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Placental Expression of Imprinted Genes and Early Childhood Neurobehavioral Performance

By

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Master of Science in Public Health

Environmental Health-Epidemiology

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University of North Carolina at Chapel Hill
2015

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An abstract of
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Abstract

Placental Expression of Imprinted Genes and Early Childhood Neurobehavioral Performance
By Sarah Menz

Imprinted genes in the placenta play a role in regulating the fetal environment, which affects fetal development and long-term health outcomes. Prior cross-sectional research has linked placental imprinted gene expression to neurobehavioral deficits at birth, and so we sought to examine if those measures also held prospective value. With available data in the New Hampshire Birth Cohort Study for 136 mother-infant pairs, we examined the relationship between placental expression of imprinted genes and social responsiveness scores (SRS-2) of 3-year old children, using linear regression. After controlling for false discovery using the false discovery rate (FDR), we found none of the imprinted genes' expression to be significantly associated with the SRS-2 T-score. The placental expression of HLA-DPB2 showed a nominally significant negative association with the SRS-2 T score (P=0.03). Because of the small amount of variation in SRS-2 scores for this cohort, larger cohorts may be better able to detect associations between placental expression of imprinted genes and developmental outcomes in the future.

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Introduction

The Placenta and Genetic Imprinting:

The Developmental Origins of Health and Disease (DOHaD) is the concept that fetal experiences can affect health outcomes over a lifetime. Observational and experimental studies support this idea (1). The placenta plays a key role for fetal development by regulating the fetal environment, by controlling the exchange of nutrients and gases, and producing growth hormones and signaling molecules including progesterone, cytokines, and chemokines (2, 3). To protect the fetus from harmful environmental exposures, the placenta can metabolize some toxic substances and can act as a barrier to others – reducing or preventing fetal exposure to these chemicals (3). Through these mechanisms, the placenta adjusts to the maternal environment to dynamically support the needs of the developing fetus, thus playing a central role in DOHaD (2, 3). Owing to its critical role in mediating fetal exposures and easy availability at delivery, the placenta is an ideal tissue for linking fetal experiences to downstream health outcomes (4).

Epigenetic mechanisms are mitotically heritable and have the potential to control gene expression without changing the DNA sequence. They control cell differentiation and embryonic development through their control of gene transcription (5). These mechanisms can be responsive to environmental stimuli (6) and represent one way in which the placenta's function is altered in response to environmental exposures (2).

Genetic imprinting, an epigenetic mechanism, reduces gene expression from biallelic expression to monoallelic expression in a parent-of-origin specific manner (7). Regulation of imprinted gene expression can occur through the methylation of DNA or

histone modification at specific imprinting control regions (8). Imprinted genes in the placenta are critical to a variety of birth outcomes, especially those involved in control of growth and neuroendocrine system effects (2). For example, increased expression of the imprinted gene NNAT has been associated with increased risk for being either large or small for gestational age (9). This type of early programming can have long-term effects, as a baby's size at delivery, whether small or large, has associations with adult diseases. Smaller babies, for example, are at higher risk for developing coronary heart disease, hypertension, stroke, Type 2 diabetes, and dyslipidemia in adulthood (2). Although these observational reports are common and robust, the mechanisms underlying them remain less well understood and, due to advances in our understanding of molecular biology and new technologies, are only now becoming possible to explore. In the recent past, cohorts were established to interrogate molecular features such as placental imprinted gene expression with newborn characteristics. As those cohorts are followed over their lifetimes, we will gain a better understanding of the relationship between placental genetic imprinting and health outcomes from childhood into adulthood.

Environmental factors, like diet, xenobiotics, drugs, smoking, and stress, can produce changes in the intrauterine environment (10, 11) and are associated with indirect epigenetic effects (2). These epigenetic effects, including alterations to the expression of imprinted genes and variation in DNA methylation, help the placenta adapt to environmental changes and protect the fetus (1, 4, 12). For example, our group has shown that increases in fine particulate matter ($PM_{2.5}$) exposures are associated with changes in expression of placental imprinted genes, and these expression patterns may be a mediator between air pollution and lower birth weight (13). Additionally, DNA methylation, which

controls gene transcription, affects the expression of proteins that are involved in the metabolism of toxic trace metals (4, 14, 15). Prenatal exposure to toxic metals affects the expression of imprinted genes in the placenta (4, 16) and has been associated with adverse birth outcomes, like abnormal fetal growth (17).

