



OPEN DNA methylation is associated with prenatal exposure to sulfur dioxide and childhood attention-deficit hyperactivity disorder symptoms

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Epigenetic influence plays a role in the association between exposure to air pollution and attention deficit hyperactivity disorder (ADHD); however, research regarding sulfur dioxide (SO₂) is scarce. Herein, we investigate the associations between prenatal SO₂ exposure and ADHD rating scale (ARS) at ages 4, 6 and 8 years repeatedly in a mother–child cohort (n = 329). Whole blood samples were obtained at ages 2 and 6 years, and genome-wide DNA methylation (DNAm) was analyzed for 51 children using the Illumina Infinium HumanMethylation BeadChip. We analyzed the associations between prenatal SO₂ exposure and DNAm levels at ages 2 and 6, and further investigated the association between the DNAm and ARS at ages 4, 6 and 8. Prenatal SO₂ exposure was associated with ADHD symptoms. From candidate gene analysis, DNAm levels at the 6 CpGs at age 2 were associated with prenatal SO₂ exposure levels. Of the 6 CpGs, cg07583420 (*INS-IGF2*) was persistently linked with ARS at ages 4, 6 and 8. Epigenome-wide analysis showed that DNAm at 6733 CpG sites were associated with prenatal SO₂ exposure, of which 58 CpGs involved in Notch signalling pathway were further associated with ARS at age 4, 6 and 8 years, persistently. DNAm at age 6 was not associated with prenatal SO₂ exposure. Changes in DNAm levels associated with prenatal SO₂ exposure during early childhood are associated with increases in ARS in later childhood.

Abbreviations

ADHD Attention deficit hyperactivity disorder
ARS ADHD rating scale

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DNAm	DNA methylation
ETS	Environmental tobacco smoking
EWAS	Epigenome-wide association study
FDR	False discovery rate
<i>GFI1</i>	Growth factor-independent 1 transcriptional repressor
GO	Gene ontology
IQR	Interquartile range
NK	Natural killer

Attention deficit hyperactivity disorder (ADHD) is a neurobehavioral disorder characterized by inattentiveness, hyperactivity, and impulsivity¹. Children with ADHD have difficulties in learning, family relationships, and social interaction². ADHD affects 5–7% of children and adolescents worldwide^{3,4}. Furthermore, at least 5% of children do not meet the full diagnostic criteria despite exhibiting ADHD symptoms⁵. ADHD in children often persists into late adolescence and adulthood, which is a risk factor of other mental health issues, including antisocial behaviors, self-harm, and substance misuse⁵.

Although the etiology of ADHD is largely unknown and complex, it is characterized by numerous gene-environmental interactions⁶. Preconceptional, gestational, and perinatal conditions have been indicated to affect ADHD⁶. Maternal nutrition during pregnancy, pesticides and heavy metal exposure during gestation, premature birth have been reported as risk factors of ADHD in children⁶. Prenatal exposure to air pollution is also a potential risk factor for ADHD^{7,8}. Previous studies have reported an association between children's behavioral development and prenatal exposure to sulfur dioxide (SO₂)^{9,10}, one of the major gaseous pollutants derived from coal-fired power plants, smelters, and industrial emissions¹¹. Although the relation between SO₂ exposure and ADHD has not been well investigated, SO₂ has been associated with various neurodevelopmental deficits, such as fine motor skills, executive function, and ADHD-related hospital admissions^{12,13}.

Epigenetic processes such as DNA methylation (DNAm) have been proposed to underly the association between environmental exposures and ADHD¹⁴. A previous study has demonstrated that methylation differences of the growth factor-independent 1 transcriptional repressor (*GFI1*) region partially mediated the association between maternal smoking during pregnancy and ADHD symptoms at age 6¹⁵. Another study found that long-term prenatal exposure to paracetamol (acetaminophen) is associated with DNAm differences in children diagnosed with ADHD¹⁶. Recent evidence suggests that air pollution may affect methylation through an oxidative stress pathway^{17,18}. Air pollution-induced reactive oxygen species oxidize 5-hydroxymethylcytosine causing DNA demethylation¹⁹. It also leads to the hypomethylation of CpG cytosine residues by inhibiting the activity of methyltransferases via alteration in their sequence alignment to the corresponding base sequences of the DNA²⁰. To date, no study has investigated the mediation effect of DNAm on the association between SO₂ and ADHD.

In this study, we aimed to (1) examine the association between prenatal exposure to SO₂ and ADHD symptoms at multiple ages during childhood and (2) investigate the effect of DNAm on the association between SO₂ exposure and ADHD symptoms. We first analyzed the association between prenatal SO₂ and ADHD symptoms at ages 4, 6 and 8, which are critical periods of symptom manifestation. Then, we identified CpG sites (CpGs) associated with both prenatal SO₂ exposure and ADHD symptoms during childhood. We utilized both candidate gene analysis approach by targeting CpGs associated with ADHD symptoms, and an epigenome-wide association study. We also aimed to detect co-methylated CpGs from a module correlated with both prenatal SO₂ exposure and ADHD symptoms using weighted gene co-methylation network analysis (WGCNA).

Results

General characteristics of study participants. There were significant differences in maternal smoking and SO₂ exposure during the 1st trimester between the entire cohort and the sub-cohort (Table 1). Other covariates were not significantly different between the entire cohort and sub-cohort. The mean age of mothers at pregnancy was 31.3 years, most of the mothers were college graduates (70.5–71.7%), while 76.9–79.6% of mothers were exposed to environmental tobacco smoking (ETS) during pregnancy. Mean maternal IQ was 116–118. Mean maternal SO₂ exposure during pregnancy was 0.0044–0.0060 ppm depending on the trimester. Among children, 51.0–53.5% were girls, 86.3–90.3% were singleton, 88.0–91.8% were born full-term. Mean postnatal SO₂ exposure ranged from 0.052 to 0.058 ppm depending on the ages. Mean ADHD rating scale (ARS) was 5.96–6.96 at age 4, 5.87–6.04 at age 6, and 5.57–6.34 at age 8. In the sub-cohort, prenatal and postnatal SO₂ exposure were not significantly different according to the trimester or ages (Fig. 1).

