Research Paper

Correlation of the methylomic signature of smoking during pregnancy with clinical traits in ADHD

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Background: Attention deficit/hyperactivity disorder (ADHD) is a highly prevalent childhood disorder. Maternal smoking during pregnancy is a replicated environmental risk factor for this disorder. It is also a robust modifier of gene methylation during the prenatal developmental period. In this study, we sought to identify loci differentially methylated by maternal smoking during pregnancy and relate their methylation levels to various behavioural and physical outcomes relevant to ADHD. **Methods:** We extracted DNA from blood samples from children diagnosed with ADHD and deeply phenotyped. Genome-wide DNA methylation was assessed using Infinium MethylationEPIC BeadChip. Maternal smoking during pregnancy was self-declared and assessed retrospectively. **Results:** Our sample included 231 children with ADHD. Statistically significant differences in DNA methylation between children exposed or not to maternal smoking during pregnancy were detected in 3457 CpGs. We kept 30 CpGs with at least 5% of methylation difference between the 2 groups for further analysis. Six genes were associated with varied phenotypes of clinical relevance to ADHD. The levels of DNA methylation in *RUNX1* were positively correlated with the CBCL scores, and DNA methylation in *MYO1G* correlated positively with the score at the Conners rating scale. Methylation level in a CpG located in *GFI1* correlated with birthweight, a risk factor for ADHD. Differentially methylated regions were also identified and confirmed the association of *RUNX1* methylation levels with the CBCL score. **Limitations:** The study has several limitations, including the retrospective recall with self-report of maternal smoking during pregnancy as well as the grouping of individuals of varying age and developmental stage and of both males and females. In addition, the correlation design prevents the building of causation models. **Conclusion:** This study provides evidence for the association between the level of methylation at specific loci and quantitative dimensions highly relevant for ADHD as well as birth weight, a measure that has already been associated with increased risk for ADHD. Our results provide further support to public health educational initiatives to stop maternal smoking during pregnancy.

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is one of the most common early-onset neurobehavioural disorders, with a worldwide prevalence of 5.9%–7.1% in the school-aged population.[1] It is a complex disorder involving both genetic and environmental factors.[2] The genetic factors include common DNA sequence variants (single nucleotide polymorphisms [SNPs]) with small effect size as well as rare copy number variants with larger effect size.[3] Genetic studies have reported the overall heritability of ADHD to be around 74%.[4] It is now well established that multiple genes contribute to the overall risk for ADHD, each with a small effect. A recent genome-wide association study (GWAS) meta-analysis identified 27 loci that showed significant association with ADHD.[5] Fine mapping of these risk loci implicates 76 potential genes with enriched expression in the brain (particularly in the frontal cortex) and during early development.

In addition, environmental factors are known to play an important role in ADHD.[6] There are consistent data indicating that the interplay between genetic and environmental factors plays a major role in the pathogenesis of this disorder, but a clear understanding of the implicated mechanisms is still lacking. Among environmental risk factors that are relatively well replicated, maternal smoking has been very widely discussed and studied. Three meta-analyses of more than 20 studies (more than 3 million participants) concluded that maternal smoking during pregnancy is associated with a

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greater than 50% increase in incidence of ADHD.[7–9] However, this association has been discussed by genetically designed studies, suggesting that genetic factors may increase both the risk for smoking and ADHD.[10,11]

Notwithstanding the question of causality, it is well documented that maternal smoking during pregnancy is associated with harmful effects on the developing fetus.[12] In a recent meta-analysis of studies conducted in the Americas, it was reported that active maternal smoking during pregnancy was associated with low birth weight (odds ratio [OR] 2.00, 95% confidence interval [CI] 1.77–2.26, I^2 = 66.3%).[13] Abraham and colleagues[14] conducted a systematic literature review and meta-analysis to better understand the gestational age at which maternal smoking during pregnancy becomes associated with reduced antenatal fetal size and growth. They noted that, by the second trimester, biparietal diameter and femur length were reduced by at least 0.06 standard deviations (SD), and all fetal measurements were reduced by 0.2 SD in the third trimester. Low birth weight has also been consistently associated with risk for ADHD. In a meta-analysis including 88 independent studies (4 645 482 participants), it was reported that low birth weight correlated with severity of ADHD symptoms.[15] These studies suggest that maternal smoking during pregnancy may exert pervasive effects on somatic and brain development of the fetus, though the mechanisms implicated in ADHD are still not well understood.

