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Metabolomics analysis of maternal serum exposed to high air pollution during pregnancy and risk of autism spectrum disorder in offspring

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.110823.

Abstract

Background: Previously, numerous epidemiologic studies reported an association between autism spectrum disorder (ASD) and exposure to air pollution during pregnancy. However, there have been no metabolomics studies investigating the impact of pregnancy pollution exposure to ASD risk in offspring.

Objectives: To identify differences in maternal metabolism that may reflect a biological response to exposure to high air pollution in pregnancies of offspring who later did or did not develop ASD.

Methods: We obtained stored mid-pregnancy serum from 214 mothers who lived in California's Central Valley and experienced the highest levels of air pollution during early pregnancy. We estimated each woman's average traffic-related air pollution exposure (carbon monoxide, nitric oxides, and particulate matter <2.5 μm) during the first trimester using the California Line Source Dispersion Model, version 4 (CALINE4). By utilizing liquid chromatography-high resolution mass spectrometry, we identified the metabolic profiles of maternal serum for 116 mothers with offspring who later developed ASD and 98 control mothers. Partial least squares discriminant analysis (PLS-DA) was employed to select metabolic features associated with air pollution exposure or autism risk in offspring. We also conducted extensive pathway enrichment analysis to elucidate potential ASD-related changes in the metabolome of pregnant women.

Results: We extracted 4022 and 4945 metabolic features from maternal serum samples in hydrophilic interaction (HILIC) chromatography (positive ion mode) and C18 (negative ion mode) columns, respectively. After controlling for potential confounders, we identified 167 and 222 discriminative features (HILIC and C18, respectively). Pathway enrichment analysis to discriminate metabolic features associated with ASD risk indicated various metabolic pathway perturbations linked to the tricarboxylic acid (TCA) cycle and mitochondrial function, including carnitine shuttle, amino acid metabolism, bile acid metabolism, and vitamin A metabolism.

Conclusion: Using high resolution metabolomics, we identified several metabolic pathways disturbed in mothers with ASD offspring among women experiencing high exposure to traffic-related air pollution during pregnancy that were associated with mitochondrial dysfunction. These findings provide us with a better understanding of metabolic disturbances involved in the development of ASD under adverse environmental conditions

Keywords

Metabolomics; Autism spectrum disorder (ASD); Air pollution exposure; Pregnancy; Mitochondria

1. Introduction

Autism spectrum disorder (ASD) is one of the most heterogeneous lifelong neurodevelopmental disabilities that typically begins in early childhood. The prevalence of ASD in the United States increased from 1 out of 150 children in 2000 to 1 out of 59 children in 2014 (Christensen et al., 2018); the etiology of ASD is still not well understood, even though there is no doubt that both genetic and environmental factors account for it. Investigations identified genetic contributions to ASD, from rare to common variants and

copy number variants, with some affecting synaptic genes (Woodbury-Smith and Scherer, 2018). Chromosomal microarray analysis and whole exome sequencing of ASD patients have been helpful (Tammimies et al., 2015); but also made it clear that there is likely no single gene candidate that can explain the phenotypic heterogeneity and variable penetrance of ASD.

A recent study indicated that high developmental activity in the cortical subplate and cerebellum occurs during the second half of gestation and the first three months post-term (Hadders-Algra, 2018). Exposure to environmental risk factors during critical periods like pregnancy has been associated with an increased risk of neurodevelopmental dysfunction in susceptible offspring. Over the past decades, numerous epidemiologic studies have shown positive associations between maternal exposure to air pollution and autism in children (Chun et al., 2019; Costa et al., 2017). However, these studies also reported inconsistent results, especially in trimesters of exposure and types of air pollutants (Chun et al., 2019). Additionally, to date, there have been no metabolomic studies of maternal serum exploring associations of prenatal exposure to air pollution with the risk of ASD development or studies that assessed differences in maternal metabolism that may reflect biologic responses to high exposure levels in pregnancies of children who later did or did not develop autism.

Metabolomics measures small molecule metabolites that reflect the underlying biochemical activity of cells and tissues in biofluids and also capture pathological and physiological alterations due to underlying conditions or environmental factors (Glinton and Elsea, 2019). Recently, an increasing number of metabolomic studies have attempted to identify ASD-related specific metabolites or metabolic perturbations in ASD using different analytical platforms in a variety of biospecimens in autistic children and adults. Most studies have been conducted by using urine as an easily accessible biological fluid (Bitar et al., 2018; Diémé et al., 2015; Gevi et al., 2016; Lussu et al., 2017) and reported changes in metabolites indicating perturbations in carbohydrate, amino acid, and oxidative stress pathways, as well as changes associated with gut microflora modifications. A few studies performed on blood identified altered metabolic profiles compatible with fatty acid metabolism, oxidative stress, and mitochondrial dysfunction (Orozco et al., 2019; Wang et al., 2016). A study using ASD brain tissue confirmed some of the altered metabolic signals found in the urine and blood of ASD patients (Kurochkin et al., 2019). Thus, to date, studies of the pregnancy environment of autistic children are generally lacking. Unlike previous epidemiologic studies showing associations with autism for high air pollution exposure, here we are aiming to not only elucidate whether this harmful environmental factor impacts the metabolome in pregnancy but whether the impact is different in those who later develop autism, thus suggesting that the maternal biologic response to the exposure contributes to the risk of ASD in offspring.