The associations between environmental stressors, placental imprinted genes, and health outcomes are complex. Genomics, epigenomics, transcriptomics, proteomics, metabolomics, and exposomics all play important roles within the placenta (18). Studying placental expression of genetic imprinting is one component in understanding DOHaD, but the associations between placental imprinted genes and neurodevelopment are not straightforward. For example, when assessing growth outcomes, there have been both positive and negative associations between the methylation status of the imprinting control region of IGF2 and birth weight (9). In addition, the regulation of an outcome like fetal growth or neurodevelopment may not have a one-to-one relationship with placental imprinted genes; other factors, like infant sex and maternal weight status, may modify the relationship (17).

Neurobehavior:

Placental expression of imprinted genes may affect neurobehavioral development. Through their role in the placental regulation of nutrients, neuroactive compounds, and signaling molecules, placental imprinted genes can influence brain development (19). Severe neurodevelopmental disorders like Beckwith-Wiedemann, Silver-Russel, Prader-Willi, and Angelman syndromes are linked to imprinting abnormalities, demonstrating the importance of imprinted genes in neurobehavioral development (20). Using both candidate gene and genome wide approaches, there have been consistent findings between epigenetic

processes and neurobehavioral development (2). Studies measuring neurobehavioral outcomes using the NICU Network Neurobehavioral Scales (NNNS), cry acoustics, and neuroendocrine system responses have also found associations between early neurobehavior and epigenetics (2, 21-24). For example, variation in placental expression of imprinted genes has been associated with differences in measured newborn neurobehavior from the NNNS (25, 26). Specifically, variation in the placental expression of the imprinted genes DLX5, DHCR24, VTRNA2-1, PHLDA2, NPAP1, FAM50B, GNAS-AS1, PAX8-AS1, SHANK2, and COPG2IT1 were most important for distinguishing infants exhibiting atypical newborn neurobehavior (25). However, these relationships are cross-sectional and measured near birth. The long-term, prospective relationships between placental imprinted gene expression and neurobehavioral development remain unclear.

Autism is an important public health concern. Autism spectrum disorder (ASD) is characterized in the Diagnostic and Statistical Manual-V (DSM-V) by difficulties with communication and interaction, and a display of restricted and repetitive behavior, interests, or activities (27). In 2010, there was an estimated 52 million cases of autism globally, which means 1 in 132 individuals has autism (28). Autism prevalence has increased globally over the past few decades. Some of this increase may be due to increased awareness and changes in diagnostic criteria. Environmental exposures, which have the potential to mediate epigenetic modifications and have a genetically-mediated effect, are the third and most controversial contributor to increased autism prevalence (29). Genetic imprinting is suspected to play a role in autism, but there has been limited research on how specific environmental factors are associated with epigenetic mechanisms and

neurodevelopment (30, 31). The causes of autism are complex, and further research is needed to understand the factors involved in the development of autism.

The DSM-V criteria encourages earlier diagnosis of autism, rather than waiting for symptoms when children are school-aged (27). Early interventions for children with autism can be beneficial to developing intelligence, communication, adaptive function, language, and socialization (32). Although, many children are diagnosed with autism as toddlers (32), high-functioning individuals tend to be diagnosed later, especially among females (33, 34). Diagnosing autism early in life can allow children to get earlier interventions for better lifetime outcomes.

Genomic imprinting has been studied as a biomarker for environmental exposures and early detection of developmental disorders in children, but these studies have used blood samples rather than placental samples (20, 35). Because of the function of the placenta in early brain development, placental tissue may provide clearer results (20). The placenta could be used to get a snapshot of the *in utero* experience and predict future neurodevelopmental outcomes (2).