Several studies have suggested that epigenetic factors may also be involved in the pathophysiology of ADHD. They may explain why exposure to environmental stressors during the pre- and postnatal periods increases susceptibility to adverse health outcomes later in life. There is much scientific evidence that suggests that epigenetic modification may be an important mechanism linking environmental exposure to the onset of neurodevelopmental disorders.[16] Of the varied epigenetic mechanisms known to be involved, DNA methylation has been the most extensively examined within the ADHD context.[17] Several studies have investigated DNA methylation in relation to ADHD diagnosis or symptoms using candidate gene approaches or epigenome-wide association studies in peripheral blood and saliva tissue. Candidate-gene approaches have focused primarily on the monoaminergic system, given that blunted dopamine reward/motivation pathways in the striatum and prefrontal cortex have been implicated in the etiology of ADHD. Alterations in DNA methylation have been noted in studies examining the dopamine transporter gene (*DAT1*),[18–24] genes encoding dopamine receptors,[18,25–27] the serotonin transporter (5-HTT),[25,28] the norepinephrine transporter,[29] and catechol-*O*-methyltransferase (COMT).[25,30] However, as with genetic studies, epigenome-wide association studies (EWAS) have been favoured since the genome can be probed in a hypothesis-free manner.

The published EWAS on ADHD have largely been case–control studies comparing peripheral DNA methylation among different age groups: children,[31–33] adolescents[34] and adults.[35] Of these, only 1 detected genome-wide significant differences in DNA methylation between groups.[35] However, some promising targets have been identified, with the most notable example being *VIPR2* methylation. This gene encodes a receptor for vasoactive intestinal peptide, a small neuropeptide that plays a role in neuronal function. While the methylation of this gene has been implicated in ADHD in multiple clinical studies,[31–33] there have been inconsistent findings.[34,35] It has been suggested that the association is complex in its sex-dependence: boys with ADHD showed lower *VIPR2* methylation and girls with ADHD had higher methylation relative to controls.[33]

In addition, several studies provide evidence that the DNA methylation patterns correlated with ADHD show significant variability with age. Examining data from more than 800 children in the Avon Longitudinal Study of Parents and Children (ALSPAC), it was noted that DNA methylation patterns at birth differed between children who went on to follow a chronic high versus low ADHD symptom trajectory from age 7–15 years. However, none of the 13 sites that met genome-wide significance thresholds at birth were significant when methylation was measured at 7 years of age.[36] Similar findings were noted in a recent EWAS meta-analysis.[37] In addition, a large-scale EWAS over the first 2 decades of life noted that half of DNA methylation sites changed significantly in a nonlinear way over time.[38]

In contrast to the inconsistent findings between epigenetic markers and ADHD, many studies have reported a strong and consistent effect of smoking on the methylation of a large number of CpG sites. The largest EWAS meta-analysis to date evaluating the association of maternal smoking during pregnancy with cord blood DNA methylation at birth was conducted within the Pregnancy And Childhood Epigenetics (PACE) consortium and included 13 cohorts.[39] These analyses showed that 6073 CpGs had significant differential methylation with sustained maternal smoking during pregnancy. The CpGs most widely identified across EWAS from diverse ancestries are within coding or regulatory regions of *AHRR*, *GFI1*, *CYP1A1* and *MYO1G*.[40–44] Differential methylation within these genes persisted from birth to 17 years of age when the ALSPAC cohort was examined.[42]

In an earlier study, we reported that maternal smoking during pregnancy was associated with differential methylation at specific sites within *AHRR*, *GFI1* and *CYP1A1* among children with a diagnosis of ADHD.[45] Furthermore, hypomethylation of these *GFI1* sites correlated with lower birth weight and an increase in the number of ADHD symptoms. In a recent study, Miyake and colleagues[46] explored the association between prenatal smoking exposure, ADHD symptoms at preschool age and cord blood DNA methylation using the Hokkaido Study on Environment and Children's Health, a prospective birth cohort study. They selected the CpG sites of 5 genes: *AHRR*, *CYP1A1*, *ESR1*, *MYO1G* and *GFI1*. They confirmed the finding that hypomethylation at sites within *GFI1* was correlated with low birth weight.

While the association of *GFI1* methylation and birth weight appears to be a well-replicated finding, there seems to be a gap in the literature on the association between maternal smoking during pregnancy, DNA methylation and behavioural outcomes in children with ADHD. Earlier we had presented a framework to help break down the complexity of ADHD in which we underscored the importance of

examining quantitative phenotypes of clinical relevance instead of looking exclusively at the disorder as a diagnostic category.[47] The objective of the present study was to conduct an EWAS to systematically examine the effects of maternal smoking during pregnancy with behavioural and clinical phenotypes of direct relevance to ADHD.