The Central Valley region in California has some of the worst air quality in the USA above the levels of state and federal air quality standards and its residents report high rates of emergency department visits and hospitalizations due to a variety of diseases from respiratory to cardiovascular disorders (Billings et al., 2016; Cisneros et al., 2017; Meng et al., 2010). We performed an untargeted metabolomics study among women living in the Central Valley of California, an agricultural region in which residents are at high risk of being exposed to pesticides and traffic-related air pollution, to investigate whether there are

discernable differences in the metabolic profiles between mothers with offspring who were later diagnosed with ASD and mothers of healthy controls under the same conditions of exposure to high levels of air pollution during pregnancy.

2. Methods

2.1. Study population

We randomly selected mothers of children born between 2005 and 2010 for a nested case-control study of autism using California birth records and matched controls in a 1:10 ratio by sex and birth year. All mothers eligible to contribute to the present study lived in the California Central Valley region according to their residential address recorded on the birth certificate (n = 1466). The offspring also had to be born at a gestational age between 21 and 46 weeks and have a birth weight between 500 g and 6800 g (n = 1433). California's Central Valley is one of the regions with the greatest pesticide use. For this reason, we limited our analyses to mothers who according to our geographic information system (GIS) and state-mandated pesticide use report system had not been exposed to suspected neurotoxic pesticides (organophosphates, pyrethroids, glyphosates, fungicides, or neonicotinoids) (Rull and Ritz, 2003). Among the women randomly selected from birth certificates, we estimate percentiles of air pollution exposure and defined as highly exposed during early pregnancy those for whom three modeled traffic-related air pollutants (carbon monoxide (CO), nitrogen oxides (NO_x), and particulate matter with aerodynamic diameter ≤ 2.5 μm ($PM_{2.5}$)) fell at or above the 75th percentile of exposures in controls using a modified version of the California LINE Source Dispersion Model Version 4 (CALINE4) model (Yan et al., 2019).

Based on air pollution estimations, we included 116 children with autism and 98 autism-free control births with high levels of traffic- related air pollution in early and mid-pregnancy for our study (Fig. S1, Supplementary Material). We utilized maternal serum samples taken mid-pregnancy (around 16th week of gestation) as part of the California Prenatal Screening Program (CPSP) in which about 74% of all California women participate. We collected the mother-child pair's demographic and other information such as maternal age, race/ethnicity, education, parity, child sex, and birth year from birth records. Other potentially confounding variables such as maternal smoking, body mass index (BMI), or diet were not available for the span of the study period.

2.2. Air pollution exposure assessment

We used OpenSource geocoding software to geocode residential addresses from birth certificates (Goldberg et al., 2008). We applied the CALINE4 model to estimate each woman's average air pollutant exposure in the first trimester until mid-pregnancy. Required input data for the prediction process included roadway geometry, hourly surface meteorological parameters (wind speed, wind direction, temperature stability class, and mixing heights), traffic counts, and emission factors. CALINE4 is a tool that models average exposures to CO, NO_x, and $PM_{2.5}$ that sources within 1500 m of residential locations generate. Emission factors for CO, NO_x, and $PM_{2.5}$, as well as year, and season (winter and summer) were obtained from California Air Resources Board's emissions factor databases (EMFAC2011) (California Air Resources Board, 2013). CALINE4 predictions do

not incorporate background levels of pollutants, thus, they solely represent the contributions from local traffic emissions in this study (Table 1S, Supplementary Material).

2.3. Autism diagnosis

For the identification of autism cases, we relied on the Department of Developmental Services (DDS) records. The agency is dedicated to providing services to individuals with developmental disabilities in California. DDS services are provided based on advice from pediatricians, teachers, or other sources. An estimated 75–80% of all California children with autism are included in the DDS records. The DDS Client Development Evaluation Report (CDER) is the assessment instrument that the DDS utilizes to collect data on diagnostic characteristics. The CDER recorded diagnoses are based on the DSM-IV criteria using US standard morbidity ICD-9-CM codes. ASD is recorded as an autistic disorder based on the ICD-9-CM code 299.0 according to the most recent CDER record in our study period.

2.4. High-resolution metabolomics (HRM)

Metabolomics analysis was performed using maternal serum samples stored by the California Biobank, which collects these from mothers who participated in the California Prenatal Screening Program. High- resolution metabolomics (HRM) was conducted following established protocols (Walker et al., 2019; Yan et al., 2019). Serum samples were prepared in batches of 40 samples by treating 65 μL of thawed serum with 130 μL of LC-MS grade acetonitrile containing a series of ^{13}C internal standards. After a 30-min equilibration on ice and centrifuging for 10 min to precipitate proteins, the supernatant was transferred to an autosampler vial and maintained at 4 °C until analysis (<24h). To evaluate system performance, we used two quality control pooled serum reference samples. Our first QC sample includes NIST 1950 which was analyzed twice, once before batch 1 and once after batch 25. The second QC sample (Q-Std) was commercially purchased serum that was pooled from an unknown number of males and females without demographic information (Equitech-Bio, Inc., Kerrville, Texas). Q-Std was analyzed at the beginning and end of each analytical batch of 40 samples for normalization, control of background noise, batch evaluation, and post hoc quantification. Each sample was analyzed in triplicate using a dual column, dual polarity approach that includes hydrophilic interaction liquid chromatography (HILIC) with positive electron spray ionization (ESI) and C18 hydrophobic reversed-phase chromatography with negative ESI (Dionex Ultimate 3000, Q-Exactive HF, Thermo Scientific).

