, respectively; the 40–59 age group included 409 and 372. None of the individuals in either age group had measures of both pollutants available. Serum concentrations of PBB-153 and PCB-153 measured in the 2003–2004 NHANES dataset were tested for correlation with 24 fatty acids that included saturated, monounsaturated and polyunsaturated fatty acids (Supplementary Table 3)

3. RESULTS

3.1 Study population

Demographics for the selected participants from generations F_0 and F_1 are provided in Table I. Excluding age at blood draw, which had an 18.6-year difference in average age, total lipid measures and sex distribution were comparable across the two age groups. For generation F_0 , metabolomic profiling results were available for 80 participants; 76 individuals were available from F_1 . Of the 76 participants in F_1 , 24 and 1 were children of mothers and fathers in the F_0 generation, respectively.

3.2 PBB-153 and PCB-153 serum levels

Summary statistics for serum levels of PBB-153 and PCB-153 are provided in Table 1; the distribution for F_0 and F_1 are shown in Figure 1. Skewness and kurtosis for all were greater than 2.5 and 7.2, respectively for both analytes. Thus, non-parametric approaches were used in all statistical testing. PBB-153 and PCB-153 had a high rate of detection for both groups. PBB-153 was detected in all individuals from the F_0 group, while five individuals from F_1 had levels below the LOD. PCB-153 was detected in all individuals from both generations. For F_0 , median lipid adjusted concentrations of PBB-153 and PCB-153 were 53.2 and 20.5 ng/g, respectively. Levels detected in the F_1 group were lower, with median lipid-adjusted concentration of 5.3 and 9.9 ng/g for PBB-153 and PCB-153, respectively. Spearman correlation between fresh-weight and lipid adjusted values showed high correlation for both PBB-153 (Spearman $r = 0.98$, $p < 0.0001$) and PCB-153 (Spearman $r = 0.96$, $p < 0.0001$); therefore, non-adjusted levels (ng/mL) were used in all further analyses, excluding comparison to NHANES data.

To evaluate similarities in PBB-153 and PCB-153 exposure, levels within and across the two generations were compared. For both groups, correlation between the two compounds exhibited a positive Spearman correlation (F_0 : $r = 0.50$, $p < 0.0001$; F_1 : $r = 0.54$, $p < 0.0001$). Pearson correlation was also calculated for PBB-153 and PCB-153 to evaluate if the relationship between the two chemicals was linear. For both F_0 ($r = 0.07$, $p = 0.53$) and F_1 ($r = 0.21$, $p = 0.07$), Pearson correlation showed no association, suggesting a monotonic, but non-linear relationship. Comparison across F_0 and F_1 indicated both pollutants were elevated in older individuals, with PBB-153 17.1-fold (Mann-Whitney $p < 0.0001$) and PCB-153 2.5-

fold (Mann-Whitney $p < 0.0001$) higher in F_0 . PBB-153 and PCB-153 concentrations in NHANES also showed an age-dependent increase (Mann-Whitney $p < 0.0001$) [49]. For NHANES, PBB-153 and PCB-153 levels increased 2.0- and 2.8-fold, respectively, in the older age group

For both F_0 and F_1 , PBB-153 levels measured are significantly higher than reported in the 2003–2004 NHANES survey (Mann-Whitney $p < 0.0001$). Relative to NHANES, the measured PBB-153 levels were 4.8-fold higher in the F_1 generation, while levels measured from F_0 were 41-fold higher. Similarly, PBB-153 is consistently higher within this cohort when compared to NHANES, which reported median concentrations for the young and older age group of 1.6 and 2.9 ng/g lipid, respectively [2]. Unlike PBB-153, median concentrations of PCB-153 were more consistent with NHANES, which were 9.5 ng/g lipid for young individuals and 29.9 ng/g lipid for older. PCB-153 levels in the F_1 cohort showed no difference when compared to the NHANES younger age group (Mann-Whitney $p = 0.84$), while levels in F_0 were lower than measured in NHANES (Mann-Whitney $p = 0.006$).

3.3 High-resolution metabolomic profiling

Following data extraction and alignment, 16,890 m/z features were detected with mean triplicate CV of 38.1%. Participant samples were then grouped by generation and m/z features filtered based on the 25% non-missing value threshold. For generation F_0 , 8,376 m/z features remained after filtering while F_1 had 8,533 m/z features meeting the missing value threshold. Comparison of the two groups showed 8,006 m/z features were detected in both groups, with 370 and 527 unique to F_0 and F_1 , respectively.