Methods

Participants

Children with a diagnosis of ADHD were selected from the pharmaco-behavioural genetic (PBG) study (n = 780) conducted at the Douglas Mental Health University Institute (DMHUI, Montréal, Canada), which has been described previously.[48] Briefly, children aged 6–12 years were referred by schools, community social workers, family doctors and pediatricians to the Disruptive Behaviour Disorders Program and pediatric outpatient clinics of the DMHUI. The research protocol was approved by the Research Ethics Board of the DMHUI. After explanation of the study, parents provided written consent, and children gave their verbal assent.

Diagnosis of ADHD was made by a child psychiatrist, according to the DSM-IV criteria, based on clinical interviews of the child and at least 1 parent. The diagnosis was supported with a comprehensive structured clinical interview (with the parents), the Diagnostic Interview Schedule for Children-version IV (DISC-IV), that covers 36 mental health disorders for children.[49]

In addition to ADHD diagnosis, the child's behaviour at home and at school were evaluated by parents using Conners' Global Index-Parents version (Conners'-P) and by teachers using Conners' Global Index-Teacher version (Conners'-T), respectively.[50] For a child to be included in the PBG study, at least 1 Conners'-P/T subscore had to be 65 or higher. The Child Behaviour Checklist (CBCL) was used to quantify several behavioural dimensions. The CBCL comprises 118 items and measures 3 dimensions (internalizing, externalizing and total problems). Internalizing behaviours refer to mood disturbance, including anxiety, depression and social withdrawal. Externalizing behaviours reflect conflict with others and violation of social norms. Finally, full IQ was measured using the Wechsler Intelligence Scale for Children (WISC).

For the present study, children with ADHD were selected based on the extreme end of the distribution: in the maternal smoking during pregnancy category, only children whose mothers had smoked 10 or more cigarettes per day throughout the duration of the pregnancy were selected. Final inclusion in our study sample was based on the DNA availability. There were no selection criteria based on symptom or behavioural severity.

Assessment of maternal smoking during pregnancy

Assessment of maternal smoking during pregnancy occurred at the baseline evaluation using the Kinney Medical and Gynaecological Questionnaire, which is a global assessment of exposure to pre- and perinatal environmental risk factors.[51] Mothers retrospectively reported smoking during each trimester of the pregnancy. The mother's report was corroborated in a separate interview with the child's father or by others who were present during the pregnancy, including grandparents, whenever possible. In order to obtain information on the number of cigarettes smoked per day, the Fagerström Test, a validated questionnaire for nicotine dependence, was added.[52] For birth weight, the vaccination booklet issued by the hospital at the birth of the child was used in the analysis.

Methylation

Genomic DNA extracted from peripheral blood was sent to the McGill University and Génome Québec Innovation Centre for treatment with sodium bisulfite. Genome-wide DNA methylation was assessed using Infinium MethylationEPIC BeadChip, which interrogates the DNA methylation profile of 867 532 CpG loci across the genome at a single-locus resolution.

Data preprocessing and clean up

Illumina GenomeStudio software was used to extract signal intensities for each probe (IDAT files). A probe corresponds to a CpG. All computations and statistical analyses were performed within the R x64 4.0.0 statistical analysis environment (http://www.r-project.org).

A first quality check was performed using the R package minfi. Samples with a bad ratio between methylated and unmethylated probes were removed (Appendix 1, Figure 1, available at www.jpn.ca/lookup/doi/10.1503/jpn.230062/tab-related-content). Then, the R package Chip Analysis Methylation Pipeline (ChAMP) was used (https://www.bioconductor.org/packages/release/bioc/vignettes/ChAMP/inst/doc/ChAMP.html). The fraction of failed positions per sample was checked (mean = 0.00034; maximum = 0.0008). The multidimensional scaling (MDS) plot is shown in Appendix 1, Figure 2, and the density plot in Appendix 1, Figure 3. No bias was detected. Filtering probes with a detection p value greater than 0.01 in 1 or more samples has removed 6183 probes from the analysis. Filtering probes with a beadcount less than 3 in at least 5% of samples removed 169 probes from the analysis. Filtering non-CPG probes removed a further 2977 from the analysis. According to Zhou,[53] 96 190 probes corresponding to SNPs were removed; 11 supplementary probes that align to multiple locations[54] were removed. Filtering probes on the X or Y chromosome removed 16621 CpGs from the analysis. Further analyses proceeded with 743 767 probes and 231 samples.

Normalization was performed using the Beta-Mixture Quantile (BMIQ) method. The singular value decomposition method (SVD)[55] for methylation data was used to identify the most significant components of variation, in particular to detect batch effect. Slide and array effects were detected and corrected using Combat package (Appendix 1, Figure 4). Cell type heterogeneity was corrected using the RefbaseEWAS method that infers changes in the distribution of white blood

cells between different subpopulations using DNA methylation signatures, in combination with a previously obtained external validation set consisting of signatures from purified leukocyte samples. All scripts are available on request from the authors.