Selection of candidate CpGs. After the systematic review, a total of 22 studies were selected^{17,21–41} (Table S1). A total of 597 CpGs were pooled from these studies (Table S2), and final 375 CpGs were selected for analysis. The detailed result of the systematic review and selection of candidate CpGs is presented in Supplementary Materials and Fig. S1.

Association between prenatal exposure to SO₂ and ADHD symptoms. In the entire cohort (n = 329), one interquartile range (IQR) increase in prenatal SO₂ exposure during the 1st trimester of pregnancy was significantly associated with 8.38% (95% CI 3.19, 13.83) increase in ARS at age 4; however, such association was not observed at age 6 or 8 (Table 2). The associations between prenatal SO₂ exposure during the entire period or 2nd trimester of pregnancy and childhood ADHD symptoms at ages 4, 6 and 8 were not significant. Prenatal SO₂ exposure during the 3rd trimester of pregnancy was not associated with ADHD symptoms at ages 4 or 6, but was negatively associated with ADHD symptoms at age 8.

Participants	Variables	Category	Total cohort (n = 329)	Sub-cohort (n = 51)	p-value
Mother	Age at pregnancy		31.5 ± 3.51	31.3 ± 3.53	0.670
	Educational level	Middle school graduate	1 (0.3%)	0	< 0.001
		High school graduate	55 (16.7%)	7 (13.7%)	
		College graduate	236 (71.7%)	36 (70.5%)	
		Graduate school	37 (11.2%)	8 (15.75)	
	Smoking status during pregnancy	Never smoked	250 (76.0%)	27 (54.0%)	< 0.001
		Smoked before pregnancy	61 (18.5%)	18 (36.0%)	
		Smoked during pregnancy	6 (1.82%)	5 (10.0%)	
	Environmental tobacco smoking	Never exposed	62 (18.8%)	10 (20.5%)	0.906
		Exposed	253 (76.9%)	39 (79.6%)	
IQ		116 ± 11.1	118 ± 11.2	0.257	
SO ₂ exposure (ppm)	1st-3rd trimester	0.0054 ± 0.0015	0.0053 ± 0.0012	0.516	
	1st trimester	0.0052 ± 0.0016	0.0044 ± 0.0014	< 0.001	
	2nd trimester	0.0056 ± 0.0019	0.0058 ± 0.0015	0.408	
	3rd trimester	0.0056 ± 0.0020	0.0060 ± 0.0014	0.088	
Child	Sex	Male	176 (53.5%)	25 (49.0%)	0.551
		Female	153 (46.5%)	26 (51.0%)	
	Season of birth	Spring	69 (21.0%)	9 (17.6%)	< 0.001
		Summer	115 (35.0%)	31 (60.8%)	
		Autumn	78 (23.7%)	11 (21.6%)	
		Winter	67 (20.4%)	0	
	Multiple birth	Singleton	297 (90.3%)	44 (86.3%)	0.381
		Multiple birth	32 (9.73%)	7 (13.7%)	
	Preterm (<37 weeks)	No	301 (91.8%)	44 (88.0%)	0.379
		Yes	27 (8.23%)	6 (12.0%)	
	SO ₂ exposure (ppm)	2 years	0.0057 ± 0.0014	0.0053 ± 0.0013	0.031
		4 years	0.0058 ± 0.0013	0.0056 ± 0.0013	0.292
		6 years	0.0052 ± 0.0011	0.0053 ± 0.00089	0.507
	ADHD rating scale	4 years	6.96 ± 5.95	5.96 ± 4.61	0.171
		6 years	5.87 ± 5.47	6.04 ± 6.55	0.863
		8 years	6.34 ± 5.53	5.57 ± 5.43	0.351
	Cell type fraction	CD4 T cell	NA	0.158 ± 0.0430	NA
CD8 T cell		NA	0.203 ± 0.0445	NA	
NK cell		NA	0.0415 ± 0.0386	NA	
B cell		NA	0.184 ± 0.0470	NA	
Monocyte		NA	0.0462 ± 0.0257	NA	
Neutrophil		NA	0.359 ± 0.0858	NA	

Table 1. General characteristics of study participants.

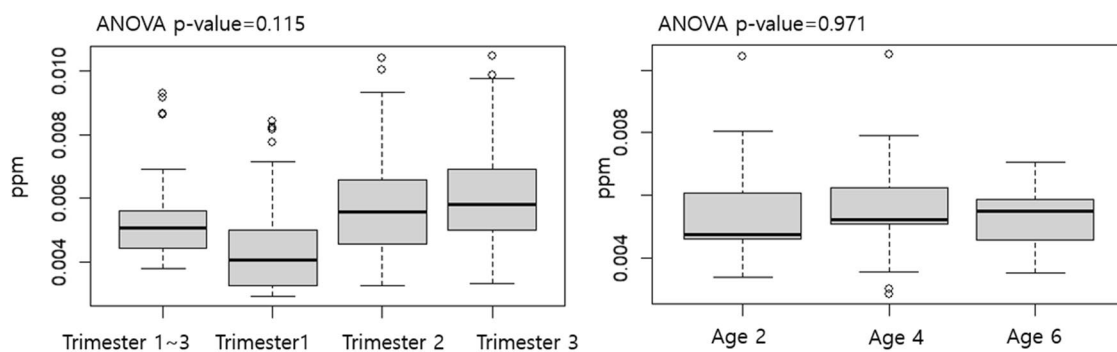


Figure 1. The prenatal SO₂ exposure according to the trimester (left) and postnatal SO₂ exposure according to the ages of 2, 4 and 6 years (right).

Trimester	% Change (95% CI)		p-value	% Change (95% CI)		p-value
	4 years	6 years		8 years	6 years	
1st–3rd	2.68 (– 0.62, 6.09)	0.113	– 1.14 (– 4.85, 2.71)	0.557	0.23 (– 3.28, 3.86)	0.901
1st	8.38 (3.19, 13.83)	0.001	3.97 (– 1.69, 9.95)	0.173	5.09 (– 0.41, 10.91)	0.07
2nd	3.68 (– 1.67, 9.32)	0.181	– 3.66 (– 9.33, 2.35)	0.227	4.04 (– 1.59, 10)	0.163
3rd	– 1.91 (– 7.14, 3.63)	0.492	– 4.68 (– 10.45, 1.46)	0.132	– 7.39 (– 12.73, – 1.73)	0.011

Table 2. Prenatal SO₂ exposure and ADHD rating scale at 4, 6 and 8 years of age in the cohort (n = 329). Adjusted for maternal age at pregnancy, mother's education levels, maternal smoking, maternal environmental tobacco smoking, season of child's birth.