3.4 F_0 metabolome-wide association study of PBB-153 and PCB-153

To assess possible metabolic variations due to PBB-153 and PCB-153 exposure for F_0 , we applied a Spearman correlation based MWAS to identify metabolites associated with the two chemicals based upon effect size measure (Spearman r) and $p < 0.01$. MWAS identified 139 and 113 m/z features correlated with PBB-153 and PCB-153, respectively (Figure 2A). Both positive and negative correlations were present for both pollutants, with the majority negative (PBB-153: 81 (58%); PCB-153: 78 (69%)). The network analysis showed the majority of m/z feature were correlated with one of the pollutants (Figure 2B); only 12 m/z were correlated with both PBB-153 and PCB-153.

Only a limited number of the m/z features matched known metabolites present in the KEGG and HMDB databases, with 65 unique metabolites matching 66 m/z features, 39 of which were correlated with PBB-153 and 24 that were correlated with PCB-153. The full list of annotated metabolites for F_0 is provided in Figure 2B and Supplementary Table 1. Metabolites correlated with PBB-153 related to co-factor metabolism, neurotransmitters, amino acids, pro-inflammatory signaling metabolites, nucleobases and mitochondrial bioenergetics are provided in Table 2. PCB-153 was correlated with metabolites that were consistent with neurotransmitters, amino acids, pro-inflammatory signaling metabolites, nucleobases and mitochondrial bioenergetics, metabolites from these processes are provided in Table 2. Only glycolate, dodecanoic acid and a phosphatidylglycerol lipid were correlated with both chemical species.

Metabolic pathway enrichment showed biological response was different for the two pollutants (Table 3). PBB-153 exposure was associated with changes to microbiome-related pathways, amino acid metabolism and catabolism. Metabolic response to PCB-153 included pathways for neurotransmitter precursors, nitrogen catabolism and drug metabolism. Only urea cycle/amino group metabolism was associated with exposure to both pollutants.

3.5 F₁ metabolome-wide association study of PBB-153 and PCB-153

MWAS of serum PBB-153 and PCB-153 for F₁ detected a greater number of *m/z* features correlated with exposure. This included 307 *m/z* features, with 208 and 124 correlated with PBB-153 and PCB-153, respectively (Figure 3A). Only 25 were correlated with both pollutants. Both positive and negative relationships were detected; however, unlike F₀, the majority of the correlations with PBB-153 were positive (n=133; 64%), while the majority were negative for PCB-153 (n= 91, 73%). As a result, the correlation networks differed for the two chemicals (Figure 3B).

Annotation of the *m/z* features provided 94 matches (31%) total, which included 75 *m/z* matching 72 metabolites for PBB-153 and 29 metabolite matches for PCB-153. The full list of annotated metabolites for F₁ is provided in Figure 3B and Supplementary Table 2. Of the 94 matches, 10 were correlated with both pollutants, and included citrate/iso-citrate, proline, nicotinamide, naphthalene, dihydrothymine, hordenine, norlaudanosoline, N2,N2-dimethylguanosine, atorvastatin and a phosphocholine lipid. Metabolite matches for PBB-153 consistent with stress hormones, polyamines, one-carbon metabolism, amino acids, pro-inflammatory signaling lipids, essential dietary fatty acids are provided in Table 4, in addition to metabolites from fatty acid metabolism, lipids and nucleobase that were correlated with PCB-153.

F₁ metabolic pathway enrichment are provided in Table 5. PBB-153 exposure was associated with changes to fatty acid metabolism, pro-inflammatory signaling lipids, antioxidants and nitrogen catabolism pathways. Metabolic response to PCB-153 included lipid metabolism, neurotransmitter precursors and nucleotide metabolism. None of the enriched pathways were associated with exposure to both PBB-153 and PCB-153.

3.6 Comparison of exposure metabolic phenotype across generations

To assess generational differences in metabolic response to exposure, we compared the PBB-153 and PCB-153 metabolic phenotypes across the two generations. Since there were *m/z* features unique to each generation, we first evaluated if *m/z* features that were only detected in one of the age groups could explain the difference. For F₀, 7 *m/z* features were specific to that generation, with four and three correlated with PBB-153 and PCB-153, respectively. Excluding a match to 5,10-methylenetetrahydrofolate, none of the other *m/z* features matched entries in the KEGG and HMDB metabolite libraries. F₁ included 12 *m/z* features that were specific to this generation, which included eight correlated with PBB-153 and four with PCB-153. Only two metabolites correlated with PCB-153 matched known metabolites, which included α -D-ribose-1-phosphate and cinnavalinate.

The *m/z* features correlated with PBB-153 included 11 that were present in both age groups; however, only three matched known metabolites (Table 6). The late retention time (>300s)

of the unidentified m/z features are consistent with lipids and other lipophilic compounds. Additional metabolites correlated with PBB-153 in both generations but detected as different adducts included glycolate, 5-acetamidopentanoate, citrate/iso-citrate, sphinganine and a phosphatidylglycerol lipid [PG(18:0/18:1(11Z))]. Only the urea cycle/amino group metabolism pathway was enriched in both generations.