Association analysis

An association analysis was conducted with the limma R package to identify differentially methylated probes (DMP) with a linear model adjusted on age and sex. Multiple testing correction using false discovery rate (FDR) was performed. Manhattan plots and QQ plots were drawn in R using the qqman package. Differential methylated regions (DMR) were detected using bumphunter and ProbeLasso, both with the default parameters. Multiple testing correction use the family-wise error (FWE) method.

Correlation analysis with clinical data

The corrplot R package was used to test the Pearson correlation of each significant DMP and the mean of the DMR with the Conners' scale (parent and teacher versions), the CBCL (internalized, externalized and total scores), the total number of items in the DISC, the total IQ and the birth weight. The correction for multiple testing was done using FDR correction (290 tests).

Results

We selected 232 children with ADHD for inclusion in our study. One child was excluded owing to DNA samples with a bad ratio between methylated and unmethylated probes (Appendix 1), leaving a final sample of 231 children. Methylomic data from 80 children exposed to maternal smoking during pregnancy was compared with methylomic data from 151 nonexposed children (Table 1). We identified 3457 CpGs significantly associated with smoking during pregnancy ($q < 0.05$ after correction for multiple testing; Manhattan plot in Figure 1; qq-plot in Appendix 1, Figure 5). We kept 30 CpGs harboring a methylation difference greater than 5% or less than –5% between the 2 groups for further analyses (Table 2). Then, we tested these 30 CpGs with the clinical data (Table 3; see an example of correlation in Appendix 1, Figure 6). Birth weight was significantly and positively correlated with the methylation levels in 1 CpG located in *GFI1* and 3 CpGs in *NRP2* and negatively correlated with 1 CpG in *ZNF536*. The significant CpGs were submitted to the web application BECon (Blood-Brain Epigenetic Concordance; https://redgar598.shinyapps.io/BECon/) to test the concordance between blood and 3 brain areas: Brodmann area (BA)10 (frontal cortex), BA20 (temporal cortex) and BA7 (parietal cortex) (Appendix 1, Figure 7). The best correlations were obtained for CpGs located in *CYP1A1* and in *RUNX1* (correlation coefficient > 0.7).

The externalizing CBCL score was significantly and positively correlated with the methylation levels in 7 CpGs located in *RUNX1* and 4 CpGs located in *MYO1G*. The level of methylation of 6 of the 7 CpGs located in *RUNX1* also correlated positively with the internalizing CBCL score. These 7 CpGs in *RUNX1* were positively correlated with the total score on the CBCL. The CpG located in *RUNX1* and harboring a very good correlation between the frontal cortex and the blood (cg00994804) was significantly associated with the score on the CBCL (Table 2 and Appendix 1, Figure 7). The CpG located in *ETS2* was negatively correlated with both the externalizing CBCL score and the total CBCL score. Regarding the Connors' scale (parent or teacher versions), the total score was significantly and positively correlated with the level of methylation of *MYO1G*. There was no correlation between the methylation levels in the DMP and IQ, nor with the number of DISC items.

Seven DMRs were identified (Table 4). We tested the mean level of methylation of the DMR with the score at the clinical scales. The mean methylation level in the DMR encompassing *RUNX1* was associated with the externalizing, internalizing and total CBCL score. Birth weight was negatively correlated with the methylation of *BHMT2* and positively

Table 1: Demographic and clinical characteristics of the study sample

Characteristic	MSDP group, mean ± SD* $n = 80$	No MSDP group, mean ± SD* $n = 151$	p value†
Gender, no. M/F	68/12	124/27	0.58
Age, yr	7.67 (1.47)	8.27 (2.53)	0.54
Birth weight, g	3244 (613)	3430 (650)	0.03
Conners' Global Index–Teachers, baseline score	68.4 (12.5)	68.3 (11.8)	0.94
Conners' Global Index–Parents, baseline score	75.2 (10.2)	73.5 (11.0)	0.27
CBCL, internalizing score	64.1 (10.3)	65.3 (9.9)	0.42
CBCL, externalizing score	69.7 (10.5)	69.0 (9.3)	0.58
CBCL, total score	69.7 (9.2)	69.9 (7.8)	0.86
DISC, total no. of ADHD items	2.3 (0.9)	2.3 (0.7)	0.88
IQ	98.5 (13.9)	95.7 (12.6)	0.14

ADHD = attention-deficit/hyperactivity disorder; CBCL = Child Behaviour Checklist; DISC = Diagnostic Interview Schedule for Children; F = female; IQ = intelligence quotient; M = male; MSDP = maternal smoking during pregnancy; SD = standard deviation.
*Unless otherwise specified.
†χ^2 for gender and t test for all other items.

correlated with the methylation of *NRP2*. The Conners' scale was significantly and positively correlated with the mean methylation level in *FRMD4*. There was no correlation between the mean level of methylation in the DMR and IQ, nor with the number of DISC items.