Association between prenatal exposure to air pollution and DNAm. DNAm at age 2 at the candidate CpGs was not associated with prenatal SO₂ exposure at the 1st–3rd, 1st, or 2nd trimester of pregnancy (Tables S3–S5). However, DNAm in children at age 2 at cg07583420 (*INS-IGF2*), cg20296524 (*TARBP1*), cg15705054 (*PBXIP1*), cg05075097 (*INS-IGF2*), and cg25163476 (*INS-IGF2*) were positively associated with an IQR increase in maternal SO₂ exposure during the 3rd trimester of pregnancy (Table 3, Fig. 2A, Table S6). DNAm at cg05951817 (*SLC6A4*) was negatively associated with SO₂ exposure during the 3rd trimester. DNAm at the 375 candidate CpGs in children at age 6 was not associated with prenatal SO₂ exposure in any trimester (Tables S7–S10). We also conducted the epigenome-wide analysis for 326,898 CpGs to investigate the association between prenatal SO₂ exposure and DNAm at age 2. We found that a total of 6,733 CpG sites were associated with prenatal exposure during the 3rd trimester with FDR-corrected p-value < 0.05 (Table S11, Fig. 2B), and the 6 CpGs listed above were included in the 6733 CpGs. Conversely, neither candidate gene analysis nor EWAS yielded significant CpGs within FDR-corrected p-value < 0.05 at age 6 (Fig. 2C).

Association between postnatal SO₂ exposure and DNAm. The associations between the postnatal SO₂ exposures at age of 2 years and DNAm at age 2, or the association between the postnatal SO₂ exposure at ages 2, 4 or 6 years and DNAm at age 6 were not significant (Tables S12–S15). The associations between the cumulative postnatal SO₂ exposures from age 2 to 4 or from 2 to 6 and DNAm at age 6 were not significant (Tables S16,S17).

Association between DNAm and ADHD symptoms. Among the 6 CpGs with significant associations at age 2 with prenatal SO₂ exposure, DNAm level at cg07583420 (*INS-IGF2*) were associated with 37.25% (95% CI 8.12, 74.22), 37.00% (95% CI 4.23, 80.08), and 34.82% (95% CI 5.44, 72.40) increase in ARS at ages 4, 6 and 8, respectively (Fig. 2D, Table S18).

Among 6733 CpGs from EWAS between prenatal SO₂ exposure during the 3rd trimester and DNAm, 58 CpGs, 219 CpGs, and 2063 CpGs were associated with ARS at ages 4, 6, and 8 years, respectively (Tables S19–S21). Among these, DNAm at 58 CpGs was persistently associated with ARS at ages 4, 6 and 8 years (Fig. S2).

Mediation analysis. The indirect effect of prenatal exposure during the 3rd trimester on ARS in childhood through DNAm at cg07583420 (*INS-IGF2*) was positive and significant at ages 4 (p-value 0.028) and 6 (p-value 0.004) but not significant at age 8 (Table 4, Fig. 3). Direct effects, from prenatal exposure to childhood ARS, were marginally significant at ages 6 (p-value 0.060) and 8 (p-value < 0.001). Particularly at age 6, indirect effect, direct effect, and total effect (indirect effect + direct effect) were all positive and significant or marginally significant.

Identifying methylation quantitative trait loci (mQTL). A total of 60 SNPs were found within 100 kb from either side from cg07583420 (*INS-IGF2*). Four SNPs showed significant association with the DNA meth-

CpGs	Chr	Gene	Gene group	Relation to CpG island	Estimate	p-value (BH corrected p-value)
cg07583420	11	<i>INS-IGF2</i>	Body	N shore	0.00683	6.48X10 ⁻⁵ (0.025)
cg20296524	1	<i>TARBP1</i>	1st exon	Island	0.00588	1.03X10 ⁻⁴ (0.025)
cg15705054	1	<i>PBXIP1</i>	5' UTR		0.00812	3.66X10 ⁻⁴ (0.039)
cg05951817	17	<i>SLC6A4</i>	5' UTR	N shore	– 0.0385	4.54 X10 ⁻⁴ (0.039)
cg05075097	11	<i>INS-IGF2</i>	Body	N shelf	0.00548	4.62 X10 ⁻⁴ (0.039)
cg25163476	11	<i>INS-IGF2</i>	Body	Island	0.00777	4.81 X10 ⁻⁴ (0.039)

Table 3. Association between prenatal exposure to sulfur dioxide during the 3rd trimester (per IQR increase) and DNAm at ADHD associated CpGs at 2 years of age* (FDR corrected p-value < 0.05) (n = 51). IQR, interquartile range, FDR false discovery rate, ADHD attention-deficit hyperactivity disorder. *Adjusted for mother's age, education level, maternal smoking, maternal environmental tobacco smoking, and season of birth.