PCB-153 correlations across F_0 and F_1 were similar to PBB-153. Only 5 of the m/z features were correlated with PCB-153 in both generations, which included four non-identifiable metabolites and 3-(4-hydroxyphenyl)pyruvate (Table 6). Excluding m/z 830.8173, the correlations were negative in both generations. Tyrosine metabolism was the only metabolic pathway associated with exposure in both F_0 and F_1 .

3.8 NHANES PCB and PBB alterations in plasma fatty acids

Both PBB-153 and PCB-153 were correlated with alterations to fatty acid metabolites for F_1 . To see if similar metabolic changes were observed in an independent cohort, we evaluated plasma fatty acid correlations with PBB-153 and PCB-153 using the 2003–2004 NHANES survey. Concentrations of PBB-153 and PCB-153 were tested for correlation with 24 fatty acids that included saturated, monounsaturated and polyunsaturated fatty acids (Supplementary Table 3) for the 20–39 and 40–59 age groups. Only three polyunsaturated fatty acids, including docosahexaenoic, docosapentaenoic-3 and eicosapentaenoic, met the correlation criteria of Spearman $|r| \geq 0.3$ and $p < 0.01$. Additional omega-3 and omega-6 fatty acids showed significant relationships with PBB-153 and PCB-153 levels at $p < 0.05$ but weaker correlations. These include α -linolenic, γ -linolenic and homo- γ -linolenic acid.

4. DISCUSSION

Metabolic phenotyping by HRM provides a framework for linking environmental exposure with internal dose and biological response in human populations. The present study demonstrates the application of HRM to a multi-generational cohort exposed to high levels of PBB-153 due to food contamination and chronic exposure to the environmental pollutant, PCB-153. MWAS of the two pollutants showed metabolites correlated with levels of PBB-153 and PCB-153 were present in the metabolome. The metabolic phenotypes for each exposure suggest response to the two pollutants differs at the metabolite and pathways level; however, overall biological function of the associated pathways was similar. Generational differences were also present; suggesting age, length and major route of exposure may contribute to metabolic changes from environmental exposures.

Biomonitoring of persistent organic pollutants, such as PBB-153 and PCB-153, provides a means to assess the current distribution within a population and prevalence of exposure. Although manufacture and use of PBB- and PCB-related commercial products has been stopped in the United States, almost all participants in this study exhibited detectable levels of the two pollutants. For both F_0 and F_1 , PBB-153 levels measured are significantly higher than reported in the 2003–2004 NHANES survey. Unlike PBB-153, concentrations of PCB-153 were more consistent with NHANES, with PCB-153 levels in the F_1 cohort showing no difference when compared to the NHANES younger age group, while levels in

F₀ were lower than measured in NHANES. This is consistent with the general population levels of PCBs reported in this population soon after the industrial accident [50].

For both pollutants, an age-dependent increase in concentration was observed. Association of age with exposure to persistent organic pollutants has been well documented, and is attributed to increased length of exposure [2, 51], changes in metabolism with age [52] and exposure during peak emission periods [53]. In this study, the large difference in concentrations of PBB-153 suggests length of exposure and changes in metabolism cannot account for the age-related differences. Compared to the NHANES data and PCB-153, the significantly greater increase in PBB-153 observed across the two generations is consistent with different routes and levels of exposure to PBB-153 for F₀ and F₁, with F₀ exposure most likely through consumption of contaminated food and F₁ *in utero* and through breastfeeding [4]. This finding is consistent with previous studies in this population showing exposure levels within families are correlated [4, 54]. Taken together, these results show greater PBB-153 exposure in Michigan PBB registry participants than is experienced in the general population. Although the greatest levels were measured in F₀, PBB-153 concentrations in F₁, which were born after the original PBB contamination, show the offspring of parents who reside in the study area also experience elevated PBB exposure.

Metabolic alterations associated with both PBB-153 and PCB-153 detected in the F₀ generation were consistent with biological changes underlying numerous chronic diseases. The PBB-153 MWAS identified alterations in key metabolic intermediates in mitochondrial energy metabolism, which included decreased creatine, acetyl-carnitine and succinate while citrate and glucose were increased with elevated PBB-153 levels. Mitochondrial dysfunction due to oxidative stress from environmental exposures is thought to underlie many age-related diseases, including neurodegenerative diseases, cardiovascular disease, diabetes, cancer and metabolic syndrome [55–57]. Mitochondrial energy production is the result of close coordination between the TCA cycle and electron transport chain, with disruption to either resulting in increased oxidative stress and mitochondrial dysfunction [58]. Changes in the TCA cycle have been identified in Alzheimer's disease (AD) and environmental exposure models of Parkinson's disease (PD), which included increases in citrate and glucose levels that were consistent with the results from this study [59, 60]. In addition to TCA cycle alterations, dopamine showed a positive correlation with PBB-153 and putrescine a negative relationship. PD is characterized by progressive loss of dopamine neurons and alterations in catecholamine metabolism [61], while previous metabolomic study of PD showed polyamine metabolism was associated with rapid disease progression [62]. These findings suggest exposure-related changes that are consistent with the pathophysiology underlying neurodegenerative diseases and are supported by previous mechanistic and epidemiology findings that have identified exposure to persistent organic pollutants, including other brominated flame retardants and PCBs, as possible etiological agents in PD and AD [10, 63, 64].

