Environmental Health

RESEARCH

Open Access

Combined effects of global DNA methylation, blood lead and total urinary arsenic levels on developmental delay in preschool children

Yuu-Hueih Hsu[1], Chih-Yin Wu[2,3], Hui-Ling Lee[4], Ru-Lan Hsieh[5,6], Ya-Li Huang[7], Horng-Sheng Shiue[8], Ying-Chin Lin[2,3,9], Mei-Chieh Chen[10,11†] and Yu-Mei Hsueh[2,7*†]

Abstract

DNA methylation is a critical step in brain development, 5-Methyl-2′-deoxycytidine (5mdC) is one of the global DNA methylation markers. Arsenic and lead exposures have been associated with neurotoxicity, which may be linked to epigenetic changes. Our research sought to investigate the correlation between 5mdC and developmental delay (DD) among preschoolers. Additionally, we assessed whether 5mdC modified the impacts of blood lead and total urinary arsenic levels on DD. We analyzed the concentrations of 5mdC, blood cadmium and lead, and total urinary arsenic in 174 children with DD and 88 healthy children. Global DNA methylation levels are expressed as the ratio 5mdC/2′-dexyguanosine (dG), called 5mdC (%). In our findings, elevated levels of blood lead and total urinary arsenic were significantly associated with DD risk among preschoolers. Furthermore, high 5mdC (%) was related with reduced risk of DD, with an odds ratio (OR) and 95% confidence interval (CI) of 0.14 (0.06 – 0.32). A notable multiplicative interaction was observed between low 5mdC (%) and elevated blood lead levels to increase OR of DD, with OR and 95% CI was 9.51 (4.18 – 21.64). The findings provide evidence of the combined effects of reduced 5mdC (%) and high blood lead concentrations, increasing the OR of DD.

Keywords 5-methyl-2′-deoxycytidine, Arsenic, Lead, Developmental delay, Preschool children

†Mei-Chieh Chen and Yu-Mei Hsueh contributed equally to this work.

*Correspondence:
Yu-Mei Hsueh
ymhsueh@tmu.edu.tw
[1] Department of Public Health, National Cheng Kung University, Tainan, Taiwan
[2] Department of Family Medicine, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan
[3] Department of Family Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[4] Department of Chemistry, Fu Jen Catholic University, New Taipei City, Taiwan
[5] Department of Physical Medicine and Rehabilitation, Su Memorial Hospital, Shin Kong Wu Ho, Taipei, Taiwan
[6] Department of Physical Medicine and Rehabilitation, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[7] Department of Public Health, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[8] Department of Chinese Medicine, College of Medicine, Chang Gung University, Taoyuan, Taiwan
[9] Department of Geriatric Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[10] Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[11] Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan

Introduction

Developmental delay (DD) are behavioral, communication, or cognitive impairments in individuals such as attention-deficit/hyperactivity disorder (ADHD), autism, and intellectual disability (mental disorders). Chen et al. used Taiwan's health insurance database from 1996 to 2001 to track 1,790,966 newborns for 3 to 8 years, and found that hyperactivity syndrome, autism, and mental retardation had cumulative incidence rates of 2.69, 0.34, and 1.29%, respectively, with boys being 3.2, 4.1, and 1.6 times more likely to develop ADHD, autism and mental retardation than girls [5]. According to Ministry of Health and Welfare data, the prevalence rate of DD among children aged 3–5 in 2007 was 1.05% and in 2010 went to 1.47% [10]. A cross-sectional study from July 2008 to December 2009 in northeastern Taiwan included 3,214 children aged 4 months to 6 years old, and found 365 children with developmental disabilities, with a prevalence rate of 11.36% [6]. In recent years, DD in preschool children has been increasing year by year. Because DD in preschool children has higher health care needs than in the general adult population with risk factors remaining unclear, it has become one of the important public health issues.