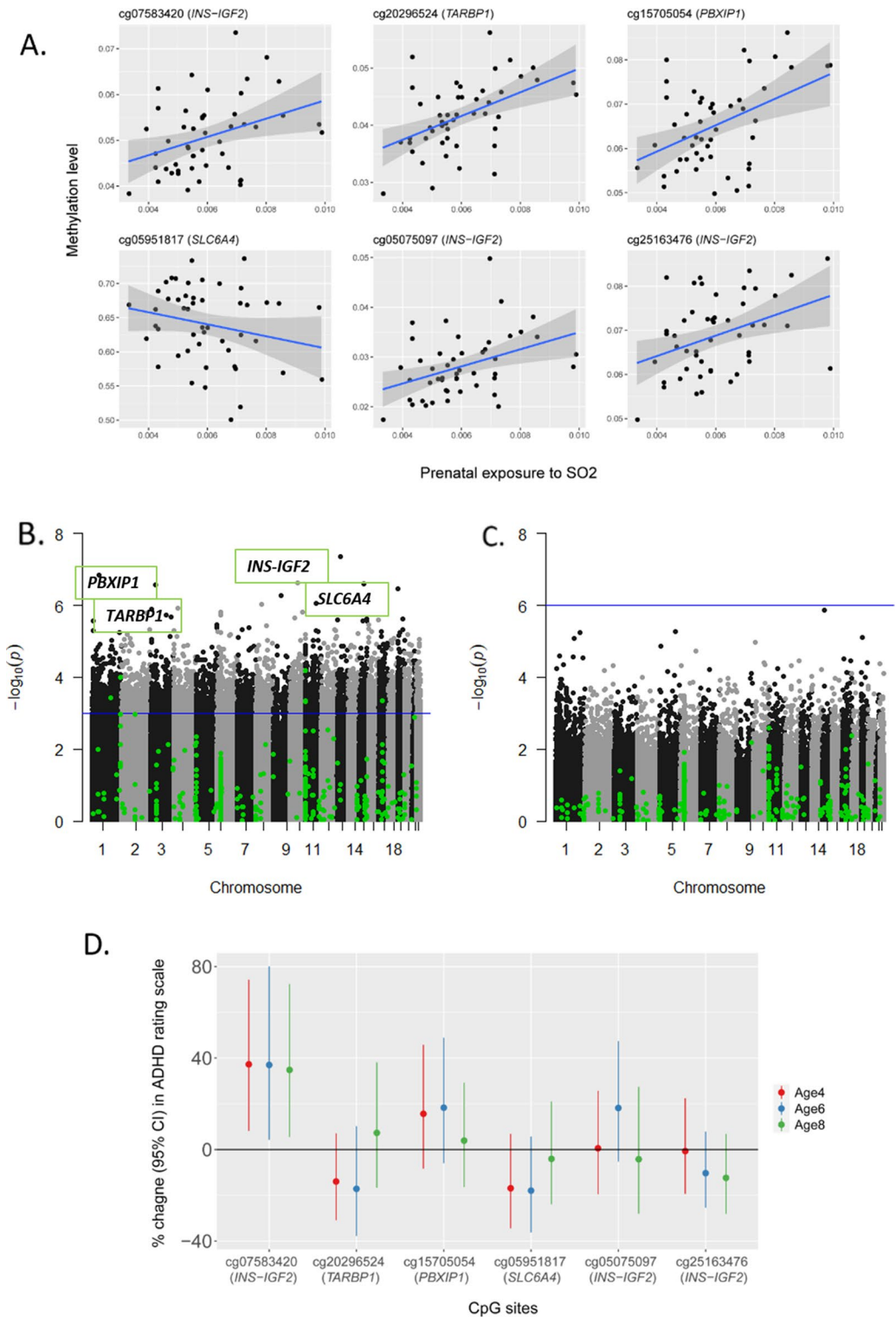


Figure 2. The association between prenatal SO₂ exposure, DNA methylation, and ADHD rating score. **(A)** The association between prenatal SO₂ exposure and DNA methylation. **(B)** Manhattan plot showing the association between prenatal SO₂ exposure during the 3rd trimester and DNA methylation at age 2. The green dots indicate CpGs selected from literature review for a candidate gene analysis. The blue line is the threshold for FDR-corrected p-value < 0.05. The CpGs significantly associated with prenatal SO₂ exposure from the candidate gene analysis were above the threshold line (cg07583420 (*INS-IGF2*), cg20296524 (*TARBP1*), cg15705054 (*PBXIP1*), cg05075097 (*INS-IGF2*), and cg25163476 (*INS-IGF2*)). **(C)** Manhattan plot showing the association between prenatal SO₂ exposure during the 3rd trimester and DNA methylation at age 6. **(D)** The association between DNA methylation at the CpGs linked with prenatal SO₂ exposure and the ADHD rating scale at ages 4, 6 and 8.

	4 years		6 years		8 years	
	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value
Indirect effect*	1.02 (0.11, 2.61)	0.028	0.43 (0.11, 1.00)	0.004	- 0.01 (- 0.17, 0.14)	0.890
Direct effect†	- 1.60 (- 6.14, 0.28)	0.144	0.45 (- 0.04, 0.77)	0.060	0.65 (0.45, 0.92)	<0.001
Total effect‡	- 0.58 (- 3.53, 0.73)	0.748	0.88 (0.65, 1.12)	<0.001	- 0.01 (- 0.24, 0.20)	0.890

Table 4. Mediation analysis of the path from prenatal exposure to SO₂ to ADHD rating scale (ARS) at 4, 6 and 8 years of age through DNAm at cg07583420 (*INS-IGF2*). *Indirect effect refers to prenatal exposure affecting ARS through DNAm at cg0783420. †Direct effect refers to prenatal exposure directly affecting ARS not through DNAm. ‡Total effect refers to the sum of indirect effect and direct effect.

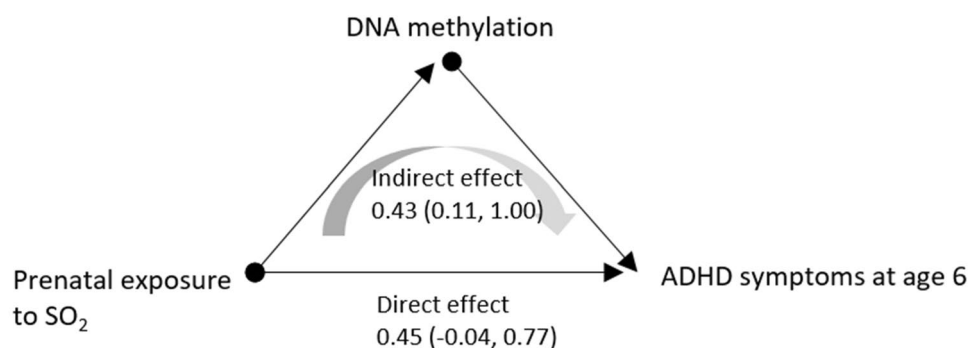


Figure 3. Mediation analysis showing the association between prenatal exposure to SO₂ and ADHD symptoms in children through DNA methylation at cg07583420 (*INS-IGF2*).

ylation levels at cg07583420 (p-value < 0.05): rs139951739, rs6578246, rs17885785, and rs3213223. However, none was significant when corrected for multiple comparisons using FDR method (Table S22, Fig. S3).

