Prenatal Cotinine Levels and ADHD among Offspring

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Abbreviations: FIPS-ADHD: Finnish Prenatal study of ADHD, FHDR: Finnish Hospital Discharge Register, FMC: Finnish Maternity Cohort, PIC: Finnish personal identity code, PRC: Finnish Population Register Center, FMBR: Finnish Medical Birth Register

Table of Contents Summary

Measuring cotinine levels from maternal serum specimens collected during pregnancy, this study investigated the association between nicotine exposure during pregnancy and offspring ADHD.

What's Known on This Subject

Exposure to maternal smoking is associated with various adverse perinatal outcomes. An association between maternal smoking and offspring ADHD has been shown across studies. However, the causality of the association has been questioned to be mostly due to familial confounding.

What This Study Adds

In this first nationwide study, objectively measured nicotine exposure through maternal cotinine levels allows us to overcome under-reporting of smoking during pregnancy. We report a strong association as well as a dose-dependent relationship between prenatal nicotine exposure and offspring ADHD.

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Contributors' Statement

Professor Andre Sourander conceptualized the study, participated in the study design and drafted the initial manuscript.

Dr Minna Sucksdorff participated in the study design, carried out the literature search and drafted portions of the initial manuscript.

Dr Roshan Chudal participated in the study design and drafted portions of the initial manuscript.

Dr Heljä-Maria Surcell conceptualized the study and participated in the study design.

Ph.Lic. Susanna Hinkka-Yli-Salomäki designed the study and carried out the analyses.

Authors Dr David Gyllenberg, Dr Keely Cheslack-Postava, and Professor Alan S. Brown conceptualized the study and participated in the study design.

All authors contributed to the interpretation of the data, critical review and revision of the manuscript, and approved the final manuscript as submitted.

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ABSTRACT

Objective
An association between maternal smoking during pregnancy and offspring attention deficit-hyperactivity disorder (ADHD) has been shown across several studies based on self-reports. No previous studies have investigated the association of nicotine exposure measured by cotinine levels during pregnancy and offspring ADHD.

Method
In this population-based study, 1079 ADHD cases born between 1998 and 1999 and diagnosed according to the International Classification of Diseases and 1079 matched controls, were identified from Finnish nationwide registers. Maternal cotinine levels were measured using quantitative immunoassay from maternal serum specimens, collected during the first and second trimesters of pregnancy and archived in the national biobank.

Results
There was a significant association between increasing log-transformed maternal cotinine levels and offspring ADHD. The OR was 1.09 (95% CI 1.06-1.12) when adjusting for maternal SES, maternal age, maternal psychopathology, paternal age, paternal psychopathology and child's birth weight for gestational age. In the categorical analyses with cotinine levels in three groups, heavy nicotine exposure (cotinine level >50ng/ml) was associated with offspring ADHD with an OR 2.21 95% CI 1.63-2.99 in the adjusted analyses. Analyses by deciles of cotinine levels showed that the adjusted odds for offspring ADHD in the highest decile was 3.34 (95% CI 2.02-5.52).

Conclusion
The study shows an association with and a dose-response relationship between nicotine exposure during pregnancy and offspring ADHD. Future studies incorporating maternal smoking, and environmental, genetic and epigenetic factors are warranted.

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Introduction

Despite its proven negative effects on fetal development, smoking during pregnancy remains a significant public health issue. Approximately 7.2 % of women who gave birth in the United Stated smoked cigarettes during pregnancy in 2016.[1] The prevalence rate was similar in Finland with approximately 7% of all pregnant women continuing to smoke throughout their pregnancy.[2] Exposure to maternal smoking has been associated with various adverse outcomes such as obstetric complications, low birth weight, preterm birth, sudden infant death syndrome and increased infections in childhood.[3] An association between maternal smoking and offspring attention-deficit/hyperactivity disorder (ADHD) has been shown across numerous studies from different populations.[3-7] However, the causality of the association has been questioned to be mostly due to familial confounding.[3,8-13] Moreover, to date the information on maternal smoking is typically based solely on maternal self-report.