The high-resolution mass spectrometer was operated in full scan mode at 120,000 resolution and mass-to-charge ratio (m/z) range 85–1275. Following instrument analyses of all samples, raw data files were processed for peak extraction and quantification of ion intensities using apL CMS with modifications by xMSanalyzer. Detected signals (referred to as metabolic features) were uniquely defined by their m/z ratio, retention time and ion intensity. Prior to data analysis, *m/z* feature intensities were batch corrected using ComBat (Johnson and Rabinovic, 2007). Only metabolic features detected in >50% of all serum samples with median coefficients of variation among technical replicates <30% and Pearson correlation >0.7 were included in further analyses. Following quality assessment,

feature intensities were summarized using the median intensity for technical replicates. Metabolomic data was then filtered to keep only features present in at least 80% of one comparison group and >50% of all samples together. Missing values were imputed using one-half of the lowest signal detected for that feature across all samples. Feature intensities were log2 transformed before analyses.

2.5. Statistical analysis

To control for potential confounders, we used residuals of intensities derived from linear regression against potential confounders including maternal age, maternal race/ethnicity, and maternal education. We performed partial least squares discriminant analysis (PLS-DA) that is a multivariate dimensionality-reduction tool and allows us to maximize the covariance between intensities of metabolic features and autism or air pollution (Ghosh et al., 2020). We selected features with a Variable Importance in Projection (VIP) scores ≥2 (Le Cao, et al, 2009). Fold change was calculated as the ratio of raw intensities between ASD case and control groups. To evaluate the performance of selected features, we conducted 10-fold cross-validation tests utilizing the support vector machine and calculated the classification accuracy of the selected features. All feature selection approaches were implemented with the R package mix Omics v6.3.1.

2.6. Pathway analysis and annotation

Annotation of discriminatory features selected by PLS-DA was performed using xMSannotator (Uppal et al., 2017). Accurate mass *m/z* for adducts formed under positive/negative ESI mode was matched to the Human Metabolome Database (HMDB), Kyoto Encyclopedia of Genes and Genomes (KEGG), and LipidMaps with a mass error threshold of 10 ppm. xMSannotator also takes into consideration the correlation of intensities and retention time, and assigns confidence scores based on a multilevel scoring algorithm which ensures the accuracy of annotation. When possible, metabolite identifications were confirmed by matching the accurate mass *m/z* and retention time to authentic chemical standards using a 5 ppm and 15-s window (Go et al., 2015)

To identify enriched metabolic pathways comparing ASD case and control groups, mummichog version 2 (http://mummichog-2.appspot.com/) was utilized to conduct pathway enrichment analysis using *m/z* features with VIP scores ≥ 2. Mummichog is an algorithm designed for high-resolution liquid chromatography-mass spectrometry. The enrichment pattern of the real metabolite subsets is compared to the null distribution on known metabolic reactions and pathways and then mummichog can predict significantly different pathways and network modules from untargeted metabolomics data. The p-value calculated by mummichog are gamma-adjusted p-values based on permutation tests as described by Li et al. (2013). Although the tentative annotation results in mummichog may include false positives (single metabolites), the enriched pathways inferred by the algorithm have been proven to be valid and to reflect the real biological activity. All annotated metabolites required the presence of the proton adduct (M+H and M-H for positive and negative mode respectively) to reduce the false positive match rate. Only enriched pathways that contained at least 3 overlapping metabolites were considered for interpretation.

3. Results

Demographics of the 214 participants are presented in Table 1. Most case and control mothers were young, with low education levels, and of Hispanic ethnicity and – as we matched by sex of autism cases - more had male offspring in this study population. In total, we identified 14,555 features (6139 in HILIC column and 8416 in C18 column) and after filtering for missing values, 8967 features remained (4022 in HILIC column and 4945 in C18 column). We identified 167 HILIC and 222 C18 metabolic features in maternal serum potentially associated with ASD development from both the HILIC and C18 column respectively (Fig. 1) using PLS-DA and VIP score greater than 2 after adjusting for maternal age, ethnicity and education. The balanced classification rate derived from 10-fold CV showed that both sets of discriminatory features effectively separate the classes (81.3% and 83.0% for HILIC and C18, respectively). Annotation of PLS-DA discriminatory features included 187 *m/z* features matching to one or more unique metabolites with medium or high confidence scores. Because compounds were present in the databases used for annotation that share the same chemical formula but different structure, we confirmed the annotation results when possible by matching the retention time and *m/z* to a database of metabolites confirmed using reference.