Metabolic alterations associated with PCB-153 were also consistent with metabolites from neurotransmitter pathways and catecholamine metabolism. Metabolites associated with PCB-153 included 4-hydroxyphenylpyruvic acid (4-HPAA), 3,4-dihydroxy-L-phenylalanine (L-dopa) and 3-methoxytyramine (3-MTT). In humans, dopamine formation is

accomplished through conversion of tyrosine (which can be synthesized from 4-HPAA by phenylalanine hydroxylase) to L-dopa by tyrosine hydroxylase (TH). L-dopa, which was positively associated with PCB-153 levels, is an immediate precursor to dopamine. Although dopamine was not associated with PCB-153, it was positively correlated with PBB-153 exposure, suggesting a possible interaction of both pollutants with catecholamine metabolism. Previous studies suggest dysregulation of TH can contribute to PD, AD and type 2 diabetes [65, 66]. *In vitro* studies have also shown that exposure to ortho-substituted PCBs can reduce TH activity [67]; however, this effect was not observed in animal models [10]. 3-MTT is a methylated metabolite of dopamine and has been identified as a neuromodulator that can affect behavior and induce intracellular signaling by activation of trace amine-associated receptor 1 (TAAR-1) [68]. Thus, PCB-153 correlated metabolic variations in F₀ were consistent with pathways implicated in neurodegenerative diseases.

In F₁, PBB-153 metabolic variations also showed changes to neurotransmitter pathways, in addition to polyamine metabolism, mineralocorticoids, oxidative stress-related metabolites, co-factors and fatty acids. Dihydrobiopterin (BH₂), tetrahydrobiopterin (BH₄) and tryptophan were all associated with PBB-153. BH₄ is an essential co-factor for biopterin-dependent aromatic amino acid hydroxylases, which include phenylalanine hydroxylase, tryptophan hydroxylase and TH. As discussed above, these enzymes provide critical functions in the biosynthesis of neurotransmitters, including dopamine, serotonin, norepinephrine and epinephrine and dysregulation has been associated with both AD and PD. While BH₄ was positively correlated with PBB-153 exposure, BH₂ was decreased. BH₂ is the oxidation product of BH₄ and produced during the conversion of phenylalanine, tryptophan and tyrosine to monoamine neurotransmitters. Polyamine metabolites associated with PBB-153 included spermine aldehyde, N-methylputrescine and N8-acetylspermidine. Polyamine metabolism has been linked to rapid PD progression in a metabolomic study of PD [62], with N8-acetylspermidine significantly elevated in patients with rapid progression PD. Cerebral spinal fluid levels of polyamines have also been associated with PD [69], while increased polyamine excretion has been observed during neuronal cell death, traumatic brain injury and neuroinflammation [70]. Oxidative stress metabolites included decreased pyridoxamine and elevated spermine dialdehyde. Pyridoxamine inhibit oxygen radical formation, reduce lipid peroxidation and block delay progression of diabetic nephropathy through blocking oxidative pathways [71, 72]. Spermine dialdehyde is the oxidized metabolite of spermine, which functions directly as a free radical scavenger and protects DNA from oxidative damage [73]. Thus, these results suggest increased oxidative stress is correlated with PBB-153 exposure. While previous studies in this cohort have identified endocrine effects of PBB exposure [13, 18–21], results from this study were largely consistent with metabolic changes associated with neurodegenerative diseases and oxidative stress. No pathways consistent with endocrine dysfunction were correlated with PBB-153 exposure. The lack of association with endocrine effects could arise due to poor sensitivity for metabolite processes that occur in distal tissues, or the results previously reported do not result in long-term metabolic changes that can be detected using current metabolomic approaches. More research is needed to understand how endocrine disrupting chemicals influence the plasma metabolome.