Globally, there is considerable concern about the deleterious effects of noxious metals on children's growth and development [26]. Toxic metals, for example, lead, mercury, and cadmium, have negative consequences that can lead to various neurodevelopmental disorders [13], neurodegenerative disease [9], and impaired growth in children [7]. A study indicated that even low levels of arsenic exposure can lead to cognitive impairment [35]. We discovered in an earlier study that insufficient ability to methylate arsenic, as indicated by elevated percentage of monomethylarsonic acid (MMA%) and lowered percentage of dimethylarsinic acid (DMA%), and increased total urinary arsenic concentration, was notably linked to the likelihood of DD among preschool children [22]. Importantly, this association persists even after considering other potential risk factors [22]. Additionally, we have observed a significant positive correlation between blood lead concentration and DD [24]. However, whether other factors influence the association between arsenic and lead exposure and DD in preschool children deserves investigation.

DNA methylation, most commonly used to study epigenetics, is where a methyl group is added to cytosine at the 5' position at a CpG site, forming 5-methylcytosine (5-MC) [3]. DNA methylation plays a crucial role in modulating gene expression in the central nervous system, with dysregulated recognition and control of DNA methylation implicated in various human brain disorders [42]. DNA methylation may serve as a biomarker for ADHD and autism in children [44]. One study showed that lower methylation status of nine CpG sites at birth was associated with the development of ADHD symptoms later in life [36]. On the basis of a large-scale study of DNA methylation of hyperactivity in children identified a significant association of hyperactivity with specific site of DNA methylation [33]. Cerebral palsy children with severe movement impairments have less DNA promoter methylation than children with less movement impairments [48]. One study showed that adults with ADHD, especially women, had lower levels of global DNA methylation as assessed by 5-MC [34]. 5'-Hydroxymethylcytosine (5hmC) oxidized from 5-MC by the 10–11 translocation (Tet) protein family is a new type of modified cytosine [27]. A recent study pointed out that reduced levels of 5hmC are critical for neurodevelopment and brain function [51]. Genomic DNA methylation levels are quantified by determining the ratio of 5-methyl-2'-deoxycytidine (5mdC) to 2'-deoxyguanosine (dG, typically measured with 2'-deoxyguanosine serving as an internal standard, where overall DNA methylation is determined by 5mdC percentage [5mdC (%) [30, 46]. We intended to use the global DNA methylation marker 5mdC (%) to explore whether it is related to DD in preschool children.

Long-term exposure to arsenic causes DNA hypomethylation [39]. Arsenic induces oxidative stress [14], chronic oxidative stress, and depletion of S-adenosylmethionine (SAM) in the folate/homocysteine pathway, leading to reduced global DNA methylation [19]. Nevertheless, a direct correlation was detected in a Bangladeshi adult epidemiological study, linking arsenic exposure through drinking water with increased global DNA methylation levels in peripheral blood monocytes [37]. Research findings indicated a positive correlation between urinary lead and cadmium concentrations and global DNA methylation levels (measured as 5mdC/dG) in young adults aged 12–30 years [29]. Global DNA methylation levels were reduced in the lead-exposed zebrafish model, compared with findings from human and rodent studies [41]. One study found that the exposure of human embryonic stem cells to lead disrupted global DNA methylation resulting in altered neuronal differentiation, affecting brain development [43]. According to the above studies, the association of arsenic, lead and cadmium exposure with global DNA methylation is inconsistent. Hence, the purpose of the present study was to investigate the correlation of 5mdC (%) with DD in preschool children. Additionally, it aimed to determine the potential associations between 5mdC (%) and blood cadmium, blood lead, and total urinary arsenic levels. Furthermore, we sought to examine whether 5mdC (%) modifies the association of blood

cadmium, blood lead, total urinary arsenic levels with DD in preschool children.