Discussion

Using an epigenome-wide association study, we first confirmed that maternal smoking during pregnancy has an effect on DNA methylation in the children. We replicated some findings (hypomethylation of *NRP2*, hypermethylation of *BHMT2*),[39] while findings for other genes, including *RUNX1*, were novel. In an earlier report with a small sample, we had noted an association between maternal smoking during pregnancy and differential methylation at specific sites within *AHRR*, *GFI1* and *CYP1A1*.[45] Again, we identified these genes among others and we further investigated the clinical consequences of maternal smoking during pregnancy by correlating the DNA methylation levels with dimensional phenotypes.

The association of birth weight with methylation at *GFI1* confirms previous reports.[45,46] Here we also report the novel association with birth weight where there was exposure to prenatal smoking during pregnancy, with *NRP2* and *ZNF536*. *NRP2* encodes a transmembrane protein, belonging to the neuropilin family of receptor proteins. It is a receptor for specific class 3 semaphorins and interacts with vascular endothelial growth factor. Semaphorins are polypeptides that are essential for axonal guidance, have been implicated in a broader role in the maintenance and stability of neuronal networks.[56] *ZNF536* is expressed in the developing central nervous system and encodes a highly conserved zinc finger protein that is most abundant in the brain, showing negative regulation of neuron differentiation.[57] Given the important role of *NRP2* and *ZNF536* in neuronal networks, molecular studies are warranted to help elucidate the epigenetic modulation that results from prenatal tobacco exposure and its connection to birth weight and ADHD.

Analysis with behavioural phenotypes implicated *RUNX1* with CBCL scores. *Runx1* is a transcription factor playing an important role in the developing nervous system and has been shown to be involved in the development of selected motor neurons, as well as gene expression programs regulating neuronal subtype specification and axonal connectivity.[58] A gene from the same family, *RUNX2*, has been associated with maternal smoking during pregnancy in a meta-analysis conducted by the PACE consortium.[39] *RUNX1* is a very interesting candidate, as the DNA methylation levels correlated well between blood and the prefrontal cortex. A correlation between methylation level and CBCL and Conners' scores was also noted with *MYO1G*. This gene has been recently highlighted as differentially expressed in ADHD by a transcriptomic analysis in the anterior cingulate cortex.[59]

The remarkable consistency of DNA methylation marks related to maternal smoking during pregnancy are in stark contrast to the more dynamic changes observed in analysis of DNA methylation and ADHD in the literature. Knowledge about the environmental factors is an asset to detect relevant