Four discriminating metabolites between mothers with ASD cases and mothers with controls were confirmed (level 1 confidence); hypotaurine, phenylalanine, 3-hydroxybutanic acid, and urate. The chemical identities and fold-change are shown in Table 2. Among these metabolites, hypotaurine and urate were positively associated, while the two other metabolites were negatively associated with ASD status in offspring. We conducted pathway enrichment analysis using mummichog and identified 17 metabolic pathways associated with ASD in offspring at $p < 0.05$ (Fig. 2). Tentative annotation results of metabolites from each pathway are presented in Supplemental Table S2.1–S2.17. As observed previously, when studying metabolic profiles in air pollution exposed and unexposed pregnancies (Yan et al., 2019), we again found perturbations in the leukotriene pathway, fatty acid metabolism and activation, butanoate metabolism, fructose, and mannose metabolism, and glycolysis and gluconeogenesis. We also found pathway perturbation that we had previously seen when comparing maternal pregnancy serum of mothers whose child later did or did not develop autism, specifically in bile acid synthesis and N-glycan degradation pathways (Ritz et al., 2020). Finally, we observed differences in several additional metabolic pathways associated with mitochondrial dysfunction including vitamin A metabolism, carnitine shuttle, aspartate and asparagine metabolism, propanoate metabolism, galactose metabolism, pyruvate metabolism, pentose phosphate pathway, and beta-alanine metabolism.

4. Discussion

In the present study, we investigated the metabolic profiles of mothers with children who later developed ASD compared to mothers with control children under high exposure to traffic-related air pollution during pregnancy. We found significant metabolic differences between mothers with ASD children and mother with control children. By conducting pathway enrichment analysis using mummichog, 17 metabolic pathways perturbations associated with ASD risk in offspring were identified. Of interest,13 pathways among

the altered pathways observed in mothers with ASD children are potentially related to mitochondrial dysfunction.

Altered amino acid pathways involving valine, lysine, phenylalanine, taurine, asparagine, and alanine are identified in maternal serum with ASD cases compared with controls. In mothers with ASD children, valine intermediate metabolite (3-methyl-2-oxobutyric acid), isoleucine metabolite (3-methyl-2-oxopentanoate), lysine, phenylalanine, and asparagine were lower than in controls, while serum hypotaurine levels were increased. Branched-chain amino acids, including leucine, isoleucine, and valine have immunomodulatory functions and exhibit anti- inflammatory effects on peripheral immune cells as well as on microglial cells (De Simone et al., 2013). Other amino acids such as taurine, threonine, lysine, and histidine also play a role as immune neuromodulators in ASD under conditions of oxidative stress (Zheng et al., 2017). However, a majority of studies reporting on amino acid metabolism in ASD have shown conflicting results (Diémé et al., 2015; Gevi et al., 2016; Lussu et al., 2017; Zheng et al., 2017). A variety of factors, such as different ages, a small number of cases, and the nutrition status of the mother may contribute to contradictory results. Despite the inconsistency, impairment of amino acid metabolism could be one potential underlying mechanism for the development of ASD. To date, increasing evidence suggests that the association between air pollution exposure and ASD risk could be explained by oxidative stress, altered lipid metabolism, and neuroinflammation as possible underlying mechanisms. The perturbation of amino acid metabolism is also associated with traffic-related air pollution (Walker et al., 2019). Thus, the metabolic perturbation of amino acids in mothers with ASD offspring may be the overlapping pathology in ASD-related to air pollution exposures.

We also found a positive association between higher levels of maternal uric acid and ASD risk in the offspring. High uric acid concentrations are regarded to be crucial for driving monosodium urate crystallization and can promote the production of inflammatory mediators. These proinflammatory mediators can damage the placenta. Placental inflammation has been linked to fetal neurodevelopment as well as adverse birth outcomes (Brien et al., 2017). Our result is consistent with other studies that showed increased levels of uric acid in patients with ASD (Page and Coleman, 2000; Vanwong et al., 2017). Additionally, a study reported that a higher incidence of hyperuricemia was associated with long-term exposure to ambient air pollution including PM_{10}, SO_2, and NO_2 among traffic police officers (Tang et al., 2019). Thus, exposure to high air pollution during pregnancy may contribute to uric acid dysregulation and possibly the development of ASD in offspring.

In our study, three of the features found to be enriched in the carnitine shuttle were annotated as L-carnitine, alpha-linolenyl carnitine, and tetracosapentaenoyl carnitine. Among these metabolites, the mothers of ASD children had lower L-carnitine, but higher levels of the other two metabolites compared to controls. Both are derivative metabolites of L-carnitine, respectively. Carnitine plays a crucial role in transporting long-chain fatty acids across the mitochondrial membrane for β-oxidation and energy production (Demarquoy and Demarquoy, 2019). Consistent with our results, a study described a 66% decrease in carnitine levels in autistic children related to gastrointestinal symptoms (Mostafa and

Al-Ayadhi, 2015). Moreover, defective synthesis or transport of L-carnitine has also been observed in ASD (Bankaitis and Xie, 2019). Overall, perturbation of the carnitine shuttle related metabolism further supports a role for mitochondrial dysfunction in ASD. However, it remains to be determined whether this is triggered by an environmental stressor such as air pollution.