Metabolic variations correlated with PCB-153 in F₁ also included tyrosine metabolism. 4-HPAA is a precursor to tyrosine and formed through hydroxylation of phenylalanine by phenylalanine hydroxylase. 4-HPAA showed a similar relationship in F₀, providing additional support that exposure to PCB-153 interacts with catecholamine metabolism. Fatty acid and lipid metabolism pathways were also associated with exposure. These pathways include glycerophospholipid metabolism, glycosphingolipid metabolism and carnitine shuttle. While these pathways were not associated with PCB-153 in F₀, other metabolomic studies of persistent organic pollutant exposures have shown similar results. In the study by Walker, Pennell [74], an MWAS framework was used to test for benzo[a]pyrene (BaP) associations with the serum metabolome. Using a pilot study of 30 individuals, untargeted HRM profiling detected changes in linoleate metabolism, carnitine shuttle, glycerophospholipid metabolism and prostaglandin formation from dihomo γ -linoleic acid, which was consistent with findings in model systems [75]. Sphinganine and linoleic acid, which were both negatively correlated with BaP, were also associated with PBB-153 exposure in the present study. Serum levels of the organochlorine pesticides p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE) and hexachlorobenzene (HCB) have shown to be associated with variation in lipid metabolism, including fatty acids, glycerophospholipids, sphingolipids and glycerolipids. MWAS of p,p'-DDE and HCB in an elderly cohort of 1,016 individuals from Sweden identified 16 metabolites associated with exposure and included lipids related to cell signaling, energy regulation and membrane composition [23]. To test for metabolic associations with exposure to β -hexachlorohexane (β -HCH), HCB, p,p'-DDE and PCB congeners 28,138 and 153, Carrizo, Chevallier [22] used an untargeted metabolomic approach to compare low- and high-exposed individuals. Identified and unidentified metabolic features were consistent with lipid species, including sphingolipids and glycerophospholipids. In addition, changes to lipid, fatty acid and carnitine metabolism were also detected in response to occupational trichloroethylene exposure [25], suggesting perturbations to these pathways may represent a general biological response to environmental exposures.

Interestingly, PBB-153 was correlated with more metabolic features for the F₁ generation than F₀, even though they experienced a shorter exposure window and lower body burden of PBB-153. In addition, different metabolites were correlated with PBB-153 exposure between the two generations, although biological processes, including pathways and disease risk, were similar across the two generations. It is well recognized that exposure during different developmental periods can influence distal health outcomes and increase disease risk later in life [76–78]. While not possible to evaluate in the present study, the results suggest exposure *in utero* could lead to greater metabolic alterations than when exposure occurs during childhood or later. Thus, age, length and major route of exposure may contribute to metabolic changes from environmental exposures. Additional studies will be needed to verify these findings.

PBB-153 and PCB-153 associations with fatty acids in F₁ were consistent with studies showing exposure-related changes to fatty acid profiles [24, 79]. Previous results have shown that co-planar PCBs can reduce synthesis of long-chain unsaturated fatty acids by inhibiting delta 5 and delta 6 desaturase activities in the liver, resulting in accumulation of omega-6 fatty acids [80]. Linoleic acid potentiates dysfunction in endothelial cells following

exposure to co-planar PCBs, which was hypothesized to be due to increased oxidative stress [81]. Arachidonic acid, which can be synthesized from homo- γ -linolenic acid and is a key intermediate in inflammation and cellular stress signaling, has also been shown to be negatively correlated with blood PCB levels during pregnancy [82]. While this was not observed in the NHANES data, the results from Grandjean and Weihe [82] support evidence of PCB interaction with desaturase activities and the observed increase in linoleic acid in the present study. Thus, these results suggest exposure to PBB-153 and PCB-153 leads to disruption to essential fatty acid metabolism and has important implications for fatty liver, cardiovascular and metabolic diseases [83, 84].

We acknowledge several limitations in this work. First, a limited sample population of 156 individuals from a specific geographical location with documented, high exposure to PBBs was used, and an independent cohort was not available to replicate many of the metabolomic findings. Second, this study only focused on a single biomarker of PBB and PCB exposure. Both of the pollutants were commercially available as mixtures; previous studies have shown some exposure to other PBB congeners at much lower levels in this population [27]. Therefore, we could not account for the metabolic effects of co-exposures. Third, this study was largely focused on changes to endogenous metabolites listed in metabolomic databases, and we did not attempt to identify additional PCB or PBB congeners detected by HRM due to the low sensitivity and unlikely ionization of these compounds by electrospray ionization [85]. Due to the limited sample availability, we could not complete in-depth structural characterization of unidentified metabolites. Lack of reference standards and low abundance will make identification of these features challenging and they were not characterized for this study. Finally, the results of this study are correlative in nature. We could also not account for unknown confounders, nor identify the exact mechanism through which these metabolic associations occurred. The results, which included alterations in lipid and fatty-acid related pathways, could arise due to the lipophilic properties of the two pollutants and we could not account for the complex interactions occurring between these two pollutants and lipids. Many of the pathways altered in association with exposure occur in specific tissues, and it is unknown how distal changes are reflected in the blood metabolome. Despite these limitations, MWAS of both pollutants identified changes to pathways previously associated with PBB-153 and PCB-153 exposure. Metabolic changes consistent with diseases linked to these pollutants were also present, and the results show HRM can detect biological response to circulating levels of environmental chemicals. In addition, comparison to an independent population showed similar patterns of exposure-associated changes in fatty acids. Taken together, the results from this study show metabolic alterations correlated with PBB-153 and PCB-153 exposure can be detected in human populations and are consistent with diseases previously linked to these exposures in epidemiological and mechanistic studies.