In previous studies maternal self-report of smoking has been shown to underestimate true smoking by 8 % to 28%.[14-16] Disclosure of smoking is lower among pregnant smokers than smoking women in general. Cotinine is the most appropriate biomarker indicating nicotine exposure due to the short half-life of nicotine, which is primarily metabolized to cotinine in the liver.[17] While previous studies are based on self-reports of active smoking, cotinine measurements enable quantifying the amount of nicotine exposure and detecting nicotine exposure from other sources such as nicotine replacement therapy or passive smoking. However, no previous studies have investigated the association of prenatal cotinine levels and the risk of offspring ADHD. Two previous studies that have examined the association between maternal cotinine level in pregnancy and childhood behavioral outcomes based on parental questionnaires reported no association.[18,19]

This is the first study to investigate the association between maternal cotinine levels during pregnancy and offspring diagnosed ADHD. In this population-based case-control study, the use of objectively measured nicotine exposure allows us to overcome under-reporting of smoking. Based on findings from previous studies showing an association between self-reported maternal smoking during pregnancy and offspring ADHD diagnoses, we hypothesized that there is an association between maternal cotinine levels and offspring ADHD. In addition, our study allows us to examine possible dose-response effects between maternal cotinine levels and offspring ADHD.

Patients and Methods

The present study is based on the Finnish Prenatal study of ADHD (FIPS-ADHD) (Figure 1), a nested case-control study derived from all singleton live births in Finland between January 1, 1998 and December 31, 1999 and followed up for any ADHD diagnosis in the Finnish Hospital Discharge Register (FHDR) by December 31st, 2011.

Finnish Maternity Cohort

All offspring in FIPS-ADHD were derived from the Finnish Maternity Cohort (FMC). The FMC consists of 2 million serum samples collected during the first and early second trimester of pregnancy (5th to 95th percentile: months 2–4 of pregnancy) from over 950,000 women since the beginning of 1983. Following informed consent, blood samples were collected at Finnish maternity clinics for the purpose of screening for congenital infections (HIV, hepatitis B and syphilis). One maternal serum sample was obtained from each pregnancy. The median gestational age of serum collection for subjects in this study was 10 weeks (interquartile range: 8–12 weeks). After the screening, approximately 1–3 mls of serum from each pregnancy are stored at -25^{o}C in a protected biorepository at Biobank Borealis in Oulu, Finland, and are available for scientific research.[20] Linkage between FMC data and the Finnish Health Registries

and other data sources is possible using the Finnish personal identity code (PIC) which has been assigned to all residents of Finland since 1971.[21]

Nationwide Registers

The Population Information System was established by the Finnish Population Register Center (PRC) in 1969 and subsequently computerized in 1971. It is a computerized national register containing basic information about Finnish citizens and foreign permanent residents. The personal data in the system include: name, PIC, address, citizenship and native language, family relations and date of birth and death (if applicable). The Finnish Medical Birth Register (FMBR), established in 1987 includes comprehensive and standardized data on the perinatal period for all live births, and stillbirths of fetuses with birth weight of at least 500 g or gestational age of at least 22 weeks.

Cases and Controls

The ADHD cases were identified by linking the information from the FHDR with the FMC using the PIC. The FHDR contains all inpatient diagnoses since 1967 and outpatient diagnoses from specialized services since 1998. The FHDR contains the patient's PIC, hospital ID and primary and secondary diagnoses. The diagnostic classification is based on the International Classification of Diseases (ICD); the 10th Revision has been used since 1996.[22] From 1987 to 1995 the diagnoses were coded according to ICD-9,[23] and from 1969 to 1986 according to ICD-8.[24] Finland has a system of regular assessment of children's physical and psychological development by well-trained health professionals. Almost all children attend free health check-ups at least 15 times during their first 6 years of life followed by annual check-ups at school. ADHD is typically diagnosed based on the assessment of a specialist in psychiatry or neurology in public outpatient services free of charge. A previous study evaluating the validity of the

ADHD diagnosis in the FHDR has shown that 88% of subjects examined met the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* diagnostic criteria for ADHD.[25]

In the present study, the ADHD cases included singletons born in Finland between 1998 and 1999 and registered in the FHDR with the ICD-10 codes of hyperkinetic disorders F90.0, until 2011. Controls were defined as singleton offspring born in Finland and without a diagnosis of ADHD, conduct disorder, or severe or profound intellectual disability in the FHDR. Each participant with ADHD was matched with one control on date of birth (±30 days), sex, and place of birth. The controls had to be alive and living in Finland at the date of diagnosis of the matched case. Cases and controls were selected from these years due to the lack of earlier availability of data from the FMBR, established in 1987 and to ensure greater uniformity of the serum specimens with regard to the time of sampling and storage. In the present study, out of the 1320 total cases and controls identified, sufficient serum was available in the FMC for 1079 cases and matched controls (n=1079). The study was approved by the ethical committees of the Hospital District of Southwest Finland and the National Institutes of Health and Welfare. Informed consent was obtained prior to collection of maternal serum.