Additional metabolic differences between mothers of ASD cases versus controls suggest alterations in cholesterol and bile synthesis pathways. These findings support our earlier observation that linked the same pathways to ASD in an independent set of ASD cases and controls, all of whom were unexposed to both air pollution and pesticides (Ritz et al., 2020). Cholesterol is an essential component of neuronal and glial membranes in the brain and excess cholesterol is converted into oxysterol through auto- or enzymatic oxidation to maintain brain cholesterol homeostasis (Zmysłowski and Szterk, 2019). A high concentration of oxysterols as a consequence of neuroinflammation associated with a brain injury can promote neuron dysfunction and enhance neuronal excitotoxicity (Ma et al., 2010). There has been growing evidence about the involvement of cholesterol dysregulation and bile acid synthesis in neurodegenerative disorders (Ritz et al., 2020; Zmysłowski and Szterk, 2019). We found unique differences of oxysterols related to bile acid synthesis in mothers with ASD cases versus controls. 7α-Hydroxy-3-oxo-4-cholestenoate (7α–OH–4-CA) was increased, but other metabolites, 7α,25-dihydroxy-4-cholesten-3-one, and 5 β-cholestane-3 α,7α,12α, 27, 27-pentaol were decreased in mothers with ASD cases compared to controls. However, there has been only one study investigating the relationships between ASD and oxysterols. 24-hydroxycholesterol levels were negatively correlated with age in autistic children and lower levels of 7α-OHC and 25-OHC were observed in autistic children compared to healthy controls (Grayaa et al., 2018).

Alterations in bile acid-related metabolites include high levels of taurine and glycocholate, and low levels of chenodeoxycholate in mothers with ASD offspring compared to controls. Cholate and chenodeoxycholate are synthesized from cholesterol as primary bile acids by a series of oxidative transformations in the liver (Chiang and Ferrell, 2018). They are further modified by conjugation of a glycine or taurine, producing glycochenodeoxycholate, taurochendeoxycholate, glycocholate and taurocholate. A mouse model of autism revealed decreased bile acid synthesis and deficient bile acid signaling in the ileum (Golubeva et al., 2017). This result and our previous results (Ritz et al., 2020) suggest that disturbances in the pathway underlying the microbiota-gut-brain axis may result in ASD and our study also showed the dysregulation of bile acid synthesis in mothers with ASD offspring. However, further investigations are needed to determine whether there is also any relationship between bile acid metabolism and air pollution, affecting those who later develop ASD differently.

Moreover, we identified perturbations in fatty acid metabolism and enrichment in the leukotriene (LT) pathways that we had previously already observed as a signal in air pollution exposed vs unexposed pregnancies (Yan et al., 2019). We found positive associations between arachidonic acid and risk of ASD in offspring. In contrast, 20-trihydroxy-leukotriene-B4 and 20-COOH-leukotriene E_4 were decreased in mothers of ASD cases compared to controls. Arachidonic acid is a key inflammatory mediator and excess liberation of arachidonic acid and its metabolite LTs responding to oxidative stress

may induce the propagation of inflammatory processes (Balboa and Balsinde, 2006). Intrahepatic ω-oxidation is regarded as a major degradative and inactivating pathway for the cysteinyl LT metabolites, LTE_4 as well as LTB_4 (Orning et al., 1987). Hepatocellular ω-oxidation of LTs by monooxygenase results in hydroxylated metabolites followed by the production of ω-carboxylated LTs (20-COOH- LTB_4, 20-trihydroxy-LTB_4, and 20-COOH-LTE_4) (Orning et al., 1987). Thus, lower levels of ω-carboxylated LTs may reflect, in part, decreased degradation of biologically active LTs. However, other studies have instead reported increased levels of LT and prostaglandin metabolites in ASD patients (El-Ansary and Al-Ayadhi, 2012) and in air pollution exposed subjects (Yan et al., 2019). Although the causes for the reverse levels are not clear, disrupted LT pathways in pregnant women exposed to high air pollution may affect the risk of ASD development in offspring.

Vitamins and minerals play an irreplaceable role in a variety of coenzyme systems and may be responsible for the activation of enzymes that synthesize neurotransmitters in the brain (Guo et al., 2018). Adequate vitamin A nutrition at delivery and during pregnancy has beneficial effects on the offspring's neurodevelopment (Chen et al., 2009). Five specific features in the metabolic profile were identified as belonging to the vitamin A metabolism in this study. Mothers with ASD offspring had lower levels of 4 retinol derivatives, 4-hydroxy-all-trans-retinyl acetate, all-trans-5,6-epoxyretinoic acid, 11-cis-Retinyl palmitate, and 13,14-dihydroxy-retinol and higher levels of 9-cis-beta-carotene compared to the controls. Although 9-cis-beta-carotene is a precursor of retinol, it is considered to be biologically inefficient in the retinol pathway. In contrast, lower levels of other 4 retinol metabolites that are bioactive metabolites of vitamin A may be associated with ASD risk. Recently, vitamin A has been suggested to enable the redox activation of protein kinase Cδ (PCKδ) with cytochrome. The PCKδ signaling system, comprising PKCδ, the adapter protein p66Shc, cytochrome c, and retinol positively regulates the conversion of pyruvate to acetyl-coenzyme A (CoA) by the pyruvate dehydrogenase enzyme (Kim and Hammerling, 2020). Vitamin A plays a key role in glycolytic energy generation in the tricarboxylic acid (TCA) cycle. Thus, we can speculate that perturbation of vitamin A metabolism is linked to mitochondrial dysfunction in ASD. In terms of air pollution, a protective role of anti-oxidant supplementation on air pollutant toxicity has been reported, suggesting that antioxidant supplementation modifies the pulmonary response to exposure to photo-oxidants such as ozone or nitrogen dioxide (NO_2) (Romieu et al., 2008). However, only a few studies have implicated perturbation of the vitamin A metabolism in those exposed to air pollution. Future studies may lead to a better understanding of vitamin A metabolism associated with ASD risk and air pollution.