Materials and methods

Participants

A total of 262 preschool-aged children were recruited for a case–control investigation; they were being care for at Shin Kong Wu Ho-Su Memorial Teaching Hospital from August 2010 to March 2014. The participating children, under the age of 7, residing in Taipei City's Shilin District, had not yet begun elementary school, and were from families where Mandarin was spoken by parents or primary caregivers. It is noteworthy that the Shilin District does not have any factories known for causing environmental pollution. All study children underwent multiple developmental assessments to confirm DD, including assessments in fine motor, gross motor, speech and language, and cognitive, emotional and social domains. Following standard practice in clinical settings, children and their parents or primary caregivers completed the Child Expression Assessment Tool, Chinese Wechsler Intelligence Scale for Children (3rd edition), Peabody Motor Development Scale, gross motor function measure, preschool language assessment tools and Bayley III Infant Development Scale assessment. The assessment data are then evaluated by pediatricians, psychiatrists, ophthalmologists, otolaryngologists, physical therapists, speech therapists, psychologists, occupational therapists and social workers. Children with DD ($n=178$) were defined as performance on an age-appropriate, standardized, norm-referenced test that was two standard deviations or more below the mean. Children with confirmed absence of DD were selected as controls ($n=88$) [22]. Our previous research has found that children with unclassified DD have significantly poorer quality of life and health outcomes, and that their condition has a significantly greater impact on their families compared to normal children [21]. Upon obtaining signed consent forms from parents or primary caregivers, questionnaire interviews were conducted, and urine and blood samples were collected from the participating children. The TMU Joint Institute Review Board (N202201083) of Taipei Medical University approved the study, and we followed the guidelines of the Declaration of Helsinki.

Questionnaires, interviews, and sample collection

Structured questionnaires were administered to all parents or primary caregivers by trained personnel to gather standardized information. Data collected included the child's age, gender, birth weight and height, and the mother's age, education, maternal gestational weeks and parity. Additionally, urine and blood samples were obtained from the children. Blood samples were processed to separate red blood cells, which were preserved in a deep freezer (-80 °C) for subsequent lead and cadmium assays. Buffy coats, obtained from the blood samples, were preserved for measuring the global DNA methylation marker 5mdC. Urine specimens were kept frozen at -20 °C for later determination of arsenic species.

Assessment of 5mdC

DNA was isolated from the buffy coat using proteinase K digestion and subsequent phenol/chloroform extraction. 5mdC was determined by liquid chromatography-tandem mass spectrometry (LC–MS/MS) using an Agilent 1260 VL system (Agilent Technologies, USA) with API 3000™ triple quadrupole detection (AB SCIEX, Canada). Explicit analytical procedures have been previously documented [8, 30]. By assuming that dG = 5mdC + 2'-deoxycytidine (dC), assessing the ratio of 5mdC to dG, termed as 5mdC (%), offers an approximation of global DNA methylation levels [8, 30]. Figure 1 displays the chromatogram of LC–MS/MS for detecting reference compounds of 5mdC.

Assessment of blood lead and cadmium levels and *urinary arsenic species*

Arsenite (As^{III}), monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}), and arsenate (As^{V}) were determined by HPLC (Merck Hitachi, Tokyo, Japan) and atomic absorption spectrometry with hydride generation (PerkinElmer, Waltham, MA, USA), as outlined previously [23]. Urinary arsenic species levels falling below the limit of detection were considered to have concentrations at half the limit of detection. Total urinary arsenic level was computed as the sum of As^{III}, As^{V}, MMA^{V}, and DMA^{V} divided by the urinary creatinine concentration to adjust for hydration status [1]. Lead and cadmium concentrations in red blood cells were determined using inductively coupled plasma mass spectrometry (Agilent Technologies, Santa Clara, CA, USA), as described by Hsueh et al. [24]. Details regarding the limits of detection as well as assay validity and reliability are presented in Supplementary Table S1.

