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Low-level maternal exposure to nicotine associates with significant metabolic perturbations in second-trimester amniotic fluid

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ABSTRACT

Decades of public health research have documented that smoking in pregnancy poses significant health risks to both mother and child. More recent studies have shown that even passive maternal exposure to secondhand smoke associates with negative birth outcomes. However, the mechanisms linking exposure to outcomes have remained obscure. As a first step toward defining the metabolic consequence of low-level nicotine exposure on fetal development, we conducted an untargeted metabolomic analysis of 81 paired samples of maternal serum and amniotic fluid collected from karyotypically normal pregnancies in the second trimester. By comparing the *m/z* and retention times of our mass spectral features with confirmed standards, we identified cotinine, a nicotine derivative, and used the calculated cotinine concentrations to classify our maternal serum samples into exposure groups using previously defined cut-offs. We found that cotinine levels consistent with low-level maternal exposure to nicotine associated with distinct metabolic perturbations, particularly in amniotic fluid. In fact, the metabolic effects in amniotic fluid of ostensibly low-level exposed mothers showed greater overlap with perturbations previously observed in the sera of adult smokers than did the perturbations observed in the corresponding maternal sera. Dysregulated fetal pathways included aspartate and asparagine metabolism, pyrimidine metabolism, and metabolism of other amino acids. We also observed a strong negative association between level of maternal serum cotinine and acetylated polyamines in the amniotic fluid. Combined, these results confirm that low-level maternal nicotine exposure, indicated by a maternal serum cotinine level of 2–10 ng/mL, is associated with striking metabolic consequences in the fetal compartment, and that the affected pathways overlap those perturbed in the sera of adult smokers.

1. Introduction

The link between maternal smoking and negative birth outcomes, including small-for-gestational age birth, preterm delivery, stillbirth, and sudden infant death syndrome (SIDS) has been well documented over past decades by careful epidemiological studies (Cnattingius, 2004; Rogers, 2009). Increased public awareness of the dangers of prenatal smoke exposure has led to a marked drop in the rate of maternal smoking in the United States (US) (Curtin and Matthews, 2016). However, the percentage of pregnant women who are passively exposed to secondhand smoke, also known as environmental tobacco smoke, remains high. In one large study of exposure patterns in the US, 30% of participating women indicated that they were exposed to secondhand smoke just before or during pregnancy (Anderka et al., 2010).

Abbreviations: SIDS, Sudden Infant Death Syndrome; LC-MS, liquid chromatography-mass spectrometry; *m/z*, mass/charge; PCA, principal component analysis; RT, retention time; KEGG, Kyoto Encyclopedia for Genes and Genomes; MS/MS, tandem mass spectrometry; PLSDA, partial least squares discriminant analysis; VIP, variable importance in projection; HCA, hierarchical cluster analysis; MS, maternal serum; AF, amniotic fluid; IQR, interquartile range; DoDSR, Department of Defense Serum Repository; AMP, adenosine monophosphate; DiAcSpm, N1,N12-diacetylspermine; HMDB, Human Metabolome Database; CV, cross-validation

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The full impact of low-level maternal smoke exposure on birth outcomes remains poorly understood, but a growing literature documents that the consequences of passive smoke exposure overlap those seen with active maternal smoking. Specifically, studies have associated maternal secondhand smoke exposure with low infant birth weight and congenital anomalies (Salmasi et al., 2010), increased risk of stillbirth, small infant head circumference (Crane et al., 2011), and SIDS (U.S. Department of Health and Human Services, 2006).

Prenatal exposure to tobacco smoke can be assessed in different ways. Historically, researchers simply asked mothers about exposure status; unfortunately, this approach was fraught with complications, especially when exposures were low or indirect (Polanska et al., 2007). A more objective approach now available involves quantifying biomarkers of tobacco exposure directly in biological samples. One such marker is nicotine, the primary addictive component in tobacco smoke. Once absorbed into the blood stream, nicotine is quickly metabolized into cotinine, which has a half-life in blood of close to 16 h. Because of its longer half-life, cotinine is often preferred over nicotine as a biomarker for tobacco exposure (Benowitz et al., 2009). Cotinine has been reliably detected at high levels (> 10 ng/mL) in serum samples from smokers, at low levels (2–10 ng/mL) in serum samples from "light" smokers or those passively exposed to secondhand smoke, and at baseline levels (< 2 ng/mL) in samples from individuals with no known smoke exposure (Hanke et al., 2004; Florescu et al., 2009).

Prior studies of both actively smoking (Luck et al., 1985) and passively exposed (Jauniaux et al., 1999) pregnant women document that nicotine and/or cotinine not only pass into the fetal compartment (Jordanov, 1990) but concentrate there. Whether this pattern reflects active concentration of nicotine metabolites across the placenta, or a lower turnover rate in the fetal compartment, remains unknown. Either way, this observation raises the alarming possibility that for any given level of maternal nicotine exposure, the fetal exposure, and therefore the fetal metabolic consequences, may be compounded.