Collectively, perturbations of most of the metabolic pathways we found are potentially related to mitochondrial dysfunction pathways. An increasing body of evidence indicates that mitochondrial dysfunction is one of the plausible underlying mechanisms contributing to the development of ASD (Hollis et al., 2017). The mitochondrial metabolome contributes to bioactive metabolites for over 90% of the whole cellular metabolome (Hu et al., 2020). In particular, neurons are the highest energy-demanding cells and more than 90% of cellular oxygen is consumed by the mitochondria to produce ATP via oxidative phosphorylation (Berndt and Holzhütter, 2013). Thus, any form of mitochondrial dysfunction related pathways such as a complex array of changes in fatty acid, glucose, and amino acid

intermediates may lead to neurological disorders. In the present study, fatty acid metabolism, carnitine shuttle, amino acid metabolism, carbohydrate metabolism, bile acid metabolism, and vitamin A metabolism all produce the metabolic intermediates that are directly or indirectly related to the mitochondrial functional network (Fig. 3).

Metabolomic studies can provide valuable information about complex pathways involving mitochondria. However, it remains to be determined how mitochondrial dysfunction that should be common to all nucleated cells can result in tissue-or organ-specific diseases. Interestingly, mothers with ASD offspring showed higher levels of urate and lower levels of vitamin A metabolites compared to controls under conditions of high air pollution exposure during pregnancy. These findings may reflect that mothers of ASD children have lower anti-oxidant activity and also are more vulnerable to a biological or environmental condition that increases oxidative stress and/or mitochondrial dysfunction. Air pollution is known to increase oxidative stress and may result in worse mitochondrial dysfunction in mothers of ASD offspring.

Our study has some limitations. Participants in our study were all exposed to high traffic-related air pollution. Although all women were exposed to possibly harmful air pollution levels, in controls this did not lead to the development of ASD, possibly due to other factors that govern individual susceptibilities, such as genetics, immunological function, and nutritional status, none of which we had data for. We also need to take into account that our specimen are from mothers during pregnancy and we do not have offspring metabolomics to explore pathways in ASD cases versus non-cases. However, maternal metabolism during pregnancy is an important predictor for the fetal/placental interface and for development. Also, we annotated features we detected via computational approaches without being able to confirm all annotations by comparison to authentic standards, which may result in inaccurate identifications. By incorporating pathway and network analysis with mummichog, we potentially reduced false positive annotations; however, there may still be incorrect matches that could introduce errors. Besides, because a metabolic pathway is a complex array of enzymes and cofactors and can reflect dynamic interactions between the host and the environment, the role of some metabolic intermediates has not been well documented for each pathway. Finally, we had a limited number of covariates available that we derived from birth certificates. The discriminative metabolic features identified in our study may be due to unaccounted for confounding from dietary, environmental, or occupational exposures, or genetic factors for which we did not have information. However, by controlling for maternal age, maternal race/ ethnicity, and maternal education, we minimized or at least partially addressed potential confounding. Nevertheless, uncontrolled or residual confounding is possible.

5. Conclusions

We demonstrated that several metabolic pathways are disturbed in mothers with children who developed ASD compared to mothers without ASD offspring among women who were all highly exposed to traffic-related air pollution during pregnancy using HRM. Interestingly, alterations in metabolic pathways involving the TCA cycle and mitochondrial function were observed in mothers with ASD offspring. Thus, increasing our understanding of

mitochondrial dysfunction related to ASD under adverse environmental conditions may inform about ASD risk in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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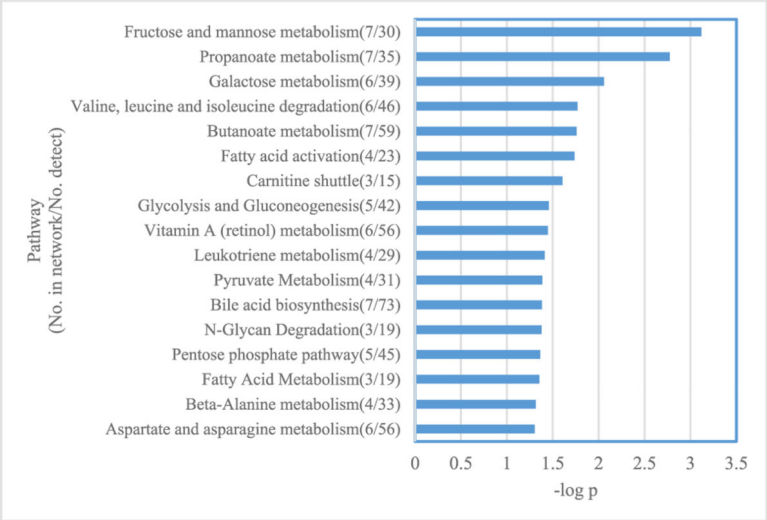

Fig. 1.

Metabolic pathways associated with ASD using HILIC with positive ESI and C18 with negative ESI. HILIC: hydrophilic interaction liquid chromatography; ESI: electron spray ionization.