5. CONCLUSIONS

Although environmental exposures have been linked to a range of diseases, the underlying mechanisms and initiating events are not well understood. In this study, we applied HRM to identify biological response to PBB-153, a brominated flame retardant with high-exposure in the selected population due to accidental contamination during the 1970's, and the

environmental pollutant PCB-153. The results show HRM is able to detect metabolic phenotypes of PBB-153 and PCB-153 exposure in both generations. Although metabolites differed among the groups, biochemical functions of many of the exposure-associated metabolic changes were similar and resulted in alterations to catecholamine metabolism, mitochondrial function and fatty acid pathways. In addition, the results show, on average, that participants in the Michigan PBB Registry who were born before or after the original contamination carry a significantly higher burden of PBB levels than experienced by the general United States population. While our study was limited to a high-exposure population, the metabolite variations detected in this study provide insight into how exposure to persistent organic pollutants influences metabolic phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

3-MTT	3-methoxytyramine
4-HPAA	4-hydroxyphenylpyruvic acid
AD	Alzheimer's Disease
BaP	Benzo[a]pyrene
BH₂	Dihydrobiopterin
BH₄	Tetrahydrobiopterin
HCB	Hexachlorobenzene
HMDB	Human Metabolome Database
HRM	High-resolution metabolomics
KEGG	Kyoto Encyclopedia of Genes and Genomes
L-dopa	3,4-dihydroxy-L-phenylalanine
LOD	Limit of detection
MWAS	Metabolome-wide association study

NHANES	National Health and Nutrition Examination Survey
PBB	Polybrominated biphenyl
PCB	Polychlorinated biphenyl
PD	Parkinson's Disease
p,p'-DDE	p,p'-dichlorodiphenyldichloroethylene
TAAR-1	Trace amine-associated receptor 1
TH	tyrosine hydroxylase

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HIGHLIGHTS

- Blood polybrominated biphenyl 153 levels are elevated in the Michigan PBB Registry.
- High-resolution metabolomics detected metabolic phenotypes of chemical exposure.
- Pathway alterations included catecholamine, mitochondrial and fatty acid pathways.
- Metabolic changes were consistent with neurodegenerative disease pathobiology.

Figure 1: PCB-153 and PBB-153 congener levels (log scale) measured in serum obtained from individuals in generation F₀ and F₁. Limit of detection (LOD): PBB-153= 0.002 ng/mL; PCB-153= 0.0016 ng/mL.

Figure 3:

Results of PBB-153 and PCB-153 metabolome-wide association study in F₁. As was observed in the older generation, metabolic changes associated with exposure were detected for both pollutants (A). Network correlation analysis for PBB-153 and PCB-153 resulted in different correlation patterns for the two pollutants in F₁, with only a small percentage correlated to both exposures (B).

Table 1:

Population summary of participants selected for MWAS

Characteristic	Total	Age 0–16 during PBB event (F ₀)	Born following PBB event (F ₁)
Number of individuals	156	80	76
Age at blood draw, mean $\pm$ SD	39.7 $\pm$ 11.0	48.8 $\pm$ 4.8	30.2 $\pm$ 6.5
Age during contamination event	9.2 $\pm$ 4.8	9.2 $\pm$ 4.8	NA
Total lipids (mg/dL)	630 $\pm$ 152	656 $\pm$ 153	602 $\pm$ 136
Sex, <i>n</i> (%)			
Male	68 (44%)	33 (41%)	35 (46%)
Female	88 (56%)	47 (59%)	41 (54%)
PBB-153			
Non-detects, <i>n</i> (%)	5 (7%)	0	5 (7%)
Plasma levels, mean $\pm$ SD (ng/mL)	0.8 $\pm$ 4.2	1.5 $\pm$ 5.9	0.1 $\pm$ 0.2
Plasma minimum - maximum (ng/mL)	ND - 50.4	0.02 - 50.4	ND - 1.5
Lipid normalized levels, 50 th percentile (ng/g)	17.26 $\pm$ 1161	53.2 $\pm$ 1612	5.3 $\pm$ 46.8
PCB-153			
Non-detects, <i>n</i> (%)	0%	0%	0%
Plasma levels, mean $\pm$ SD (ng/mL)	0.15 $\pm$ 0.19	0.22 $\pm$ 0.23	0.08 $\pm$ 0.08
Plasma minimum - maximum (ng/mL)	0.01 - 1.2	0.04 - 1.2	0.01 - 0.5
Lipid normalized levels, 50 th percentile (ng/g)	15.1 $\pm$ 31.0	20.5 $\pm$ 38.3	9.9 $\pm$ 14.4