Pathway enrichment analysis. Reactome pathways analysis showed that mostly NOTCH signalling was involved in genes annotated to the 58 CpGs associated with prenatal SO₂ exposure during the 3rd trimester and ARS through 4, 6 and 8 years (Table S23, Figure S4).

WGCNA. A total of 375 CpGs were analyzed for co-methylation. Two modules were detected: grey (316 CpGs) and turquoise (59 CpGs) (Fig. 4A,B). The correlation between the grey module and prenatal exposure to SO₂ during the 3rd trimester was not significant (Fig. 4C). CpGs from the grey modules were analyzed again for co-methylation, and the turquoise module was correlated with ARS at age 4 (Pearson's coefficient correlation 0.38, p-value 0.003), and the grey module was correlated with ARS at age 8 (Pearson's coefficient correlation 0.28, p-value 0.03) (Fig. 4D). CpGs from the final turquoise module were assessed, and the CpGs associated with SO₂ exposure during the 3rd trimester of pregnancy including *INS-IGF2*, *PBXIP1*, and *SLC6A4* (Table 3) were found from the turquoise module (Fig. 4E), suggesting that these CpGs are co-methylated in relation with ARS at age 4.

Discussion

To the best of our knowledge, this is the first study to report the epigenetic effect on the positive association between prenatal SO₂ exposure and ADHD symptoms at ages 4, 6 and 8 years in a mother–child cohort. For a candidate gene analysis, we selected 375 CpGs from a systematic literature review of EWAS of ADHD. In the sub-cohort with available DNAm measurements at age 2, an IQR increase in prenatal SO₂ exposure during the 3rd trimester was associated with an increased level of DNAm at CpGs of *INS-IGF2*, *TARBP1*, *PBXIP1*, and *SLC6A4*. Among these, DNAm level at cg07583420 (*INS-IGF2*) was positively associated with ARS at ages 4, 6 and 8, persistently (Fig. 5). When significant CpGs (n = 6733) from EWAS were tested for the association with ARS, 58 CpGs were persistently associated with ARS at ages 4, 6 and 8 years, which were involved in NOTCH signalling pathway. DNAm at age 6 was not associated with prenatal SO₂ exposure. We further investigated the association between postnatal exposure to SO₂ and DNAm by several different approaches, which showed no significant result. This implies that prenatal SO₂ exposure may affect DNAm more significantly than postnatal SO₂ exposure.

Prenatal environmental exposure may affect ADHD development through DNAm, which may lead to long-term phenotype changes⁴². The fact that DNAm was associated with prenatal SO₂ at age 2 but not at age 6 may be related to the trajectory of global DNAm over the developmental stages, which fluctuates at an earlier stage of development and later stabilized⁴³. This implies that a sensitive window period of DNAm may exist in which DNAm at earlier childhood is more susceptible to environmental triggers than later childhood⁴⁴. For example, DNAm at several CpGs at age 7 was mostly predicted by early childhood adversity before age 3⁴⁵. To investigate