Study Objectives:

The placenta plays a key role in regulating the fetal environment, and some placental functions are regulated through genomic imprinting. Variation in the expression of imprinted genes is associated with abnormal newborn growth and neurobehavior patterns as well as with environmental exposures during pregnancy. The relationship between environmental exposures, placental expression of imprinted genes, and health outcomes is not completely understood. Based on the DOHaD, it would be expected that placental expression of imprinted genes could affect various health outcomes over a

lifetime. This study will assess if variation in placental imprinted gene expression can be prospectively associated with early childhood neurobehavioral performance. These analyses will be performed using an established birth cohort, the New Hampshire Birth Cohort Study, which has examined placental imprinted gene expression and has followed children, assessing neurobehavioral performance using the Social Responsiveness Scale-2, a measure of autism spectrum features, at age 3. Using linear regression, we will first assess if placental expression of the imprinted genes *DLX5, DHCR24, VTRNA2-1, PHLDA2, FAM50B, GNAS-AS1, SHANK2,* and *COPG2IT1,* as hypothesized candidates (25), is associated with variation in social responsiveness at 3 years old, and will then expand to examine all assessed imprinted genes in the placenta.

Methods

Study population:

This study includes mother-infant pairs from the New Hampshire Birth Cohort Study (NHBCS), a longitudinal pregnancy and birth cohort in New Hampshire, USA. Pregnant women were eligible if they were between the ages of 18 and 45, had English literacy, expected a singleton birth, and had a private well at their home within the study region. Women were recruited at 24 to 28 weeks gestation from prenatal study clinics from 2009 to 2013. Participants completed demographic questionnaires at enrollment. Participants provided informed consent, in accordance with the Institutional Review Board of Dartmouth College (17).

Parents completed the Social Responsiveness Scale-2 (SRS-2) by mail when the child was 3 years old. This 65-item assessment is used to assess communication, social

awareness, social anxiety, social information processing, interpersonal behaviors, and repetitive or stereotypic behaviors. From this, eight measurements are obtained: a summary scale based on these observed behaviors, two scales related to the DSM-V for autism spectrum disorder diagnostic criteria, and five subscales for social awareness, social cognition, social communication, social motivation, and restricted interests and repetitive behaviors (36, 37). Although cohort enrollment is ongoing, this analysis focuses on those infants having both placental molecular profile data (n=330 mother-infant pairs) and SRS-2 questionnaire completion at age 3 (total n= 136 children).

Placenta collection and RNA extraction:

Samples were taken from each placenta, shortly after delivery. Placental tissue was collected next to the umbilical cord insertion to minimize heterogeneity and was free of maternal decidua (38). These samples were stored at 4°C in RNAlater (Life Technologies, #AM7020). Placenta samples were removed after at least 72 hours, dried, snap-frozen in liquid nitrogen, homogenized, and stored at -80°C until examination. RNA was extracted using RNeasy kit, followed by double DNasel (Qiagen, #79254) to remove DNA contamination. Then the extracted RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., #ND-2000) and stored at -80°C until analysis(25).

RNA Expression Quantification:

RNA expression quantification was completed using a custom-designed code-set with 144 known and putative imprinting genes on the Nanostring nCounter system. For hybridization, RNA (100 ng) was incubated overnight with reporter and capture probes at 65°C. Unbound probes were removed, and the remaining complexes were captured on

imaging cartridges with nCounter Prep Station II. The sealed cartridges were scanned in nCounter Digital Analyzer for code count detection. The code counts were first normalized using the geometric mean of spike-in controls; this accounted for differences in hybridization and recovery. The dataset was then normalized against the geometric mean of housekeeping genes *GAPDH*, *RPL19,* and *RPLP0* to account for differences in sample content. Two other genes on the array *ACTB* and *B2M* were not used for normalization because of the high variability between individuals. The background threshold of detection was set at 2 standard deviations above the mean of the negative controls. Anything below the background was set to the limit of detection divided by square root of 2.