Table 2:Select metabolites correlated with PBB-153 and PCB-153 in F₀

Metabolite Annotation	Spearman <i>r</i>	<i>p</i>	Spearman <i>r</i> 95% confidence interval	Metabolite Class Description
<i>PBB-153</i>				
Asparagine	−0.41	0.0013	(−0.64, −0.13)	Amino Acid
Threonine	−0.32	0.0086	(−0.51, −0.1)	Amino Acid
Retinyl beta-glucuronide	0.31	0.0053	(0.07, 0.51)	Co-factor metabolite
25-Hydroxyvitamin D2	−0.33	0.0027	(−0.53, −0.12)	Hormone
1alpha,24R,25-trihydroxyvitamin D3	0.31	0.0055	(0.09, 0.50)	Hormone
Leukotriene B4	0.32	0.0033	(0.09, 0.53)	Inflammatory Lipid
Sphinganine	−0.54	0.0007	(−0.74, −0.28)	Lipid
Creatine	−0.42	0.0005	(−0.59, −0.22)	Mitochondrial
Acetylcarnitine	−0.37	0.0032	(−0.59, −0.1)	Mitochondrial
Succinate	−0.33	0.0041	(−0.52, −0.11)	Mitochondrial
Citrate; Iso-Citrate	0.38	0.0039	(0.12, 0.58)	Mitochondrial
Glucose	0.38	0.0005	(0.14, 0.57)	Mitochondrial
Cytosine	−0.34	0.0019	(−0.53, −0.11)	Nucleotide
5-Hydroxy-N-formylkynurenine	−0.53	0.0020	(−0.73, −0.24)	Neurotransmitter metabolite
Dopamine	0.44	0.0023	(0.16, 0.65)	Neurotransmitter metabolite
Putrescine	−0.41	0.0016	(−0.61, −0.16)	Polyamine
<i>PCB-153</i>				
N-Acetyl-L-glutamate 5-semialdehyde	−0.43	0.0019	(−0.64, −0.21)	Amino acid metabolite
Picolinic acid	−0.30	0.0071	(−0.5, −0.08)	Amino acid metabolite
5,10-Methylenetetrahydrofolate	−0.56	0.0053	(−0.81, −0.15)	Co-factor metabolite
Prostaglandin B1	−0.40	0.0070	(−0.66, −0.09)	Inflammatory lipid
N-Acetyl-L-glutamate 5-phosphate	0.34	0.0023	(0.11, 0.52)	Mitochondrial
Uridine triphosphate	0.33	0.0036	(0.09, 0.53)	Nucleotide metabolite
3-(4-Hydroxyphenyl)pyruvate	−0.32	0.0038	(−0.5, −0.11)	Neurotransmitter metabolite
3,4-Dihydroxy-L-phenylalanine	0.46	0.0076	(0.1, 0.72)	Neurotransmitter metabolite
3-Methoxytyramine	0.30	0.0066	(0.12, 0.5)	Neurotransmitter metabolite

Table 3:Metabolic pathway enrichment results for PBB-153 and PCB-153 exposure in F₀

Pathway ^a	Number overlapping metabolites ^b	Total number metabolites ^c	Mummichog <i>P</i> ^d
<i>PBB-153 MWAS pathways</i>			
Butanoate metabolism	4	25	0.003
Heparan sulfate degradation	2	6	0.005
Chondroitin sulfate degradation	2	6	0.005
Glyoxylate and Dicarboxylate Metabolism	2	9	0.011
Urea cycle/amino group metabolism	4	41	0.014
Glycosphingolipid metabolism	3	28	0.018
Aspartate and asparagine metabolism	5	62	0.021
TCA cycle	2	13	0.023
Hexose phosphorylation	2	15	0.031
Arginine and Proline Metabolism	3	35	0.039
<i>PCB-153 MWAS pathways</i>			
Tryptophan metabolism	4	61	0.005
Tyrosine metabolism	4	87	0.016
Urea cycle/amino group metabolism	2	41	0.044

^aPathways in bold were associated with PBB or PCB exposure in both the F₀ and F₁ age groups.^bNumber of metabolites from pathway correlated with PBB or PCB.^cTotal number of metabolites detected from pathway using HRM^dMummichog *P* represents the adjusted *p*-value when comparing pathway enrichment *p*-values for significant *m/z* to permuted results generated by randomly sampling the full feature table.