Statistical analysis

Differences in continuous variables between preschool children with and without DD were assessed using the Wilcoxon rank sum test. Goodness-of-fit χ^2 tests were used to examine differences in categorical variables. Multiple logistic regression models served to determine the odds ratio (OR) and 95% confidence interval (CI) for DD related to the global DNA methylation marker 5mdC (%), metals, or their interaction, after adjusting for birth weight, age, sex, gestational age, and maternal education level. The distribution of variables in the control group was divided into tertiles,

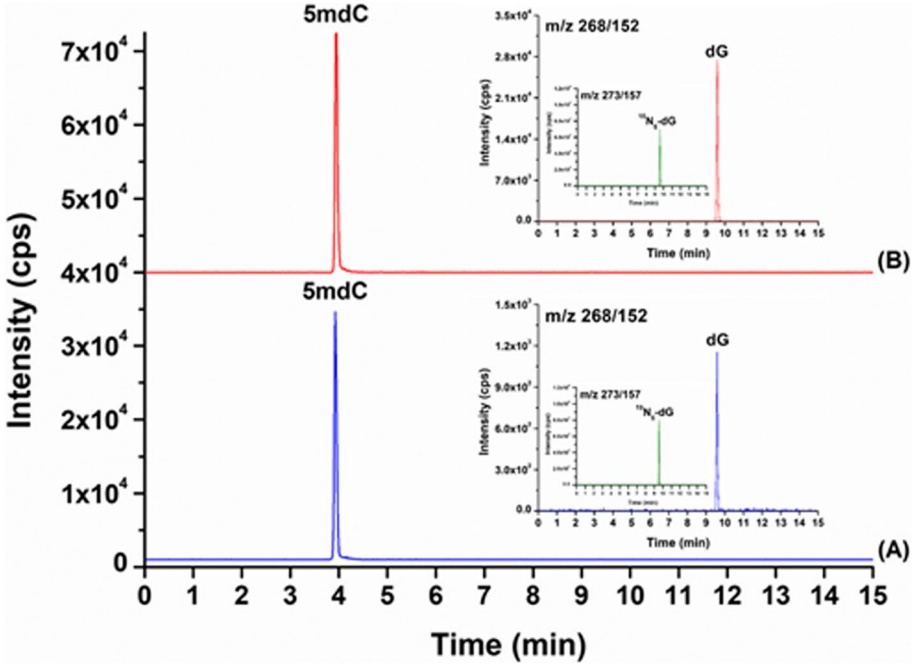

Fig. 1 LC–MS/MS chromatogram of reference compounds using MRM (multiple reaction monitor) mode. **A** 10 ng/mL 5mdC and 20 ng/mL $^{15}N_5$-dG were dissolved in 5% MeOH + 0.1% FA. **B** sample spike with 20 ng/mL $^{15}N_5$-dG was dissolved in 5% MeOH + 0.1% FA

where the tertile with the lowest values served as reference. Tertiles were treated as ordinal variables in the model to determine the linear trend of the OR in the stratification of the independent variables. Interaction analyses were performed using the median of control variables as a cutoff point. The multiplicative interaction between blood lead, blood cadmium, and total urinary arsenic levels with 5mdC (%) on DD was examined through product terms in multiple logistic regression models. Additionally, the additive interaction of total urinary arsenic or blood lead and 5mdC (%) on DD was estimated using the synergy index [20]. SAS 9.4 (SAS Institute, Cary, NC, USA) was used for statistical analysis. Statistical significance was considered when $p < 0.05$.

Results

Comparison of sociodemographic characteristics between DD cases and controls

Table 1 shows the sociodemographic characteristics of children, along with maternal information, stratified by the presence or absence of DD. Preschoolers experiencing DD tended to be younger and predominantly male and have lower birth weight, shorter gestational age, and mother with lower educational level, compared to their counterparts without DD.