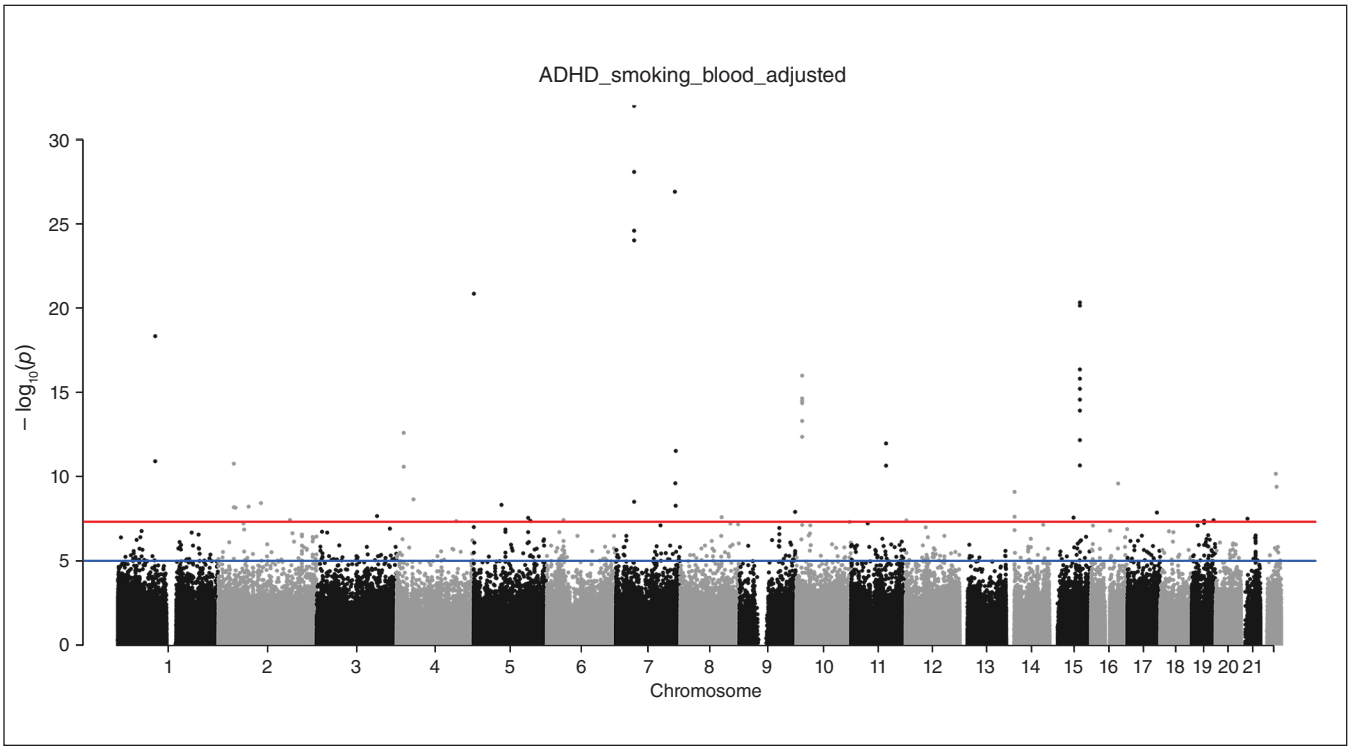

Figure 1: Manhattan plot of the methylation epigenome-wide association study comparing children with attention-deficit/hyperactivity disorder (ADHD) who were exposed or not exposed to maternal smoking during pregnancy.

Table 2: Differential methylation probe analysis between children who have been exposed to maternal smoking during pregnancy and those who have not been exposed

CpG	CHR	BP	Gene	Feature	Localization	*p* value	*q* value	Δ_β, %
cg14179389	1	92947961	*GFI1*	Body	island	$3.93 \times E^{-19}$	$3.25 \times E^{-14}$	–5.9
cg04198471	2	38325317		IGR	opensea	$1.05 \times E^{-11}$	$2.90 \times E^{-7}$	5.3
cg14157435	2	206628692	*NRP2*	Body	opensea	$3.59 \times E^{-7}$	0.0022233	–6.1
cg20351668	2	206628737	*NRP2*	Body	opensea	$8.26 \times E^{-7}$	0.00359426	–5.6
cg25715429	2	206628747	*NRP2*	Body	opensea	$3.94 \times E^{-7}$	0.00234309	–6
cg07616871	2	218843504		IGR	island	$8.82 \times E^{-7}$	0.00366351	5.4
cg05857999	6	31650760		IGR	island	$5.92 \times E^{-5}$	0.02859968	5.4
cg01856384	6	41376904		IGR	island	$2.09 \times E^{-8}$	0.00028735	5.1
cg19089201	7	45002287	*MYO1G*	3′UTR	island	$8.56 \times E^{-25}$	$1.27 \times E^{-19}$	7.6
cg04180046	7	45002736	*MYO1G*	Body	island	$3.90 \times E^{-33}$	$2.90 \times E^{-27}$	10
cg12803068	7	45002919	*MYO1G*	Body	shore	$7.46 \times E^{-29}$	$2.77 \times E^{-23}$	13.3
cg05009104	7	45002980	*MYO1G*	Body	shore	$1.93 \times E^{-25}$	$4.79 \times E^{-20}$	7.9
cg19796617	7	147983431	*CNTNAP2*	Body	opensea	$7.54 \times E^{-5}$	0.03175132	6.8
cg05640346	7	148038174	*CNTNAP2*	Body	shore	$2.30 \times E^{-12}$	$7.12 \times E^{-8}$	6.6
cg13750264	10	134910540	*GPR123*	Body	shore	$9.20 \times E^{-7}$	0.00371914	–7.2
cg21199085	15	59469479	*MYO1E*	Body	opensea	$2.07 \times E^{-8}$	0.00028735	6.4
cg05549655	15	75019143	*CYP1A1*	TSS1500	island	$2.93 \times E^{-22}$	$3.63 \times E^{-17}$	5.2
cg13570656	15	75019196	*CYP1A1*	TSS1500	island	$3.16 \times E^{-15}$	$1.47 \times E^{-10}$	5.7
cg12101586	15	75019203	*CYP1A1*	TSS1500	island	$1.48 \times E^{-14}$	$6.12 \times E^{-10}$	5.2
cg22549041	15	75019251	*CYP1A1*	TSS1500	island	$5.67 \times E^{-17}$	$3.51 \times E^{-12}$	7.3
cg18092474	15	75019302	*CYP1A1*	TSS1500	island	$2.20 \times E^{-16}$	$1.26 \times E^{-11}$	6.1
cg23458168	19	30864867	*ZNF536*	5′UTR	shore	$2.47 \times E^{-8}$	0.00032232	5.2
cg03142697	21	36258497	*RUNX1*	Body	shore	$3.00 \times E^{-7}$	0.00199603	6.8
cg26974661	21	36258596	*RUNX1*	Body	shore	$1.38 \times E^{-7}$	0.00115178	6.5
cg02869559	21	36259067	*RUNX1*	Body	island	$2.75 \times E^{-6}$	0.00687953	5.3
cg12477880	21	36259241	*RUNX1*	1stExon	island	$2.44 \times E^{-6}$	0.00648155	6.7
cg00994804	21	36259383	*RUNX1*	1stExon	island	$7.24 \times E^{-7}$	0.00338816	6.9
cg06758350	21	36259460	*RUNX1*	1stExon	island	$3.74 \times E^{-7}$	0.00228233	5.1
cg09889857	21	36259618	*RUNX1*	1stExon	shore	$2.05 \times E^{-6}$	0.00579365	5.4
cg01359822	21	40176597	*ETS2*	TSS1500	shore	$9.00 \times E^{-5}$	0.03422828	–5.3

