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INTERACTIONS BETWEEN ENVIRONMENTAL POLLUTION AND NUTRITION-BASED BIOMARKERS OF METABOLIC DISEASE RISK IN RESIDENTS OF ANNISTON, ALABAMA

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Introduction

Cardiovascular and related metabolic disorders are largely caused by genetic (heritable) and environmental factors. Understanding how these factors intersect to determine individual disease risk is a critical challenge in developing personalized approaches to the diagnosis and treatment of the disease. Well-studied "lifestyle-dependent" determinants of increased cardiovascular disease risk include smoking, physical inactivity, and poor nutrition, but emerging data now implicate exposures to persistent environmental pollutants as an important contributor to inter-individual variability in cardiovascular disease risk. Diets that promote obesity and hyperlipidemia are well established to increase cardiovascular disease risk but recent studies point to specific dietary constituents as precursors for metabolites that may increase or protect from cardiovascular disease risk. Indeed, our research using cell culture and animal models broadly supports the paradigm that nutrition is a critical player in the overall vulnerability to environmental stressors [1-4]. These studies support the concept that poor nutrition may exacerbate the toxicity of environmental pollutants whereas healthful nutrition offers a viable modulator that could markedly buffer the body against toxic chemical insult from persistent chlorinated organics such as PCBs. Extrapolation of these studies to investigate toxicant/nutrient interactions in the pathology of human cardiovascular disease will eventually require longitudinal studies to relate environmental exposures and nutritional status to cardiovascular disease risk in suitable populations.

Interestingly, emerging diet-derived biomarkers such as trimethyl n-oxide (TMAO), carnitine, certain fatty acids, and choline have strong positive relationships with heart disease risk, whereas plasma levels of other nutrients, for example plant-derivided carotenoids, sterols and polyphenols are correlated with reduced risk. Quantitating levels of these nutrient biomarkers in individuals with well defined environmental exposures and well documented metabolic disease histories may shed light on why certain people are more or less prone to environmentally induced diseases. It is also conceivable that PCBs and related persistent organic pollutants may increase cardiovascular disease risk by directly altering levels of pro-atherogenic biomarkers such as TMAO [5].

To investigate the importance of toxicant/nutrient interactions in determining overall cardiovascular disease risk, archived blood plasma samples from individuals of the Anniston, AL cohort (ACHS Follow-up Study; ACHS II) [6] have been examined for nutritionally relevant biomarkers of disease risk. Although multiple biomarkers of diet and nutrition are being examined as part of this study, the focus of this abstract is on recent studies of the relationship between TMAO and exposure to dioxin-like PCBs.

Recently, we published that in preclincial models, exposure to dioxin-like PCBs (PCB 126 or PCB 77) can increase circulating levels of TMAO after administration of dietary precursors [5]. Plasma TMAO levels are strongly associated with coronary artery disease risk in humans and may be increased in individuals with diabetes, and kidney disease. Importantly for our study, TMAO is generated from dietary precursors and may therefore be reflective of dietary choices. TMAO is synthesized in the liver by the enzyme flavin-containing monooxygenase 3 (FMO3). The substrate for TMAO generation is trimthylamine (TMA). In humans, the major systemic source of trimethylamine is food containing carnintine and choline, predominantly choline containing glycero- and sphingo- phospholipids. The gut microbiota express lyase enzymes that can cleave the C-N bond in choline and carnitine to generate TMA. In our preclinical studies, dioxin-like PCBs at environmentally relevant levels strongly increase FMO3 mRNA, protein and enzyme activity resulting in amplified increases in TMAO levels after dietary intake of phosphatidylcholine or TMA in comparison to vehicle treated mice [5]. This ongoing collaborative work utilizing the Anniston, AL samples was undertaken to determine if our observations linking PCB exposure to circulating TMAO levels in preclinical models can be translated to human subjects.

We have used HPLC coupled electrospray ionization tandem mass spectrometry methods to quantitate TMAO and related trimethylamine containing metabolites in archived plasma samples. We then examined associations between levels of TMAO and related metabolites, PCB levels, and disease risk. Eventually, we expect that definition of interactions between toxicology, nutrition, and cardiovascular disease risk will allow for a better understanding of the negative impacts of multiple environmental stressors on physiological systems, as well as opportunities for intervention/prevention measures, ultimately leading to improvements in environmental health in afflicted communities.

