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LINKING PCB CONGENER CONCENTRATIONS AND METABOLOMIC PROFILES IN ANNISTON RESIDENTS: A PILOT STUDY

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Introduction

Between 1929-1971, Anniston, Alabama housed a major PCB manufacturing plant. Factory releases during this period have resulted in elevated PCB concentrations in the environment and subsequently in the Anniston population. Health effects of PCBs in animals are known to vary by congener, but although substantial monitoring of PCB levels and resident health has been performed through the Anniston Community Health Survey (ACHS), only 35 ortho-substituted congeners have so far been evaluated [1,2]. Metabolomics, the study of endogenous small molecules indicative of biological state can provide additional insights into system-wide changes in human health resulting from factors such as disease, diet and age. The objective of this pilot study was to apply targeted metabolomics to assess changes in the serum metabolome of Anniston residents and to perform preliminary, pilot scale linkages to concordantly measured concentrations of all 209 PCB congeners.

Materials and methods

140 serum samples from Anniston residents, 90 collected in 2005-7 (a subset of ACHS) and 50 in 2014 (a subset of ACHS II) [3], were analyzed for PCB congener levels. All samples received were stored at -80°C prior to analysis. A subset of 50 samples, equally distributed across the collection time-points, was selected for metabolomics analysis.

AXYS analyzed 209 PCB congeners using procedures from AXYS method MLA-010 [4] based on US EPA Method 1668 C. 2 mL per sample was used for the ACHS cohort and 10 mL for the ACHS II cohort. The samples were fortified with an internal standard mix containing 29 13C-labeled PCBs and liquid-liquid extracted using ethanol, hexane and ammonium sulfate. The extract was further processed using a combination of size-exclusion (biobead) and other column chromatographic processes including acid/base silica, alumina and florisil solid phases and concentrated to 300 µl for GC-HRMS analysis. PCB congener concentrations were measured using an HP 6890 gas chromatograph coupled with a Micromass Ultima magnetic sector high resolution mass spectrometer operated at 10,000 resolution. Chromatographic separation was achieved using an SPB-Octyl column. One blank and one spiked matrix (SPM) sample was extracted and analyzed in conjunction with each analytical batch of 20 or fewer samples. Lipids in the samples were measured in parallel using standardized enzymatic colorimetric assays.

Targeted metabolomics was performed by AXYS using procedures modified from Benskin et al, [5]. A total of 219 metabolites including 21 amino acids (AA), 22 biogenic amines (BAs), 13 bile acids, Σhexose, 18 fatty acids (FAs), 40 acylcarnitines (ACs), 89 phosphatidylcholines (PCs), and 15 sphingomyelines (SMs) were measured. 50 µl per sample was added to a 96-well filter plate which had been fortified with an internal standard mix. The plates were dried under liquid nitrogen and the AAs and BAs were derivatized using Edman's Reagent [6]. After drying, 250 µl of 5 mM ammonium acetate in methanol was added to each wells and the plate shaken for 30 min. The samples were then eluted by centrifugation and diluted with an equivalent volume of water (methanol for ACs, PCs, and SM) prior to analysis.

Metabolite concentrations were measured using an Agilent 1100 high performance liquid chromatography (HPLC) coupled to an API4000 triple quadrupole mass spectrometer (Applied Biosystems/Sciex, Concord, ON, Canada). Amino acids, biogenic amines, hexose, fatty acids and bile acids were quantified by isotope dilution/surrogate quantification using a 5-7 calibration curve generated from authentic native standards. ACs, SMs and PCs were measured using flow-injection MS/MS (FI-MS/MS). After deconvolution of overlapping isotopic peaks [7], the lipid analytes were quantified relative

to an internal standard. Three blanks and 3 internal reference human serum samples were processed and analyzed with the samples. In addition, a calibration sample was analyzed once every 20 samples to assess instrument stability, and instrument methanol blank samples were run after high concentration calibration samples to assess sample carryover. The method was previously validated at two different spiking levels (n=5) in human plasma.

After primary and secondary validation of the analytical data, further statistical processing of the PCB and metabolite concentration data was performed using the R statistical and programming environment [8].

Results and discussion

PCB congener data indicated that the method successfully measured a large number of congeners even for low volume samples (< 2 mL) from ACHS. Similar numbers of PCBs, 135 and 130, were detected in the 2005-7 and 2014 samples, respectively, indicating no significant detection frequency difference by year or sample size.