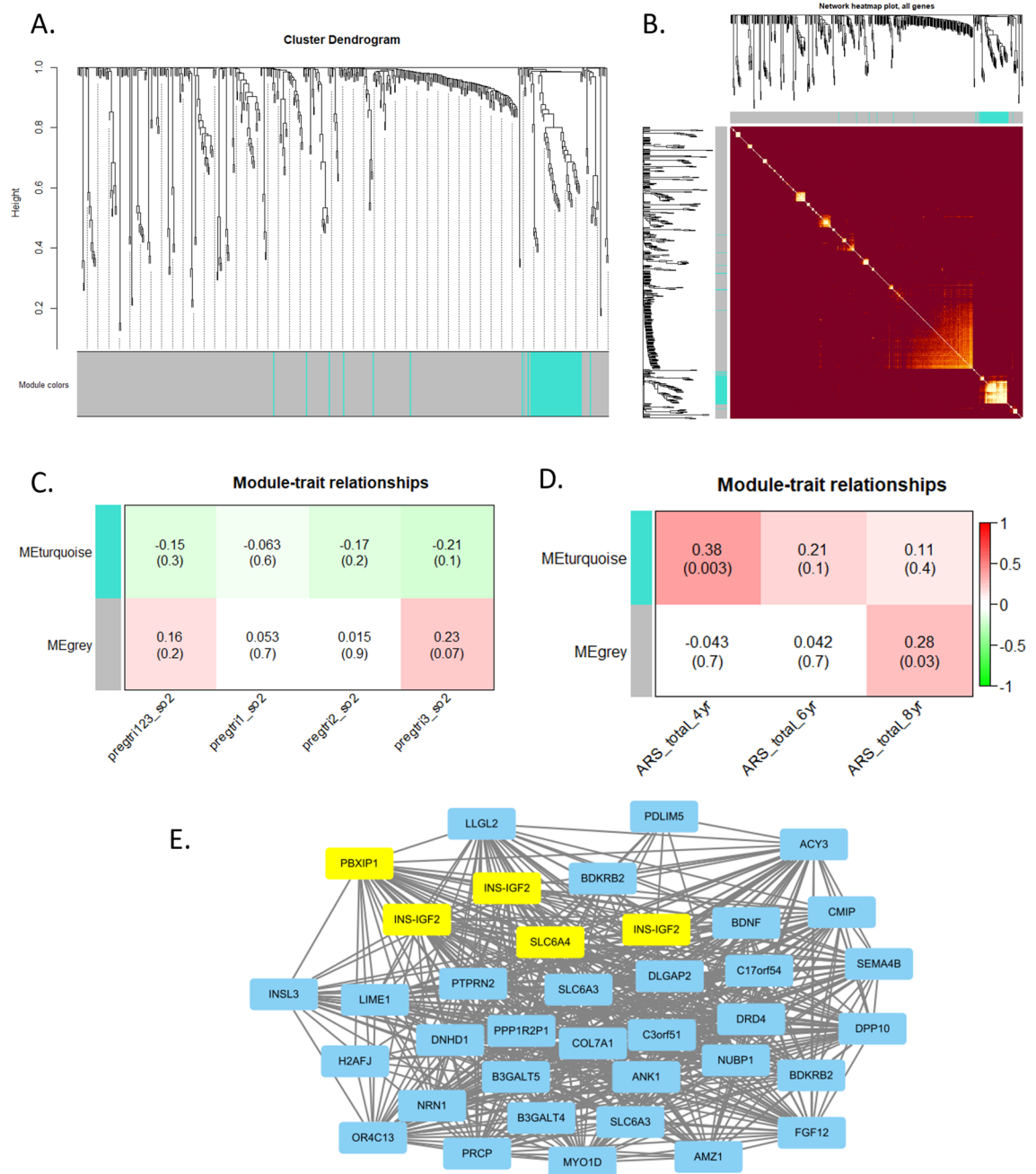


Figure 4. Weighted gene co-methylation network analysis of DNA methylation at CpGs for candidate gene analysis. **(A)** Dendrogram produced by hierarchical clustering of samples. Modules of co-methylated CpGs are indicated by different color (grey and turquoise) beneath the dendrogram. **(B)** Network heatmap plot. Dendrograms from hierarchical clustering are corresponded to the color-coded modules (grey and turquoise). In the heatmap, bright yellow color indicate high co-expression inter-connectedness. **(C)** Correlating modules with exposure variables (prenatal SO₂ exposure by trimester). **(D)** Correlating modules with outcome variable (ADHD rating scales at age 4, 6 and 8). **(E)** Network between CpGs from the turquoise module.

the period of clinical manifestation of ADHD with respect to DNAm, we examined ADHD symptoms at ages 4, 6 and 8. Surprisingly, DNAm was associated with ADHD symptoms during the wide range of early childhood from ages 4 to 8 consistently rather than focal stage of childhood, suggesting that the effect of DNAm can extend to a long period.

DNAm is also under genetic control if mQTL is identified adjacent to cg07583420 (*INS-IGF2*). In the present study, we could not identify statistically significant mQTL, possibly due to the small sample size. Further studies with a larger sample size are therefore warranted to elucidate the complex interplay between genetic, epigenetic, and environmental factors in the etiology of ADHD in children.

Although ADHD is associated with various compounds of air pollution, studies on the relationship between SO₂ and neurodevelopmental outcomes, like ADHD, are limited. SO₂ exposure prenatally up to 12 months was

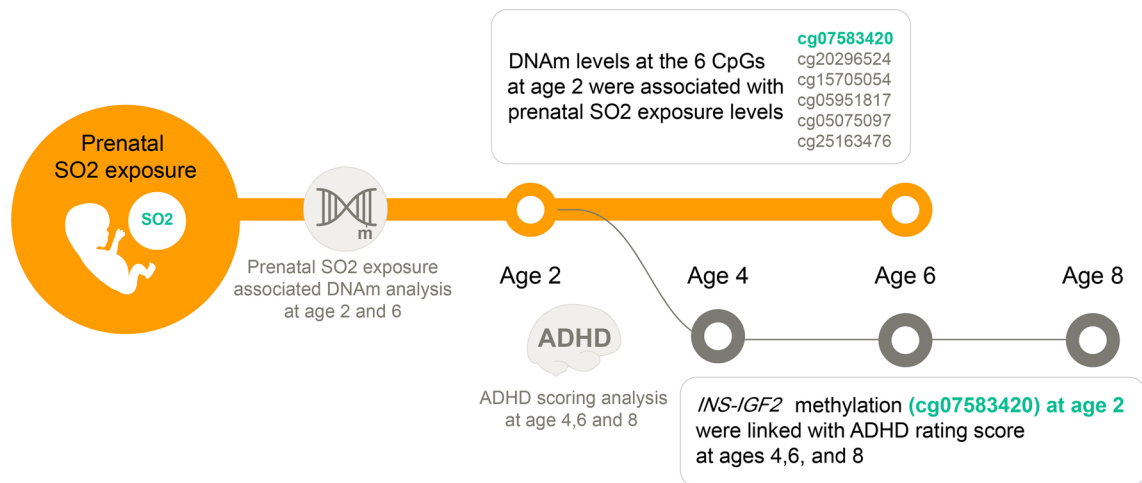


Figure 5. The overview of the study design and results (Candidate gene analysis).

negatively associated with fine motor scores at 18 months of age¹², and SO₂ exposure was related to poor executive function in 6–12-year-old children in a cross-sectional study in China¹². Another study reported an association between short-term exposure to SO₂ and ADHD-related hospital admission in adolescents aged 10–19 years¹³.

The biological mechanism underlying the association between SO₂ exposure and ADHD remains unknown. Therefore, only a few hypothetical mechanisms can be inferred from previous studies. SO₂ can increase lipid peroxidation in the brain and generate reactive oxygen species⁴⁶, as well as various inflammatory cytokines. These reactive oxygen species and inflammatory cytokines can relocate to the central nervous system via systemic circulation, thereby inducing neuroinflammation. SO₂ has also been reported to induce neurotoxicity through protein oxidation, DNA protein cross-links, apoptosis, and damage of cell constituting the central nervous systems including cerebral cortex neurons, glial cell and nerve fibers^{47,48}.

Another possible link is the association between SO₂ and other regional pollutants. The region close to power plants or industrial facilities using fossil fuel shows higher SO₂ level⁴⁹. A cohort study with 5193 children including 116 patients with ADHD reported that the proximity to an industrial estate in the study area was associated with an increased risk of ADHD, and suggested prenatal exposure to organochlorine compound released from the industrial estate as a potential explanation for the finding⁵⁰.

Notably, the result of WGCNA showed that the CpGs (cg07583420 (*INS-IGF2*), cg15705054 (*PBXIP1*), cg05075097 (*INS-IGF2*), and cg25163476 (*INS-IGF2*)) associated with prenatal SO₂ exposure were co-methylated, corresponding to the module simultaneously correlated with prenatal SO₂ during the 3rd trimester and ARS at age 4. *IGF2* plays an integral role in brain development after birth⁵¹. *IGF2* is associated with developmental abnormalities in the structure and/or function of the cerebellum and the hippocampus^{52,53}, both of which are associated with ADHD. Higher *IGF2* methylation can predict ADHD symptoms in youth with conduct disorders³³. In mice, *IGF2* enhancer deletion disrupted levels of striatal dopamine, which has been suggested to be involved in the pathophysiology of ADHD⁵⁴.