Materials and methods

For details regarding methods from the preclinical FMO3 study, please refer to Petriello et al., 2016 [5]. For more information related to the ACHS-II methods, please refer to Birnbaum et al., 2016 [6]. The study design and statistical analyses utilized for the human portion are outlined below:

Study Participants:

A total of 359 participants were originally involved in the cross-sectional study. Participants that lacked TMAO levels were excluded for subsequent analyses which ultimately totaled 340. Demographic information, which included age, sex, race, BMI, and various self-reported medical conditions was collected. Brief demographic information is shown in Table 1.

Overview of statistical analyses:

Exploratory analyses included the use of descriptive statistics (e.g., means, standard deviations), were examined as continuous variables and frequencies, and percentages were examined for categorical variables (Table 1), unless otherwise indicated. Results were examined by total population only.

TMAO, TMA and choline were all log transformed to account for non-normality prior to subsequent analysis. To examine associations between TMAO and disease outcomes, univariate logistic regression analysis was performed on selected self-reported medical condition outcomes which included Heart Attack, Heart Failure, Heart Disease, High Blood Pressure, Abnormal Lipid Levels, Diabetes, High Blood Sugar, Stroke, Liver Disease, Kidney Disease, and Hyper/Hypo Thyrodism. A subset of these results related to cardiovascular disease risk factors is included in Table 2. For self-reported medical conditions, “do not knows” were recoded as missing variables. SAS version 9.4 was used for epidemiological analyses and JMP 12 was used for statistical interaction studies.

To initially examine if TMAO levels associate with pollutant body burden (i.e., TMAO can be used as a surrogate biomarker of dioxin-like pollutant exposure), a logistic regression model to predict log (PCB 126-lipid weight) was built examining race, sex, age, BMI, and log (TMAO) (see Table 3).

Plasma analysis of TMAO [5]

Analysis of TMAO, TMA and choline was carried out using a Shimadzu UPLC coupled with an AB Sciex 6500-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. d9-TMAO, d9-TMA and d9-choline were used as internal standards. TMAO, TMA and choline were analyzed using a Primesep 100, 3 μm, 2.1 X 100 mm column (from SIELC) with a flow rate of 0.5 ml/min and column temperature of 30°C. The mobile phase consisted of water with 0.1% TFA as solvent A and acetonitrile as solvent B. Analysis of TMAO, TMA and choline was achieved using an isocratic flow of 80% solvent A and 20% solvent B for 12 min. Sample injection volume was 10 μL. The mass spectrometer was operated in the positive electrospray ionization mode with optimal ion source settings determined by synthetic standards of TMAO, d9-TMAO, TMA, d9-TMA, choline and d9-choline with a declustering potential of 66 V, entrance potential of 10 V, collision energy of 31 V, collision cell exit potential of 6 V, curtain gas of 20 psi, ion spray voltage of 5500 V, ion source gas1/ gas2 of 40 psi and temperature of 550°C. MRM transitions monitored were as follows: 60.1/44.4 for TMA, 69.1/49.2 for d9-TMA, 76/59.1 for TMAO, 85.1/66.0 for d9-TMAO, 104.2/60.1 for choline and 113.1/69.1 for d9-choline.

Results and discussion

Preclincial study examining dioxin-like PCB modulation of FMO3 and TMAO [5]:

Dioxin-like pollutants can upregulate a critical enzyme responsible for TMAO formation, hepatic flavin containing monooxygenase 3 (FMO3), but a link between dioxin-like PCBs, upregulation of FMO3, and increased plasma levels of TMAO has not been previously reported. Thus, we designed multiple animal