Global DNA methylation index-5mdC (%) and metals and DD risk

Table 2 presents the relationships between 5mdC (%), blood lead, blood cadmium and total urinary arsenic levels, and DD in preschool children. Children with DD exhibited significantly lower 5mdC (%) levels, alongside significantly elevated blood lead and total urinary arsenic levels compared to those without DD. We noted a trend indicating a progressively lower OR for DD as 5mdC (%) increased. Following multivariate adjustment, children with 5mdC (%) > 5.45 demonstrated significantly decreased odds of DD compared to those with 5mdC (%) ≤ 4.28 (OR = 0.14, 95% CI, 0.06 – 0.32). Additionally, the OR for DD displayed a significant dose–response relationship with increasing blood lead and total urinary arsenic levels. However, no association was observed between blood cadmium level and DD.

Interaction of metals and 5mdC (%) and effect on DD risk

We examined the correlation between blood cadmium, blood lead, and total urinary arsenic concentrations and 5mdC (%), finding no discernible relationship between metals and 5mdC (%) (Supplemental Table S2). Given that 5mdC (%) and blood lead and urinary total arsenic concentrations were individually associated with DD, we proceeded to investigate their combined effects on DD in pairs and analyze their interactions. The reference group consisted of children with 5mdC (%) > 5.03, total arsenic

Table 1 Sociodemographic characteristics of preschool children with and without developmental delay and maternal information

Variable	Children with developmental delay ($n=174$)	Children without developmental delay ($n=88$)	Age-sex-adjusted OR (95% CI)
Sex			
Male	121 (69.54)	50 (56.82)	1.00
Female	53 (30.46)	38 (43.18)	0.62 (0.36 –1.08) $^{+,a}$
Age	4.83 (3.54, 6.08)***	6.29 (3.74, 9.16)***	0.79 (0.71 – 0.88) ^{***,b}
Birth height (cm)	50.00 (48.00, 51.00)*	50.00 (49.00, 51.00)*	0.93 (0.85 – 1.02)
Birth weight (g)	3060.0 (2705.0, 3345.0)*	3100.0 (2820.0, 3350.0)*	0.99 (0.99 –1.00)*
Maternal age (years)	29.00 (26.00, 33.00)	30.00 (27.00, 34.00)	0.97 (0.92 – 1.02)
Maternal gestational weeks (weeks)	38.00 (37.00, 40.00)**	39.00 (38.00, 40.00) **	0.84 (0.74 – 0.94) **
Parity			
One	95 (55.56)	48 (55.17)	1.00
Two	56 (32.75)	34 (39.08)	0.78 (0.43 – 1.39)
Three or more	20 (11.70)	5 (5.75)	0.25 (0.83 – 7.58)
Maternal educational level			
Illiterate/elementary school	7 (4.02)	0 (0.00)	1.00
Junior/senior high school	80 (45.98)	31 (35.23)	
College and higher	87 (50.00)	57 (64.77)	0.48 (0.27—0.84)*

Values expressed as the median (first quartile, third quartile) or number (%) of cases and controls

Abbreviations: OR, odds ratio and CI, confidence interval

p-values were tested using the Wilcoxon rank-sum test for continuous variables and χ^2 test for the rest of categorical variables

[a] Age-adjusted OR and 95% CI

[b] Sex-adjusted OR and 95% CI

$^{+}$ $0.05 \le p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

concentration ≤ 12.55 μg/L, and blood lead concentration ≤ 3.00 μg/dL. The odds of DD significantly increased gradually in children with no risk factor, either one, or both (5mdC (%) ≤ 5.03, blood lead > 2.96 μg/dL, or total urinary arsenic > 12.55 μg/L). Compared to the reference group, children with low 5mdC (%) and high blood lead or total urinary arsenic exhibited a significant rise in odds of DD, with OR (95% CI) of 9.51 (4.18 – 21.64) and 8.09 (2.49 – 26.34), respectively (Table 3). A significant multiplicative interaction between high blood lead levels and low 5mdC (%) on DD in children was observed, where *p* value was 0.011 for multiplicative interaction following adjustment for age and sex and 0.021 for multivariate adjustment, while other interactions showed no significance. Additionally, we discovered that low 5mdC (%) together with high blood lead and total urinary arsenic concentrations significantly increased DD risk in preschool children, with an OR (95% CI) of 18.82 (5.72 – 61.85) after controlling for multiple variables, compared to those with high 5mdC (%) and low blood lead and total urinary arsenic concentrations (Table 3).