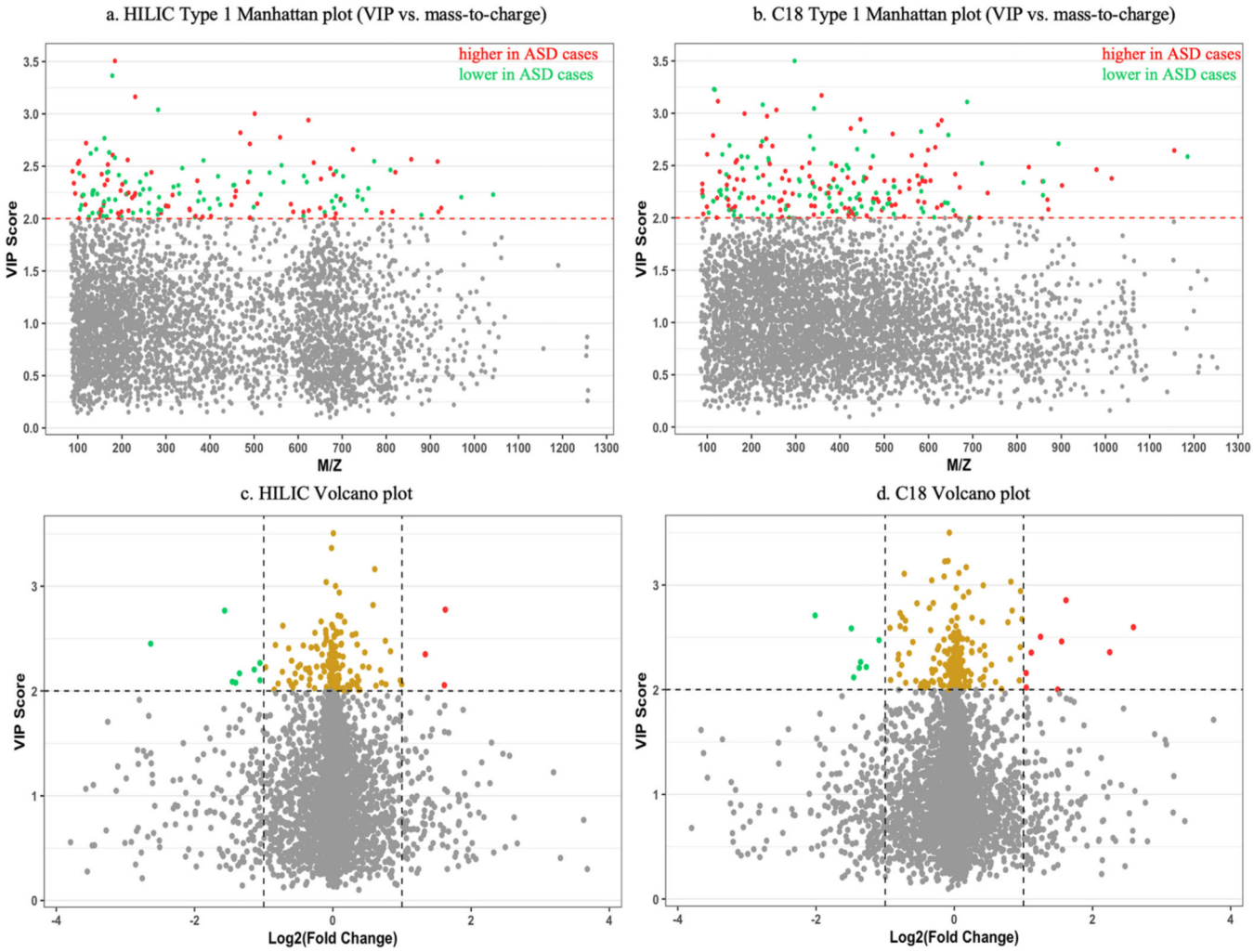

Fig. 2.

Identification of metabolic features associated with ASD risk in offspring during pregnancy. A) Type 1 Manhattan plot for features in the HILIC column (positive ion mode), VIP score vs *m/z*. 167 *m/z* features were found above the VIP≥2 threshold. Red dots indicate features that were increased, and green dots features that were decreased in maternal mid-pregnancy serum of offspring with autism; B) Type 1 Manhattan plot for features in the C18 column (negative ion mode), VIP score vs *m/z*. 222 *m/z* features were found above the VIP≥2 threshold. C) Volcano plot for features in the HILIC column; D) Volcano plot for features in the C18 column. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