studies to examine the impact of PCB exposure on hepatic FMO3 expression and subsequent TMAO levels. We determined that mice exposed acutely to dioxin-like PCBs exhibit increased hepatic FMO3 mRNA, protein, as well as an increase in circulating levels of TMAO following oral administration of its metabolic precursors. Adult male C57BL/6 mice (8 weeks old; 23.8 g average weight) were exposed to 5 µmol PCB 126/kg mouse weight (1.63 mg/kg) via gavage. At 48 h post-PCB exposure, mice were subsequently given a single gavage of phosphatidylcholine dissolved in corn oil. Exposure to 5 µmole/ kg PCB 126 resulted in greater than 100-fold increase in FMO3 mRNA expression, robust induction of FMO3 protein, and a 5-fold increase in TMAO levels compared with vehicle treated mice. We made similar observations in mice exposed to PCB 77 (49.6 mg/kg two times); stable isotope tracer studies revealed increased formation of plasma TMAO from an orally administered precursor trimethylamine (TMA). With this evidence of increased TMAO in mice exposed to dioxin-like PCBs, we next examined if our results could be mirrored in a highly exposed human population.

Preliminary results linking dioxin-like pollutants and TMAO in residents of Anniston, Alabama:
Our preliminary data from the Anniston cohort support our animal studies, i.e., that elevated plasma TMAO levels can be correlated with elevated plasma levels of PCB 126, and future analysis will determine if this trend continues for different environmental pollutants and TEQ values. Further statistical analysis is necessary before determining if specific classes of PCBs or other toxicants identified in this population associate with higher levels of TMAO, or if TMAO can be used as a biomarker of total pollutant body burden.

Targeted analysis of TMAO and related nutrient metabolite biomarkers is complete, and statistical analysis and model building is on-going. Our initial analysis using univariate logistic regression showed crude positive associations between levels of circulating TMAO and the probability of self-reported high blood pressure, diabetes, and high blood sugar (Table 2). After adjusting for race, sex, age and BMI, we found a positive and statistically significant association with serum levels of PCB 126 and TMAO (Table 3).
It is important to stress that while the association between plasma TMAO levels and human coronary artery disease risk is strong and has been observed in several independent studies, the underlying mechanism accounting for this association is presently unclear. While TMAO can accelerate atherosclerosis in mouse models and TMAO apparently has additional effects on blood and vascular cells, other data implicate FMO3 as a determinant of hepatic insulin sensitivity, providing an indirect mechanism for modulation of atherosclerosis and suggesting an alternate hypothesis that TMAO associates with cardiovascular disease risk because it is a biomarker of FMO3 activity. It is also noteworthy that the association between TMAO levels and the cardiovascular disease risk discussed above, and the observations linking TMAO levels to circulating PCB levels in some individuals reported here were made using plasma samples that were collected from fasted individuals or from individuals without controlling for differences in diet. Measurements of acute TMAO production after dietary challenge of fasted individuals with TMA, choline or other FMO3 substrates will be needed to definitively link disease risk, FMO3 activity and exposure to environmental pollutants.

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Table 1. Demographic Information

	Total Population (n=340)
Age (Mean ± SD)	62.66 ± 13.12
Sex	
Male	91 (27.33)
Female	242 (72.67)
Race	
White	162 (48.65)
Black	170 (51.05)
American Indian	1 (0.30)
BMI Category	
Underweight	1 (0.30)
Normal	64 (19.22)
Overweight	96 (28.83)
Obese	171 (51.35)
Missing	1 (0.30)
TMAO	
Mean + SD	6.47 + 8.15
Median (Q1, Q3)	4.26 (2.87,6.97)
Min-Max	0.90 – 86.32
Choline	
Mean + SD	29.80 + 19.14
Median (Q1, Q3)	24.66 (15.22, 38.81)
Min-Max	7.75 – 135.24

Table 2. Univariate logistical regression of disease outcomes with TMAO as the predictor

	High Blood Pressure		Diabetes		High Blood Sugar	
	β-Estimate	P-value	β-Estimate	P-value	β-Estimate	P-Value
TMAO	**0.4342**	**0.0069**	**1.0551**	**<0.0001**	**0.9157**	**<0.0001**

Table 3. Multiple linear regression model for PCB 126.

Term	β-Estimate	P-Value
Intercept	-0.179	0.6812
Race	0.490	<0.0001
Sex	0.320	<0.0001
Age	0.040	<0.0001
BMI	0.027	0.0001
TMAO	**0.156**	**0.0375**

R^2=0.40