Stepwise multiple logistic regression analysis

We used stepwise logistic regression analysis to identify negative or positive predictors of DD (Table 4). Our findings suggested that age, gestational age, and 5mdC (%) were notable negative predictors, whereas blood lead levels emerged as a significant positive predictor for DD in preschool-aged children.

Discussion

We found a significant positive correlation between blood lead and total urinary arsenic concentrations and the risk of DD in preschool children, which aligns with findings from our prior research [22, 24]. This study marks the first examination of the combined impacts of the global DNA methylation marker 5mdC (%) alongside blood lead and/or total urinary arsenic concentrations on DD in preschoolers. Higher levels of 5mdC (%) were associated with decreased risk of DD in this age group. Furthermore, low 5mdC (%) showed a significant multiplicative interaction with high lead levels, increasing the OR of DD. In addition, we found that with more risk factors (low 5mdC (%), high blood lead and high total urinary arsenic concentration), the risk of DD among preschoolers gradually increased dose-dependently.

In a review paper, it was pointed out that epigenetic regulation, especially changes in gene methylation patterns, is related to the progression and outcome of neurological diseases [52]. Global DNA methylation measurements in leukocytes showed that global DNA methylation levels decreased with age, but in contrast,

Table 2 Association of 5mdC (%), total urinary arsenic and blood lead and cadmium levels with developmental delay in preschool children

Variable	Children with developmental delay (*n* = 174)	Children without developmental delay (*n* = 88)	Age-sex-adjusted ORs (95% CI)	Multivariate ORs (95% CI)[b]
5mdC (%)	3.68 (3.19, 4.24) [#,***]	5.03 (3.91, 5.70) [#,***]		
£ 4.28	131 (75.29	30 (34.09)	1.00 [§,***]	1.00 [§,***]
>4.28 – 5.45	21 (12.07)	28 (31.82)	0.13 (0.06 – 0.28) [***]	0.09 (0.04–0.25) [***]
> 5.45	22 (12.64)	30 (34.09)	0.15 (0.07 – 0.31) [***]	0.14 (0.06–0.32) [***]
Total urinary arsenic(μg/L)	15.98 (8.00, 28.05) [#,+]	12.55 (5.38, 23.41)[#,+]		
≤ 7.06	36 (20.69)	30 (34.09)	1.00[§,a,+]	1.00 [§,c,*]
>7.06 – 18.46	65 (37.36)	29 (32.95)	1.71 (0.84 – 3.46)	1.79 (0.78 – 4.09)
> 18.46	73 (41.95)	29 (32.95)	2.21 (0.94 – 3.17)+	2.44 (0.89 – 6.70)+
Blood lead(μg/dL)	5.89 (3.59, 8.46) [#,***]	3.00 (2.37, 4.34) [#,***]		
≤ 2.68	21 (12.64)	31 (35.23)	1.00[§,***]	1.00 [§,***]
>2.68 – 3.91	30 (17.24)	28 (31.82)	1.69 (0.76 – 3.75)	1.13 (0.44 – 2.89)
>3.91	122 (70.11)	29 (32.95)	6.35 (3.08 – 13.09)[***]	4.46 (1.92 – 10.39)[***]
Blood cadmium(μg/L)	0.90 (0.70, 1.35) [#]	0.85 (0.60, 1.35) [#]		
≤ 0.70	45 (25.86)	30 (34.10)	1.00[§,+]	1.00
>0.70 – 1.15	68 (39.08)	29 (32.95)	1.67 (0.85 – 3.26)	1.75 (0.80 – 3.86)
>1.15	61 (35.06)	29 (32.95)	1.92 (0.96 – 3.85)[+]	1.38 (0.61 – 3.14)