Statistical Analysis:

Statistical analyses were done using R. Analysis was initially restricted to the subset of 8 expressed genes (*DLX5, DHCR24, VTRNA2-1, PHLDA2, FAM50B, GNAS-AS1, SHANK2*, and *COPG2IT1*) previously found to be associated with newborn neurobehavior (25). Given that the distributions of SRS-2 summary scores are defined to have a normal distribution with a mean of 50 and standard deviation of 10, we used linear regression to examine the association between each of the imprinted genes and the SRS-2 summary scale (39). Maternal age, maternal smoking status, maternal education level, infant sex, gestational age, and birth weight were considered as potential confounders, and in final models, we controlled for infant sex and maternal age, as the other potential confounders were shown to not impact the estimates.

After completing the analysis for the candidate genes, we performed an imprintome-wide association study. We examined all 82 expressed imprinted genes quantified by the panel, utilizing linear regression models to examine the association for

each imprinted gene and the SRS-2 summary scale, controlling for maternal age and infant sex. We used a false discovery rate (FDR) of 0.05 for multiple testing corrections.

Results

Study Population:

The demographics for the total group of samples with available placental molecular data and the subset of 136 children with complete expression and SRS-2 data are included in Table 1. The average mother's age in both groups was 31.5 years old. The majority of mothers reported not smoking during pregnancy, with 81.2% in the full sample and 89.0% in the subset with SRS-2 data. There was a roughly even split of male and female infants, but there were slightly more females (51.5%) with complete SRS-2 data compared to the full sample, which had a majority of male infants (53%). Most mothers had completed more than a high school education, with 91.2% in the complete SRS-2 data group. Overall, those with complete SRS-2 data appeared to have very similar characteristics to those without complete SRS-2 data.

In this subset, the mean SRS-2 T-score was 44.49, with a standard deviation of 4.95. The distribution of SRS-2 T-scores is shown in Figure 1. The distribution was roughly normal, with only one score that may be considered clinically significant, with a score above 60 (40).

Candidate Gene Regression:

Using linear regression, we analyzed the association between 8 candidate genes and SRS-2 T-score (Table 2). We did not identify any significant associations between the expression of these individual genes and the SRS-2 summary score.

Imprintome-Wide Association Study:

The full complement of 82 expressed imprinted genes were subsequently analyzed for their association with the SRS-2 T-score (Table 3). The placental expression of HLA-DPB2 showed a nominally significant negative association with the SRS-T score (P=0.03). None of the imprinted genes' expression was considered to be significantly associated with the SRS-2 T-score, when we controlled for false discovery using the FDR.

Discussion

In this study, we used a prospective study design to assess the association of imprinted gene expression levels in placenta with social responsiveness in three-year-old children from a mother-infant cohort. We did not find the expression of any imprinted genes to be significantly associated with SRS-2 scores, including eight candidate genes that were previously associated with neonatal neurobehavior. Placental expression of one gene, HLA-DPB2, had a suggestive association with social responsiveness, but demonstrated a high false discovery rate, suggesting this may likely be a false discovery.

HLA-DPB2 is a pseudo-gene involved in the major histocompatibility complex (MHC) and genetic association studies have linked variation in this gene to rheumatoid conditions involving auto-antibody presence (41, 42). The MHC is essential to CNS development, including neuronal connectivity and plasticity. Changes to the MHC, from genetic mutation or maternal immune activation, can alter brain neuronal connections, which is an underlying mechanism of ASD development (43). In addition, recent evidence suggests that the presence of maternal autoantibodies during pregnancy is a risk factor for

ASD. So, although the finding of HLA-DP2 expression linked to SRS-2 scores may lack reliability, there is biologic plausibility to this finding warranting further study (44).

The infants' SRS-2 scores were measured when the child was at 3 years of age. The SRS-2 is an appropriate test at this age and has been found to be reliable for identifying autistic symptoms, but it is not consistently used for autism diagnosis at this age. Studies have used different cut-off points for an autism classification, depending on cultural context, whether a parent or teacher completed the form, and infant sex (45). For a low-risk, healthy, term newborn population, we would not expect to see a wide range of SRS-2 T-scores, and in this cohort, there was very little variation in our outcome. The cohort's mean SRS-2 T-score was 44.5, which is below the normative average, indicating a low-risk population. Only one score was above the normal range and might be classified as mild autism (40).