Cotinine measurements

Cotinine measurements from the FMC samples were conducted blind to case/control status. Serum cotinine levels were measured using a commercially available quantitative immunoassay kit (OraSure Technologies, Bethlehem, PA, USA) (sensitivity=96-97%, specificity=99-100%). Intra- and interassay variation are 3.5-6.2%, and 6.0-9.6%, respectively. The results are reported in ng/ml.

Covariates

The covariates included variables that have been previously shown to be associated with both maternal smoking as well as offspring ADHD: number of previous births, maternal socioeconomic status (SES), maternal psychiatric history, maternal history of ADHD diagnosis, maternal history of substance use disorder, paternal psychiatric history, paternal history of ADHD diagnosis, gestational age of the child, birth weight for gestational age (WGA) (based on national sex-specific growth curves)[26], maternal age, paternal age and gestational week of blood draw. Information on number of previous births, maternal SES, maternal age and gestational age was obtained from the FMBR. Maternal and paternal psychiatric diagnoses were obtained from the FHDR. A parent was defined as having a psychiatric history if he or she had any psychiatric diagnoses registered in the FHDR during his or her lifetime: F10–F99 based on the ICD-10 (corresponding diagnoses in the ICD-9 [291–316] and the ICD-8 [291–309]) but excluding intellectual disability (F70-79) or a diagnosis of ADHD (ICD-10: F90.X or ICD-9: 314.X). For mothers, the association with disorders due to alcohol or substance abuse (ICD-10: F10–19; ICD-9: 291–292, 303–305; and ICD-8: 291, 303, and 304) was tested separately from other psychiatric disorders. Paternal age was obtained from the PRC and gestational week of blood draw from the FMC. The covariates were considered for inclusion in the models based on associations with both cotinine exposure and ADHD at P $\leq$0.1, in accord with standard texts.[27]

Statistical analysis

The analysis was based on a nested case-control design, in which the controls for each case were identified from the population at risk and matched on selected factors (see "Cases and controls"). Initially, cotinine was examined as a continuous measure. Due to the skewed distribution of cotinine, the variable was log-transformed before analysis.

In order to further facilitate data interpretation, we examined maternal cotinine as categorized into deciles and as a three-class categorical variable: reference (<20ng/ml); moderate exposure (20-50 ng/ml); and heavy exposure (>50 ng/ml). The deciles for the case and control groups in the analyses were derived from the cut-points of maternal cotinine levels that defined the deciles for this biomarker in the control group. We hypothesized that a significant association would be observed for maternal cotinine classified in the highest decile compared with the reference group, which was defined as the lowest decile. The cut-off points for the three-class categorical variable were based on the manufacturer's recommendation and have been used in previous studies based on the FMC serum bank.[28,29] These levels also corresponded well with the frequencies of self-reported maternal smoking. Among mothers of cases, smoking was reported by 56 of 702 (7.9%) in the reference category of <20ng/ml, 62 of 80 (77%) mothers with cotinine levels of 20–50 ng/ml, and 196 of 214 (91.5%) mothers with cotinine levels >50 ng/ml (P<.001). Appropriate to the nested case-control study design, point and interval estimates of odds ratios were obtained by fitting conditional logistic regression models for matched pairs. Statistical significance was based on P <0.05. All the statistical analyses were performed with SAS software (SAS 9.4, SAS Institute, Cary, N.C.).

Results

The mean age of the cases at the time of the ADHD diagnosis was 7.3 years (SD 1.9, range 2-13.7 years). As shown in Supplementary table 4, there was no difference in covariates between 1079 cases included in the analysis and 241 cases not included due to insufficient serum except for paternal age. The mean paternal age among cases not included was 1.2 years (SD 6.9) older than that of included cases (p=0.02). The mean cotinine level among cases was 27.4 ng/ml (SD 54.8, range 0.0-427.7 ng/ml) and 11.3 ng/ml (SD 34.5, range 0.0-320.0 ng/ml) among controls. Supplementary Table 1 shows the distribution of serum cotinine levels and corresponding self-

reported smoking status in the sample (see Table S1, available online) As shown in the table, the prevalence of self-reported smoking status increased with increasing serum cotinine levels.