In the metabolomic data, 197 of the 219 metabolites were detected in one or more of the 50 samples and 162 were detected in >80% of samples. Principal Component Analysis (PCA) of metabolite concentration levels showed that, whilst there are differences between the samples collected in different years, this factor explains a relatively small proportion of the variability and shows that there is a significant overlap between the cohorts. This indicates that while stability of metabolites in stored samples can be an issue, meaningful metabolomic results could be generated with 10-year archived samples.

Metabolite differences were observed between subjects with low and high serum PCB concentrations (lipid adjusted). Figure 1 shows results from an Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) where subjects were binned into three groups based on their total PCB congener levels. The OPLS-DA models indicated significant separation between the low total PCB samples and the high PCB samples with the model explaining a significant portion of the total variation and passing quality and permutation tests. The highest Variable Importance in Projection (VIP) scores from the O-PLS-DA model created using samples from bin 1 (lowest PCB levels) and bin 3 (highest PCB levels) were observed for omega-3 and omega-6 fatty acids docasohexaenoic acid, cis-4,8,12,15,19-docosapentaenoic acid and cis-4,7,10,13,16-docosapentaenoic acid, essential fatty acid docosatetraenoic acid, phospholipids PC ae C38:0, PC aa C36:0 and PC ae C42:0, and amino acid ornithine.

Kruskal-Wallis analysis identified 8 metabolites as statistically significantly different between samples from the three total PCB bins. In this metabolite list there are 3 fatty acids, 3 lipids and 2 amino acids and a number of them overlap with metabolites with high VIP scores in the O-PLS-DA models. The statistically significant differences were seen between bin 1 and 3 and between bin 2 and 3.

These data provide a preliminary indication that the metabolite profiles in subjects with higher PCB concentrations can be distinguished from those in subjects with lower PCB concentrations Further analysis incorporating subject metadata including race, sex, age and health indicators, and specific congener metabolite correlations, is ongoing. . While these preliminary findings are promising, given the expected biological diversity of this population and the ranges of metabolite concentrations observed, further validation with a larger dataset would provide confirmation.

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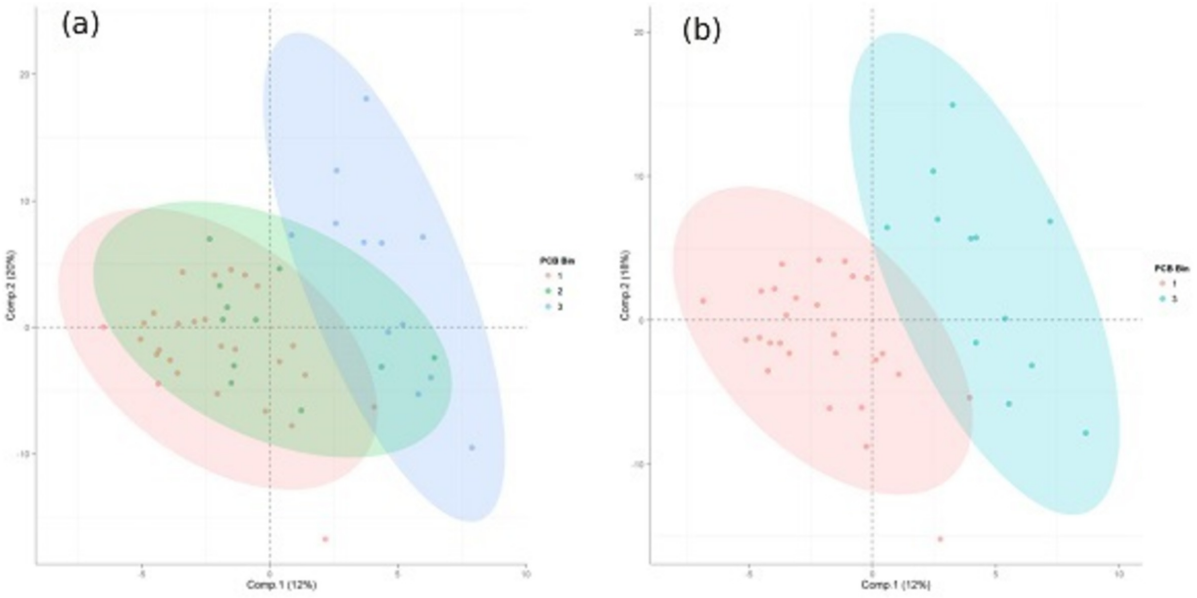

Figure 1. OPLS-DA model of metabolite values created using (binned) total PCB levels. The image on the left shows the model using all bins and on the right the model without bin 2 samples; this model has a Q2 value >0.4, and permutation validation