In our cohort, both infant sex and mother's age were associated with the child's SRS-2 T-score. This is consistent with research finding that both of these variables are considered risk factors for autism (46). This suggests that, although we have not captured much variability in SRS-2 T-scores in our cohort, our data are in line with previous research on risk factors. To avoid confounding in our regression models, we included infant sex and mother's age as covariates in the analyses.

From the original cohort of mother-infant pairs with complete placental gene expression data, we had a smaller number of infants with complete SRS-2 scores. This subset appears representative of the larger cohort, but we may not be able to detect small differences due to the lower number of people and reduced power. As shown in Table 1, some subgroups were very small. Very few mothers smoked during pregnancy. The

majority of mothers had received some formal education after high school. These small groups would affect the sensitivity of our analysis, and it also shows that the majority of the cohort did not have many risk factors for autism. The mothers were selected based on the use of private wells and may have consumed more toxic trace metals in their water, which is a risk factor for autism (47, 48). Overall, this is a cohort that would not be expected to have a high prevalence of autism.

The genes selected for the candidate gene analysis were based on a previous study that measured the association between NNNS scores and imprinted gene expression. The NNNS measures newborn neurobehavioral performance, including neurologic and reflex functioning, active and passive muscle tone, quality of movement, signs of stress, responses to visual and auditory stimuli while awake and asleep, and infant state (49). The SRS-2 measures communication, social awareness, social anxiety, social information processing, interpersonal behaviors, and repetitive or stereotypic behaviors. These two assessments measure different behaviors, during a time of rapid development. Some development problems appear later, and some infants that appear high-risk at birth develop normally (50). There are NNNS profiles that are associated with medical and behavioral problems through 4.5 years old, but these are not necessarily the social responsiveness behaviors that are measured with the SRS-2 (50). Because these two assessments measure different behaviors and aim to predict aspects of development, we suspect that the candidate genes from the NNNS analysis affect different pathways than the pathways involved in social responsiveness development. Both of these may be affecting neural connections, but they may be along different biologic pathways.

This study had several strengths. The population is a prospective cohort, and we have a complete measurement of placenta imprinted gene expression. The analysis included data from 136 mother-infant pairs, which represents a reasonably large cohort with placental imprinted gene expression information. The mothers were selected for the study based on having private wells, with no specific exclusions based on race, ethnicity of socioeconomic status, and so it is a relatively representative cohort of mothers in rural New Hampshire.

To better understand the relationship between imprinted gene expression and health outcomes, future studies may benefit from a different study design. Because high and low SRS-2 scores are not a common outcome, a study design that selects participants with high and low SRS-2 scores would be better able to identify any possible relationships with placental expression of imprinted genes. With a case-control or nested cohort study, a larger number of infants with high SRS-2 scores could be selected, so there would be a larger range of SRS-2 scores for analysis. A much larger children's cohort study, like the National Institutes of Health's Environmental influences on Child Health Outcomes (ECHO) project, could allow for better detection and understanding of the association between imprinted gene expression and health outcomes (51).

Conclusion and Recommendations:

Neurodevelopment is complex. Placental expression of imprinted genes is one component in understanding how fetal experiences affect lifetime neurodevelopmental health outcomes. In a normative population, we did not identify any placental imprinted genes that were associated with measures of social responsiveness. Future cohorts, with a

wider range of social responsiveness scores, may be better powered to detect potential differences. Larger cohorts will allow for a better understanding of the association between placental imprinted gene expression and development.