As shown in Supplementary Table 2 maternal SES, psychopathology, substance abuse, ADHD, age and number of previous births; paternal psychopathology, ADHD and age; child's gestational age and WGA were associated with P<0.1 with offspring ADHD (see Table S2, available online). As shown in Supplementary Table 3 maternal SES, psychopathology, substance use and age, paternal psychopathology and child's WGA were associated with maternal serum cotinine level. Gestational week of blood draw was not associated with these outcomes (see Table S3, available online). Maternal SES, psychopathology, substance use, age, paternal psychopathology and WGA were associated with both offspring ADHD and maternal cotinine level and therefore fulfilled criteria for confounding. Furthermore, adjustment was made for paternal age to address any potential selection bias, due to its difference among included and missing ADHD cases.

The main findings of the present study are shown in Table 1. The adjusted analyses were performed using two models. Model 1 contained WGA, maternal SES, maternal age, maternal psychopathology, maternal substance abuse, paternal age and paternal psychopathology as confounders. Model 2 contained the same confounders as in model 1 except for paternal psychopathology to specifically assess the effect of maternal factors. There was a significant association between increasing log-transformed maternal cotinine levels and offspring ADHD both in unadjusted analyses (OR 1.14; 95% CI 1.11-1.17) as well as adjusted analyses. In model 1 the OR was 1.09 (95% CI 1.06-1.12) and in model 2 the OR was 1.10 (95% CI 1.06-1.13).

In the categorical analyses cotinine levels were categorized into three groups: heavy (cotinine level >50ng/ml), moderate (20-50ng/ml) and no or low nicotine exposure (<20nmg/ml). As shown in Table 1 heavy exposure was associated with offspring ADHD in the unadjusted

analyses (OR 2.95; 95% CI 2.25-3.88) as well as in the adjusted analyses both in model 1 (OR 2.21; 95% CI 1.63-2.99) and in model 2 (OR 2.27; 95% CI 1.68-3.07). Moderate cotinine levels were associated with offspring ADHD in the unadjusted analyses (OR 1.92; 95% CI 1.33-2.77) but did not remain significant in the adjusted models (In model 1: OR 1.27, 95% CI 0.84-1.92; in model 2: OR 1.31, 95% CI 0.87-1.96).

The distribution of cotinine exposure in deciles by case-control status is presented in Table 2. As shown in Figure 2, the strongest association was in the highest decile (90%-100%). The odds for offspring ADHD in the highest decile in the unadjusted analyses was 4.90 (95%CI 3.10-7.76) while in the analyses adjusting for WGA, maternal SES, maternal age, maternal psychopathology, maternal substance abuse, paternal age paternal psychopathology it was 3.34 (95% CI 2.02-5.52). For the second highest decile (80-89%) the association in the unadjusted analysis showed an OR of 2.71 (95% CI 1.70-4.31) and in the adjusted analyses an OR 1.91 (95% CI 1.15-3.18). (Table 3). The difference between the deciles (80-89% vs. 90-100%) was significant showing that the risk of ADHD was higher with a higher level of nicotine exposure.

Discussion

This study provides evidence for the association between nicotine exposure during pregnancy and increased risk for ADHD in the offspring, as previous population-based studies have been based on self-report of smoking during pregnancy. The association between fetal exposure to nicotine, quantified as cotinine during gestation, and ADHD were similar when cotinine was classified as a continuous variable and when classified in three categories based on cotinine levels. Most importantly, the findings suggest a dose-dependent relationship between fetal exposure to nicotine and risk for ADHD. The findings persisted in all analyses after adjusting for potential confounders including SES, maternal and paternal psychopathology, maternal and

paternal age and WGA. To the best of our knowledge, this is the first biomarker-based study to show a relationship between fetal nicotine exposure and later offspring ADHD.