Notch signalling pathway plays an essential role in embryogenesis and organogenesis⁵⁵, particularly in regulation of neurogenesis⁵⁶, and is also known to be involved in schizophrenia and bipolar disorder⁵⁷. In a mice model, damages in neural stem cells affected cognitive impairment during Pb exposure, which was dependent on the Notch pathway⁵⁸. Prenatal exposure to ketamine in rats, which increased the expression level of Notch1, inhibited the proliferation and differentiation of neural stem cells in hippocampus and impaired neurocognitive function including learning and memory in adulthood⁵⁶.

Our study presents certain limitations. First, the small sample size limits the statistical power of the results, hence, further replication of these results in larger population is warranted. ADHD symptoms were assessed by a parent-rated questionnaire on a continuum, rather than analyzed dichotomously according to a formal diagnosis by a specialist. Therefore, the results of this study may differ in a clinical sample. SO₂ gaseous exposure could have greater temporal variability, therefore, the results should be interpreted cautiously. In addition, DNAm measured in the peripheral blood may not reflect the methylation signature in the brain, as epigenetic markers are tissue specific. In the absence of gene expression data, direct conclusions about the transcriptional consequences of the DNAm changes could not be made. Despite these limitations, our study also has notable merits. We employed a prospective study design, with prenatal exposure assessment, methylation profiles at age 2 and 6, and longitudinal neurocognitive outcome measurements at ages 4, 6 and 8. Investigation of the effect of methylation provided insight into the mechanism underlying the association between air pollution and ADHD. The longitudinal study design enabled identification of susceptible window period of DNAm changes in association with prenatal environmental exposure, which was earlier childhood in this study. We adjusted for potential covariates related to both the exposure and outcome, including both maternal and childhood characteristics.

Conclusion

In this study, we found that prenatal exposure to SO₂ contributed to differential methylation at a CpG site located within *INS-IGF2* at age 2, which, in turn, was associated with ADHD symptoms at ages 4, 6 and 8 years. These findings indicate that an epigenetic mechanism involving methylation could underlie the relationship between the toxicity of SO₂ and neurodevelopment in children.

Methods

Study population. Study participants were selected from the Environment and Development of Children (EDC) Study, a prospective cohort study that investigated the environmental effects on growth and neurodevelopment⁵⁹. Mothers participating in the Congenital Anomaly Study cohort were contacted during 2012–2015 after birth, and a total of 726 mother–child pairs were recruited for EDC study, followed by regular follow-up at 2-year intervals (i.e. 2, 4, 6 and 8 years). We collected epidemiological information, anthropometric characteristics, and neurocognitive outcomes biennially. ADHD symptoms were evaluated at ages 4, 6, and 8. Among these children, 329 had information on ADHD symptoms all at ages 4, 6 and 8.

In a sub-cohort, we selected 60 children at age 2 and analyzed DNAm from whole blood at age 2 and 6 repeatedly. Similar to the main EDC cohort, we examined neurocognitive functions including symptoms of ADHD at ages 4, 6 and 8. Among 60 children, 54 at age 4, 60 at age 6, and 57 at age 8 were assessed for ADHD symptoms. Those assessed at these ages were included in the main analysis (n = 51).

Ethical statements. The methods of this study were approved by the Institutional Review Board of Seoul National University College of Medicine (IRB No. 1201-010-392) and was conducted according to the guidelines and regulations of the Declaration of Helsinki. Informed consent was obtained from mothers according to the Institutional Review Board of Seoul National University College of Medicine (IRB No. 1201-010-392).

Measurement of air pollution. We used the levels of air pollution including district-specific monthly SO₂ as the main exposure. The levels of SO₂ were collected from the publicly available data from Air Korea (<http://www.airkorea.or.kr/eng>) from the Korea Ministry of Environment, which monitors the hourly air pollution concentration levels at 257 stations nationwide. We assigned air pollution levels to the various pregnancy stages, such as the 1st, 2nd, 3rd trimester, as a proxy of exposure to pregnant women based on the residential address. The SO₂ levels were measured from the nearest monitoring station from the residential address of each participant. The nearest monitoring stations was assigned according to the Euclidean distance between each residential address and the closest monitoring station using ArcGIS.

DNAm analysis. *Bisulfite sequencing and microarray.* The Illumina Infinium HumanMethylation EPIC BeadChip (850 K) was used for the samples from the 2-year-olds, and the Illumina Infinium HumanMethylation 450 K BeadChip was used for the samples of the 6-year-olds (Illumina, San Diego, CA, USA). Detailed experimental procedures are presented in the Supplementary Materials.

Quality control and probe filtering. Array CpG probes which had a detection p-value > 0.05 in more than 25% of samples were filtered out. Filtered data were normalized using the Beta Mixture Quantile (BMIQ) method⁶⁰ and corrected for batch effect using ComBat package in R. CpGs with at least one “not-available” (NA) values for the normalization were excluded, leaving 865,688 CpGs from the EPIC BeadChip (850 K) at age 2, and 460,960 CpGs from the 450 K BeadChip at age 6. Among these CpGs, we selected the overlapping 430,101 CpGs. After excluding SNP-associated CpGs (SNP distance ≤ 1 or SNP minor allele frequency ≥ 0.05), CpGs corresponded to non-CpG loci or the X or Y chromosomes, and CpGs at cross-reactive probes, and multimodal CpGs, 326,898 CpGs were finally selected for analysis (Fig. S1). We identified multimodal CpGs via the Dip test⁶¹ using dipTest R package⁶² (Dip test’s p-value < 0.05).

Systematic review of literature and selection of candidate CpGs. We selected candidate CpGs through a systematic literature review. The detailed procedure of systematic review is shown in the Supplementary Materials.

SNP Genotyping. We used Axiom 2.0 Reagent Kit (Affymetrix Axiom 2.0 Assay User Guide) according to manufacturer’s protocol; the detailed procedure is provided in the Supplementary Materials. We used Korean Chip (K-CHIP) available from the K-CHIP consortium to produce Genotype data. K-CHIP was designed by the Center for Genome Science, Korea National Institute of Health, Korea (4845-301, 3000-3031).