To test this hypothesis, we conducted high-resolution untargeted metabolomic analysis of 81 pairs of maternal serum and amniotic fluid samples collected from women in the second trimester of pregnancy. During most of the second trimester, the fetal skin remains un-keratinized, allowing for rapid and bi-directional diffusion across the fetal skin and surfaces of the umbilical cord, placenta, and amnion. The composition of amniotic fluid is therefore similar to that of fetal plasma during this time (Underwood et al., 2005), and metabolites found in amniotic fluid collected during the second trimester can serve as a good indicator of metabolic status of the fetal compartment.

We compared the pathway profiles of samples characterized by maternal serum cotinine level and found clear perturbations that associated with maternal cotinine in the range of 2–10 ng/mL. Our data therefore confirmed that even low-level nicotine exposure associates with significant changes in fetal metabolism related to aspartate and asparagine metabolism, pyrimidine metabolism, and amino group metabolism, and these perturbations show striking overlap with the dysregulated pathways previously observed in the sera of actively smoking adults (serum cotinine > 10 ng/mL). Although this is an implied causality framework in which the associations do not establish a cause-effect relationship, the results presented here are both compelling and disturbing, and extend from previous studies to provide a first glimpse into potential mechanisms of fetal consequence following even low-level maternal exposure to nicotine.

2. Materials and methods

2.1. Paired maternal serum and amniotic fluid samples from women with karyotypically normal pregnancies

We conducted this study using 81 pairs of second-trimester amniotic fluid and matched maternal serum samples collected in the US between 2004 and 2014; these samples derived from pregnant women who underwent amniocentesis and prenatal testing and whose results confirmed normal fetal karyotype (46,XX or 46,XY). Specifically, samples were obtained as de-identified banked clinical laboratory discards from the Greenwood Genetic Center (GGC, Greenwood, SC, USA), which served as a referral lab for the samples. All women whose samples were used in this study had previously consented to have their de-identified clinical sample leftovers made available for research.

Of the 81 women whose samples we studied here, 62 had been referred for amniocentesis and testing due to an increased risk of Down syndrome (often because of a positive screening result), and 19 were referred due to advanced maternal age. For all paired samples, we were provided the following information: maternal age at serum collection, gestational age at the time of both serum and amniotic fluid collection, serum and amniotic fluid collection year, maternal race/ethnicity, reason for referral, maternal self-reported smoking status, fetal gender, and fetal karyotype. Of note, the 81 pairs of samples used in this study were selected from a larger set to serve as matched controls for a separate study of chromosomally abnormal pregnancies.

Individual samples were collected at the location of the woman's referring physician or laboratory and transported the same day, or overnight, at ambient temperature to the prenatal testing laboratory at GGC. Maternal serum samples were collected most commonly in Becton-Dickinson (BD) red top, red/black top (serum separator, SST), or gold top (SST) vacutainer tubes. Amniotic fluid samples were collected in standard BD plastic syringes and transported in clear or amber polystyrene tubes. Upon arrival in the laboratory at GGC, all samples were inspected for correct identification, sample type, and sample condition, and each received a unique sample identification number in compliance with accession protocols.

Serum samples were received at GGC either as isolated serum, which had been previously removed from the red blood cell clot by centrifugation, or as a clotted whole blood sample. Clotted whole blood samples were centrifuged at 2200 rpm for 10 min and the serum transferred to polypropylene vials for storage. Serum samples were stored at 2–8 °C for up to 48 h before clinical testing and then frozen at − 20 °C (± 10 °C) for long-term storage. Amniotic fluid samples received in the cytogenetic laboratory were centrifuged at 1000 rpm for 10 min. The supernatant was removed and maintained at − 20 °C (± 10 °C) for long-term storage.

The maternal serum and amniotic fluid samples selected for this study were thawed, aliquotted to fresh vials, and shipped on dry ice to Emory University by overnight courier. At Emory, the samples were stored at − 80 °C and then thawed and subjected to liquid chromatography-mass spectrometry (LC-MS), as described below.

2.2. High-resolution liquid chromatography-mass spectrometry

Sample analysis was performed as previously described (Jones et al., 2016; Soltow et al., 2013). Serum samples were analyzed with three technical replicates on a Thermo Scientific LTQ Velos Orbitrap mass spectrometer, coupled with dual liquid chromatography, alternating data collection between HILIC and C_{18} columns. Analyses were performed with positive electrospray ionization mode, an injection volume of 10 μL, mass-to-charge ratio (m/z) scan range of 85 to 2000, and resolution of 60,000 (FWHM). Serum samples were randomized and run in batches of 20, with pooled reference plasma (Q-Standard) samples analyzed prior to and following each batch to enable quality control and metabolite quantification, as described previously (Go et al., 2015b). Data extraction was performed using apLCMS (Yu et al., 2009) and xMSanalyzer (Uppal et al., 2013). Amniotic fluid samples were processed and analyzed separately from serum samples but under parallel protocols. We performed principal component analysis (PCA) to evaluate potential batch effects (Yang et al., 2008) and corrected for these effects, where necessary, using ComBat (Johnson et al., 2007) and xMSanalyzer.

The resulting data matrices contained individual features defined by