Δ_β = difference in methylation β values in group with maternal smoking during pregnancy minus group with no maternal smoking during pregnancy; BP = base pair (locus); CHR = number of the chromosome; IGR = intergenic region; *q* value = false discovery rate–corrected *p* value; UTR = untranslated region.

DNA methylation marks and correlate them with clinical symptoms. Notwithstanding the causality issue of maternal smoking during pregnancy and ADHD, there is a need to better understand the effects of this important prenatal exposure on the developing fetus for its long-term effects. Previously we had presented a framework to help break down the complexity of ADHD and suggested that stratification of a sample based on exposure to environmental factors, including maternal smoking during pregnancy, could help provide unique insight into the disorder.[47] We also underscored the importance of examining quantitative phenotypes of clinical relevance. The present study encapsulates both these features, and sheds novel insight of pathways that may be involved in the etiology of ADHD, in the subgroup of children whose mothers smoked during pregnancy. One of the specific advantages of this study is that the EWAS was conducted with multiple quantitative assessments of ADHD diagnosis, parent/teacher reports of behaviour using the Conners' and the CBCL. These analyses extend our understanding of pathways that may be affected in children who were exposed to maternal smoking during pregnancy and hint at implicated mechanisms in adverse behavioural outcomes.

Table 3: Correlation between clinical dimensions and the level of methylation of the significant differential methylated CpG

Clinical item	DMP	Gene	Pearson correlation	*p* value	*q* value
birthwt	cg14179389	*GFI1*	0.25	$1.30 \times E^{-5}$	0.0001421
birthwt	cg04198471	intergenic	–0.25	0.0017	0.01036346
birthwt	cg23458168	*ZNF536*	–0.21	0.0047	0.02327969
birthwt	cg14157435	*NRP2*	0.15	0.0017	0.01036346
birthwt	cg20351668	*NRP2*	0.14	0.0018	0.01076604
birthwt	cg25715429	*NRP2*	0.15	0.0012	0.00826957
cbclext	cg00994804	*RUNX1*	0.26	0.00051	0.00414539
cbclext	cg02869559	*RUNX1*	0.23	0.00057	0.00451725
cbclext	cg09889857	*RUNX1*	0.21	0.00063	0.00483048
cbclext	cg26974661	*RUNX1*	0.22	0.00072	0.00530791
cbclext	cg12477880	*RUNX1*	0.24	0.00077	0.0055475
cbclext	cg03142697	*RUNX1*	0.21	0.0013	0.00876809
cbclext	cg06758350	*RUNX1*	0.22	0.0016	0.01035102
cbclext	cg12803068	*MYO1G*	0.27	0.0021	0.01210364
cbclext	cg19089201	*MYO1G*	0.25	0.0044	0.02249677
cbclext	cg05009104	*MYO1G*	0.23	0.0049	0.02353485
cbclext	cg04180046	*MYO1G*	0.23	0.0085	0.03742361
cbclext	cg07616871	intergenic	0.13	0.0091	0.03951644
cbclext	cg01359822	*ETS2*	–0.24	0.0016	0.01035102
cbclint	cg00994804	*RUNX1*	0.22	0.00093	0.00655133
cbclint	cg12477880	*RUNX1*	0.18	0.0038	0.01974754
cbclint	cg26974661	*RUNX1*	0.19	0.0047	0.02327969
cbclint	cg02869559	*RUNX1*	0.19	0.0049	0.02353485
cbclint	cg09889857	*RUNX1*	0.18	0.0053	0.02507612
cbclint	cg06758350	*RUNX1*	0.18	0.0067	0.03078116
cbcltot	cg00994804	*RUNX1*	0.28	$1.00 \times E^{-4}$	0.00105667
cbcltot	cg02869559	*RUNX1*	0.26	0.00022	0.00199257
cbcltot	cg12477880	*RUNX1*	0.25	0.00033	0.00290583
cbcltot	cg09889857	*RUNX1*	0.23	0.00046	0.00383737
cbcltot	cg26974661	*RUNX1*	0.24	0.00046	0.00383737
cbcltot	cg06758350	*RUNX1*	0.24	0.00064	0.00483048
cbcltot	cg03142697	*RUNX1*	0.22	0.0017	0.01036346
cbcltot	cg01359822	*ETS2*	–0.19	0.0021	0.01210364
conntop	cg05009104	*MYO1G*	0.2	0.0033	0.01849167
conntop	cg12803068	*MYO1G*	0.19	0.0056	0.02610588
conntop	cg01856384	intergenic	0.18	0.0082	0.03713429
conntop	cg04180046	*MYO1G*	0.19	0.0085	0.03742361
conntop	cg19089201	*MYO1G*	0.18	0.011	0.04712162