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Table 1. Selected characteristics of 330 mothers and their infants from a New Hampshire cohort for those with complete SRS-2 data and for all participants

Characteristic	Complete SRS-2 Data (n=136)	Full Sample (n=330)
SRS-2 T-score[1]	44.49 ± 4.95	
Mother's age[1]	31.51 ± 4.85	31.50 ± 4.93
Gestational age[1,3]	39.15 ± 1.46	38.99 ± 1.59
Birth weight[1,4]	3395.46 ± 543.48	3432.74 ± 518.76
Maternal Smoking During Pregnancy[2]		
Yes	9.6% (13)	11.2% (37)
No	89.0% (121)	81.2% (268)
Infant Sex[2]		
Male	48.5% (66)	53.0% (175)
Female	51.5% (70)	47.0% (155)
Education[2]		
High school or less	8.1% (11)	10.9% (36)
More than high school	91.2% (124)	81.8% (270)

[1]Mean ± Standard Deviation; [2] % (n); [3]in weeks; [4]in grams

Figure 1. Distribution of Total SRS-2 T-scores

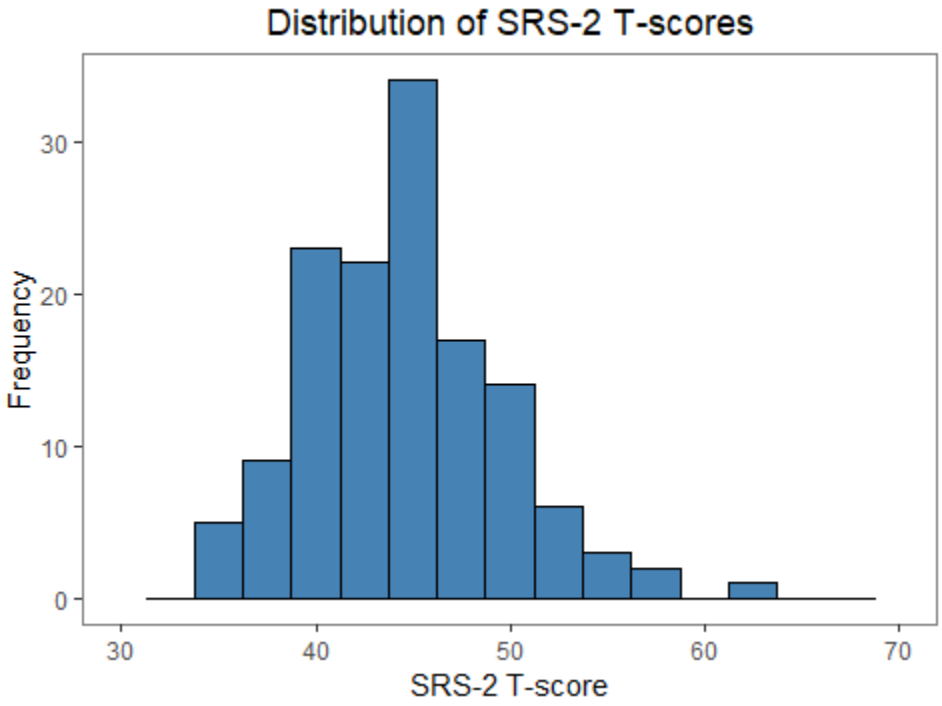

Table 2. Linear regression coefficients for the expression of the candidate imprinted genes in their association with the SRS-2 summary scale, adjusted for infant's sex and mother's age

Gene	Estimate	Standard Error	p-value
DLX5	-0.04	0.59	0.94
PHLDA2	0.01	0.61	0.98
DHCR24	0.60	0.58	0.30
FAM50B	0.79	0.72	0.27
GNAS	0.90	1.26	0.47
COPG2	-1.00	0.78	0.20
SHANK2	-0.31	0.54	0.57
VTRNA2-1	0.03	0.44	0.94

Table 3. Linear regression coefficients for top 10 genes, adjusted for infant's sex and mother's age

Gene	Estimate	Standard Error	p-value	FDR
HLA-DPB2	-0.91	0.41	0.03	0.98
ZNF597	-1.08	0.56	0.06	0.98
SLC22A3	-0.69	0.43	0.11	0.98
DLK1	-0.77	0.49	0.12	0.98
CDKN1C	1.26	0.83	0.13	0.98
PEG3	-0.88	0.60	0.14	0.98
SLC22A18AS	0.83	0.57	0.14	0.98
CPXM2	-0.36	0.27	0.18	0.98
COPG2	-1.00	0.78	0.20	0.98
ITUP1/MIMT1_(Usp29)	0.86	0.69	0.21	0.98