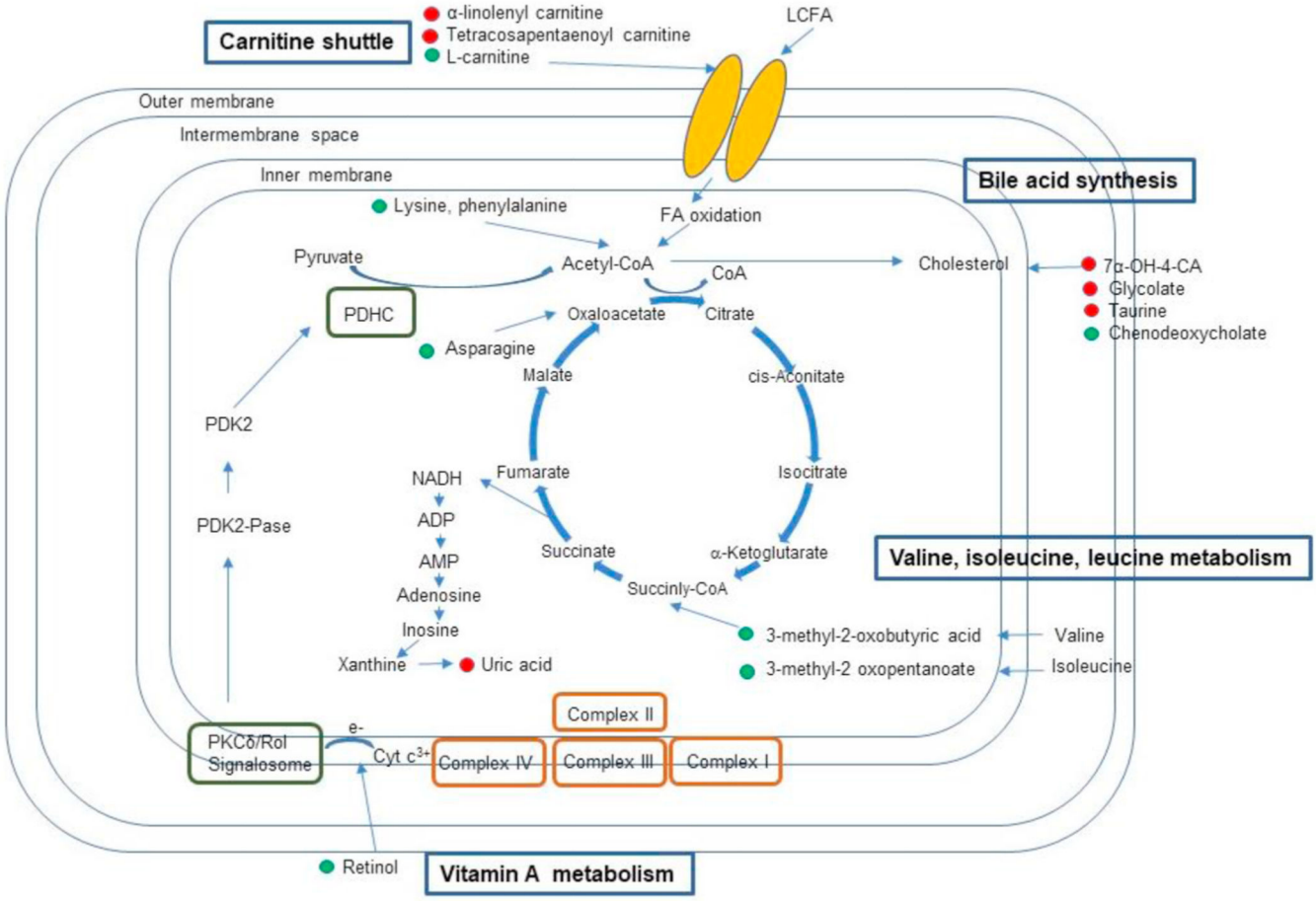

Fig. 3.

Altered metabolic pathways observed in mothers with ASD offspring under exposure to high levels of air pollution during pregnancy. Red circles represent higher metabolite levels, while green circles indicate lower metabolite levels. ADP: adenosine diphosphate; AMP: adenosine monophosphate; LCFA: long-chain fatty acids; NADH: nicotinamide adenine dinucleotide; PKCδ: protein kinase Cδ; Rol: retinol complex; PDHC: pyruvate dehydrogenase; PDK2: pyruvate dehydrogenase kinase 2; PDK2-Pase: pyruvate dehydrogenase kinase 2-phosphatase; Cyt c^{3+}: cytochrome C^{3+}; TCA: tricarboxylic acid cycle; 7α–OH–4-CA: 7α-Hydroxy-3-oxo-4- cholestenoate.

Table 1

Demographic characteristics of mothers and children.

	ASD (n = 116)		Control (n= 98)	
	n	**%**	**n**	**%**
Maternal age at time of pregnancy (years)				
<= 18	8	6.9	18	18.4
19–25	45	38.8	42	42.9
26–30	35	30.2	24	24.5
>30	28	24.1	14	14.3
Maternal race/ethnicity				
Non-Hispanic White	31	26.7	11	11.2
Hispanic	63	54.3	70	71.4
Others (African American/Black, Asian)	22	18.9	17	17.4
Maternal education				
Less than 12th grade	31	26.7	47	48
High school graduate or equivalent	37	31.9	24	24.5
Some college	37	31.9	20	20.4
College or more	11	9.5	7	7.1
Mother born in the US				
Yes	88	75.9	61	62.2
No	27	23.3	37	37.8
Missing	1	0.86	0	0
Preterm birth				
Yes	16	13.8	12	12.2
No	100	86.2	86	87.8
Child sex				
Male	102	87.9	79	80.8
Female	14	12.1	19	19.2

Table 2

Maternal mid-pregnancy serum metabolites with confirmed[a] chemical identity.

m/z	RT (s)	Adduct Form	Metabolite	Fold Change (Log2)	Column
110.027	76.8	M + H	Hypotaurine	0.097186946	HILIC
166.086	51.6	M + H	Phenylalanine	− 0.025956396	HILIC
103.04	29.9	M-H	3-hydroxybutanoic acid	− 0.196530973	C18
167.0211	23.2	M-H	Urate	0.251494418	C18

[a]Chemical identification 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.