birthwt = birthweight; CBCL = Child Behaviour Checklist; cbclext = CBCL, externalizing score; cbclint = CBCL, internalizing score; cbcltot = CBCL total score; conntopb = Conners' Global Index–Parents; DMP = differential methylation probe; *q* value = false discovery rate–corrected *p* value.

Limitations

This study has limitations. The correlative design prevents us from establishing causality and leaves it unclear whether the identified DNA methylation patterns represent an antecedent (e.g., reflecting genetic or environmental risk factors for ADHD), a mere correlate (e.g., due to smoking or other behaviours associated with ADHD) or a consequence (e.g., as a result of medication use or as part of the disease process itself). The other limitation of the study was that the information on maternal smoking during pregnancy was obtained through self-reports and was based on retrospective recall. While there was an attempt to obtain partner and/or family member verification of maternal smoking, the information may still be subject to recall bias. Other potential biases can emerge from the cohort that groups samples of varying age and developmental stage, as well as both males and females. We adjusted for sex and age, and excluded sex chromosome, but we cannot exclude sex-specificity. Moreover, we did not assess the type of medications the children were taking, the duration of the illness or of the drug exposure. All these factors can influence DNA methylation,[59,60] but it is unlikely that

Table 4: Differential methylated regions analysis between children exposed to maternal smoking during pregnancy and those who were not exposed, and correlation with the clinical scales

Method	CHR	Start	End	Length	Δ_β, %	DMR *p* value	Gene	Clinical score	Pearson correlation	*p* value correlation	*q* value correlation
Bumphunter	chr7	27183133	27184853	1720	8.4	0.00022837	*HOXA5 HOXA-AS3*				
Bumphunter	chr21	36259067	36259797	730	34.0	0.000262203	*RUNX1*	cbclext	0.24	0.00077	0.0040425
								cbclint	0.16	0.0058	0.02342308
								cbcltot	0.23	0.00049	0.00270789
Bumphunter	chr6	31650735	31651411	676	22.1	0.000312952	*LY6G5C*				
Bumphunter	chr15	75018700	75019376	676	21.1	0.000405992	intergenic				
ProbeLasso	chr10	14371289	14373249	1961	3.1	$2.19 \times E^{-57}$	*FRMD4A*	conntopb	0.2	0.0079	0.02860345
ProbeLasso	chr2	206627089	206630312	3224	–2.2	0.001044314	*NRP2*	birthwt	0.12	0.0022	0.01004348
ProbeLasso	chr5	78365574	78366027	454	3.1	$5.36 \times E^{-33}$	*DMGDH BHMT2*	birthwt	–0.19	0.0062	0.02411111

birthwt = birthweight; CBCL = Child Behaviour Checklist; cbclext = CBCL, externalizing score; cbclint = CBCL, internalizing score; cbcltot = CBCL total score; conntopb = Conners' Global Index–Parents; DMR = differential methylated regions; *q* value = false discovery rate–corrected *p* value.

they dysmethylate the same genes as maternal smoking during pregnancy. Future longitudinal studies should consider detailed environmental recording. In addition, the results obtained here relate to a clinical sample of children with a diagnosis of ADHD; we did not assess a control sample at the same time.

Conclusion

While our study results are interesting, they need to be confirmed in large, independent samples of children with ADHD. It will also be interesting to examine whether methylation changes in these peripheral blood markers are observed across the lifetime, in longitudinal studies of individuals with ADHD. Although further work is warranted, our results provide additional empirical evidence to help underscore the importance of preventing maternal smoking during pregnancy.

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