ADHD symptom screening. To evaluate ADHD symptoms in children at ages 4, 6, and 8, the Korean version of the ARS IV (K-ARS) was completed by the parents^{63,64}. K-ARS is a standardized screening tool for ADHD symptoms in Korean children⁶⁵ and has shown validity and reliability⁶⁶. K-ARS, which has also been used in relation to environmental exposure⁶⁷, is composed of 18 questions corresponding to the diagnostic criteria of ADHD according to the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition. Nine questions evaluate inattention, and the other 9 are related to hyperactivity and impulsivity. Each item is rated from 0 to 3, with a total score ranging from 0 to 54⁶⁸.

Covariates. Covariates were pooled from the literature review^{69–71}. Covariates included maternal age at pregnancy, maternal educational level (middle school graduate, high school graduate, college graduate, or

graduate school attendance), maternal smoking status during pregnancy (current smoker, ex-smoker, never-smoker), ETS during pregnancy (yes/no), child's sex, gestational age (weeks), multiple births (singleton or twin/triplet), season of child's birth, cell type fraction, and maternal IQ evaluated by the short version of the Korean Wechsler Adult Intelligence Scale⁷². Cell type distribution, defined as the fraction of CD8 + T cells, CD4 + T cells, natural killer (NK) cells, B cells, monocytes, and neutrophils, was estimated by using the adult leukocyte reference panel⁷³ and Minfi R package⁷⁴.

Statistical analysis. We investigated the association between trimester-specific prenatal exposure to SO₂ (ppm) and ARS at ages 4, 6 and 8 in the cohort of 329 mother–child pairs by linear regression analysis by adjusting for maternal age at pregnancy, mother's education levels, maternal smoking, maternal ETS, and season of child's birth. Covariates were selected based on directed acyclic graph⁷⁵ using the publicly available program (<http://www.dagitty.net/>) (Fig. S5).

In a sub-cohort (n = 51), we investigated the association between prenatal trimester-specific exposure to SO₂ (ppm) and DNAm at the CpGs selected from the literature review (n = 375) at ages 2 and 6, using multivariable linear regression. We expressed the changes in DNAm per interquartile range (IQR) increase in SO₂. The regression model for the association between prenatal SO₂ and DNAm was adjusted for maternal age at pregnancy, mother's educational level, maternal smoking, ETS during pregnancy, and season of the child's birth (Fig. S6). To investigate the association between postnatal SO₂ exposure and DNA methylation levels, we used a linear regression model for the average SO₂ level during age 2 (12 months) and DNAm at age 2, and the average SO₂ level during age 2, 4 and 6 each and DNAm at age 6, adjusted for mother's age, postnatal ETS, postnatal mother's smoking, and the season of birth. To evaluate the effects of cumulative SO₂ exposure, we have analyzed the association between SO₂ exposure at ages 2–4 years and 2–4–6 years and DNAm at age 6. SO₂ exposure at ages 2–4 years was taken from the average of SO₂ exposure at ages 2 and 4 years, and SO₂ exposure at 2–4–6 years was taken from the average of SO₂ exposures at age 2, 4 and 6 years. Benjamini–Hochberg method⁷⁶ was used to correct the effects of multiple comparisons by using FDR. We also performed EWAS for 326,898 CpGs at ages 2 and 6 with prenatal SO₂ exposure by trimester, adjusted by the same covariates.

Furthermore, we investigated the association between an increase in DNAm at prenatal SO₂ exposure-associated CpGs at ages 2 and ARS at ages 4, 6, and 8, after adjusting for mother's age at pregnancy, mother's education level, maternal smoking, ETS during pregnancy, season of the child's birth, maternal IQ, the child's sex, preterm birth, and multiple births (Fig. S7).

WGCNA was used to detect co-methylated modules (clusters of CpGs) using WGCNA R package^{77,78}. Using beta-value of methylation at the CpGs from candidate gene analysis, weighted co-methylation networks (module) were identified. For each module, hierarchical clustering was performed for all samples, and the dendrogram were grouped into modules. Then the correlation was tested between the modules and trimester-specific SO₂ exposure during pregnancy, using Pearson correlation coefficient. We selected the module with a significant correlation with exposure variables, then pooled CpGs corresponding to the module of interest. Next, the methylation levels at these CpGs were used to detect co-methylation module, and the correlation between the modules and ADHD symptom scores at ages 4, 6 and 8 were then analyzed. The networks between CpGs were visualized using Cytoscape ver 3.8.2. (<https://cytoscape.org/>).

We investigated the mediation effect of DNAm at the significant CpGs on the association between prenatal SO₂ and ARS in childhood using Mediation R package⁷⁹. Indirect effect indicates the effect of prenatal exposure to SO₂ affecting ARS through DNAm, whereas direct effect refers to the effect of prenatal SO₂ exposure directly affecting ARS, not through DNAm. The total effect is defined as the sum of indirect and direct effects.

To identify mQTL, we searched for SNPs positioned within 100 kb window to either side of the significant CpGs, then analyzed the correlation between DNA methylation levels at the CpGs and SNP genotypes. The p-value for the correlation between genotype and DNAm levels was calculated using the Kruskal–Wallis test. Multiple comparisons were corrected via FDR.

For functional enrichment analysis, Gene Ontology (GO) terms and Reactome cell signaling pathways were identified using topGO R package⁸⁰ and ReactomePA R package⁸¹.

Data availability

The data that support the findings of this study are available from Environment and Development of Children (EDC) cohort. The datasets used during the current study are available from the corresponding author on reasonable request.

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Author contributions

Y.J.C., J.I.K., Y.H.L. and Y.C.H. conceived and designed the study. Y.C.H., B.J.K., C.H.S., Y.A.L., Y.H.L. acquired funding and established the data. Y.J.C., J.C., S.M. and K.S.L. performed the data analysis. Y.J.C., J.C., J.I.K., D.W.L., S.J.P. drafted the original manuscript. J.I.K. and Y.H.L. supervised and reviewed the writing. Z.K., Y.C.H., B.N.K., C.H.S. and Y.A.L. made critical revisions of the manuscript for important intellectual contents. The authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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