Table 4:Select metabolites correlated with PBB-153 and PCB-153 in F₁

Metabolite annotation	Spearman <i>r</i>	<i>P</i> -value	Spearman <i>r</i> 95% confidence interval	Metabolite Class Description
<i>PBB-153</i>				
Glycine	−0.35	0.0074	(−0.57, −0.08)	Amino acid
Selenohomocysteine	0.38	0.0098	(0.09, 0.63)	Amino acid metabolite
Tryptophan	0.49	0.0066	(0.23, 0.68)	Amino acid/neurotransmitter
Pyridoxamine	−0.29	0.0086	(−0.54, −0.08)	Co-factor
Retinyl beta-glucuronide	0.32	0.0046	(0.12, 0.51)	Co-factor metabolite
Linoleic acid	0.41	0.0003	(0.2, 0.59)	Fatty acid
Glycolate	−0.51	4.9E−05	(−0.67, −0.29)	Hydroxy acid
Sphinganine	0.36	0.0015	(0.15, 0.54)	Lipid
Citrate; Iso-Citrate	−0.31	0.0076	(−0.52, −0.07)	Mitochondrial
Dihydrobiopterin	−0.31	0.0072	(−0.51, −0.08)	Neurotransmitter metabolite
Tetrahydrobiopterin	0.36	0.0020	(0.15, 0.56)	Neurotransmitter metabolite
Spermine dialdehyde	0.31	0.0097	(0.06, 0.52)	Polyamine
N-Methylputrescine	0.43	0.0005	(0.2, 0.61)	Polyamine
N8-Acetylspermidine	0.50	0.0090	(0.09, 0.78)	Polyamine
Cortisol	0.41	0.0049	(0.17, 0.6)	Steroid hormone
<i>PCB-153</i>				
Serine	−0.32	0.0071	(−0.52, −0.08)	Amino acid
Eicosadienoic acid	−0.69	0.0004	(−0.83, −0.44)	Fatty acid
Phosphoethanolamine	−0.34	0.0027	(−0.53, −0.13)	Lipid
Cer(d18:0/22:0)	−0.31	0.0074	(−0.52, −0.07)	Lipid
PI(16:0/20:0)	0.39	0.0013	(0.16, 0.57)	Lipid
Palmitoylcarnitine	0.35	0.0040	(0.13, 0.53)	Mitochondrial
Uracil	−0.30	0.0085	(−0.52, −0.08)	Nucleobase
Urocortisol	−0.34	0.0079	(−0.58, −0.09)	Steroid hormone

Table 5:Metabolic pathway enrichment results for PBB-153 and PCB-153 exposure in F₁

Pathway ^a	Number overlapping metabolites ^b	Total number metabolites ^c	Mummichog <i>P</i> ^d
<i>PBB-153 MWAS pathways</i>			
Linoleate metabolism	5	20	0.003
Prostaglandin formation from dihomogamma-linoleic acid	2	6	0.017
Limonene and pinene degradation	2	6	0.017
Selenoamino acid metabolism	3	18	0.024
Fatty acid activation	3	18	0.024
Urea cycle/amino group metabolism	5	41	0.028
Alkaloid biosynthesis II	2	8	0.029
Glutathione Metabolism	2	9	0.037
Porphyrin metabolism	3	22	0.045
<i>PCB-153 MWAS pathways</i>			
Glycerophospholipid metabolism	3	42	0.005
Pyrimidine metabolism	3	48	0.007
Carnitine shuttle	2	21	0.009
Sialic acid metabolism	2	26	0.013
Glycosphingolipid metabolism	2	29	0.016
Tyrosine metabolism	3	89	0.040
Glycine, serine, alanine and threonine metabolism	2	49	0.048

^a Pathways in bold were associated with PBB or PCB exposure in both the F₀ and F₁ age groups.

^b Number of metabolites from pathway correlated with PBB or PCB.

^c Total number of metabolites detected from pathway using HRM

^d Mummichog *P* represents the adjusted *p*-value when comparing pathway enrichment *p*-values for significant *m/z* to permuted results generated by randomly sampling the full feature table.

Table 6:Overlapping features correlated with PBB-153 or PCB-153 in both F_0 and F_1

<i>m/z</i>	Retention time (s)	Identity	F_0 Spearman <i>r</i>	F_1 Spearman <i>r</i>
<i>PBB-153</i>				
114.0738	391	No match	−0.43	−0.34
166.1227	491	Hordenine	−0.37	−0.39
127.0392	349	2-Hydroxyadipic acid	−0.36	−0.34
171.0347	439	No match	−0.31	−0.36
235.2058	295	No match	−0.31	−0.43
480.2956	587	Retinyl beta-glucuronide	0.31	0.32
317.6602	519	No match	0.31	0.35
317.6551	526	No match	0.31	0.33
416.3716	548	TG(16:0/16:0/18:2(9Z,12Z))[iso3]	0.38	0.31
263.2318	561	No match	0.39	0.55
548.3595	562	No match	0.45	0.53
<i>PCB-153</i>				
688.7503	95	No match	−0.35	−0.37
1072.7635	118	No match	−0.34	−0.53
114.0738	391	No match	−0.33	−0.42
181.0502	113	3-(4-Hydroxyphenyl)pyruvate	−0.32	−0.33
830.8173	57	No match	0.35	−0.43