The findings add to the evidence of a complex association between exposure to nicotine during pregnancy and later ADHD. A neurobiological mechanism via intrauterine effects seems possible as nicotine crosses the placenta during pregnancy.[30] Animal studies have demonstrated an association between in utero nicotine exposure and increased locomotor activity as well as changes in the neurotransmitter systems in the brain.[31,32] Human studies have shown structural and functional changes in the brain associated with prenatal smoking exposure.[33] The mechanisms behind these effects are thought to develop through nicotine modulating the maturation of the developing central nervous system. In addition, other components of smoking, such as carbon monoxide, are thought to lead to fetal hypoxia and affect fetal brain development.[34]

The association between smoking during pregnancy and offspring ADHD may be explained by genetic or social factors.[9,35] Based on several sibling and family studies, the association between fetal exposure to smoking and ADHD has been suggested to be mostly explained by familial confounding.[8-13] These studies, as well as a novel study design on children born with assisted conception,[36] suggest that inherited effects and unmeasured household-level confounding seem more likely contributors behind the association than direct intrauterine effects of smoking**.** There are also contradictory sibling study findings including results from a Finnish study with over 150 000 sibling pairs showing that if the mother stopped smoking between the pregnancies, the second sibling did not have an increased risk of externalizing diagnoses including ADHD. Correspondingly, if the mother started smoking between the pregnancies, a significantly higher risk was observed in the second sibling.[37] A Dutch family study found that maternal compared to paternal smoking during pregnancy was associated with

a greater effect with offspring externalizing problems. The same study found that quitting smoking was associated with less externalizing problems among offspring.[38] Furthermore, an American sibling study found that familial confounding explained inattentive, but not hyperactive/impulsive ADHD behaviors, suggesting that the association may vary by phenotype.[39]

Despite several strengths, sibling comparison studies should also be interpreted with caution given their limitations. It has been suggested that within-sibling estimates will be biased towards the null by measurement error and that they may be either more or less biased than between-family estimates depending on the extent to which siblings share confounders versus the exposure.[40] In addition**,** mothers who vary in their smoking habits during different pregnancies can perhaps not be generalized to all smoking population.[40]

It is possible that the association between nicotine exposure during pregnancy and ADHD might partially be explained also by gene-environment interaction. Accumulating data provides evidence that prenatal smoking may act through epigenetic changes via altered DNA methylation and microRNA expression.[41,42] The exposure to nicotine may increase the risk of ADHD particularly among children with genetic vulnerability for ADHD. It is also possible that maternal smoking during pregnancy is a proxy risk factor leading to ADHD by independent mechanisms. Smoking during pregnancy is associated with poorer parenting skills which are associated with child behavioral problems.[43]

The present study has several strengths including being based on a large nationwide sample, assessing nicotine exposure with objective biological measurement and including several potential confounders. However, when interpreting the findings several limitations should be considered. A key question is if smoking during pregnancy is causally associated with ADHD or whether it is a proxy of another risk factor, e.g. familial confounding. Even if most of the

effect is due to familial or genetic confounding, the present study shows that the association between smoking and ADHD has a dose-response effect. The limitation of observational data is that we cannot examine causal processes. We did not have access to biomarkers of sibling pregnancies that would have shed more light on a possible causal link between nicotine exposure during pregnancy and offspring ADHD. However, in the present study we were able to adjust for several confounders including WGA, maternal SES, age, psychopathology and substance use disorder as well as paternal psychopathology. Information about possible substance use during pregnancy was restricted to register based substance use diagnoses. In the present study, 5.3 % of mothers of children with ADHD had a diagnosis of substance use disorder, which was similar to the estimated prevalence in Finland of 6.4% for the exposure to maternal alcohol and drug dependence.[44] The number of parents diagnosed with ADHD in this sample was low, which is a limitation of the study (Supplementary Tables 2,3). The underdiagnoses among parents could be primarily because ADHD was not a widely used diagnosis in the parental generation. In addition, it is unlikely that ADHD among parents was treated in inpatient care. Since the FHDR doesn't cover outpatient diagnoses before 1998, the diagnosis of ADHD among parents is likely underestimated. Finally, the ADHD subjects in this study included only those referred to specialized services, and likely represent the more severe cases with ADHD. However, a previous study has reported an 88% validity of the ADHD diagnoses in the FHDR examined against DMS-IV criteria for ADHD.[7]

Conclusion

According to the WHO, smoking is considered one of the main public health concerns worldwide.[45] The present study shows a strong association between prenatal nicotine exposure and a dose-dependent relationship with offspring ADHD. This study adds two important aspects; first, the use of cotinine as a documented measure of nicotine exposure during pregnancy and second, the finding of a dose-response effect in the association. Given the high

prevalence of both smoking during pregnancy and ADHD among children, these findings warrant future studies on the interplay between maternal smoking, and environmental, genetic and epigenetic factors.

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