Note: Values are expressed as the median (first and third quartile) or the number of cases (%)

Abbreviations: *OR* = odds ratio, *CI* = confidence interval. 5mdC (%) = ratio of 5-methyl-2'deoxycytidine

[a] Adjusted for age, sex, and urinary creatinine

[b] Adjusted for age, sex, birth weight, maternal education level, and number of gestational weeks

[c] Adjusted for age, sex, birth weight, maternal education level, number of gestational weeks, and urinary creatinine

[#] Calculated using the Wilcoxon rank-sum test

[+] $0.05 \leq p < 0.1$, [*] $p < 0.05$, [***] $p < 0.001$

[§] for trend tests

global DNA hypermethylation was observed in autism, a neurodevelopmental disorder [47]. A Brazilian study found that global DNA methylation (5mdc%) was lower in adults with ADHD than normal adults, but if they had bipolar disorder (BD) at the same time, 5mdc% was higher than in those without BD [34]. One study pointed out that the use of 5-MC ELISA kit in cord blood assessment of global DNA methylation showed a significant positive correlation with the mental scores of 18-month-old infants [18]. The content of 5hmC in neurons is about 10 times more abundant than in other tissues, showing that 5hmC, as an important and stable epigenetic marker, may play an important role in the brain [28]. Global levels of 5hmC were significantly lower in a study of Mcph1-del mice, suggesting that reduced 5hmC levels are critical for neurodevelopment and brain function [51]. Nonetheless, an investigation involving 50 individuals diagnosed with autism spectrum disorder (ASD) and 45 controls revealed a slight reduction in Line1, serving as a global DNA methylation indicator, within the cohort of children with ASD compared to the control group [16]. Another study pointed out that hypomethylation of specific gene cg01271805 will increase the expression of ERC2 (ELKS/RAB6-Interacting/CAST Family Member 2), thereby increasing the release of neurotransmitters and adversely affecting the development of ADHD symptoms; other changes in DNA methylation of a specific gene, CREB5 (cyclic AMP response element binding protein 5) (cg25520701), may alter the risk of ADHD during development [36]. In our investigation, we discovered that the global DNA methylation marker 5mdC (%) was notably lower in preschool children with DD compared to those exhibiting typical development. This observation suggests a potential correlation between decreased global DNA methylation and DD in preschool-aged children. Perhaps DNA methylation dysregulation corresponds to de novo mutations, increased expression of genes encoding epigenetic machinery, epigenetic silencing, aberrantly methylated genes, and genome-wide hypomethylation

Table 3 The combined effects of 5mdC, total urinary arsenic, and blood lead levels on with developmental delay in preschool children

Total urinary arsenic(μg/L)	Blood lead(μg/dL)		Children with /without developmental delay	Age-sex-adjusted ORs (95%CI)	Multivariate ORs(95%CI)[b]
≤ 12.55	≤ 3.00		10/26	1.00[§,a,***]	1.00[§,c,***]
>12.55	≤ 3.00		22/18	2.22 (0.75 – 6.55)	1.26 (0.36 – 4.42)
≤ 12.55	>3.00		61/18	8.06 (3.12 – 20.78)[***]	4.89 (1.69 – 14.21)[**]
>12.55	>3.00		84/26	7.56 (2.90 – 19.76)[***]	4.27 (1.48 – 12.27)[**]
			Synergy index	0.79 (0.34 – 1.84)	0.79 (0.27 – 2.33)
			p interaction	0.2047	0.097
5mdC (%)	Total urinary arsenic(μg/L)				
>5.03	≤ 12.55		9/23	1.00[§,a,***]	1.00[§,c,***]
>5.03	>12.55		15/21	1.29 (0.38 – 4.33)	1.21 (0.30 – 4.91)
≤ 5.03	≤ 12.55		62/21	7.12 (2.73 – 18.55)[***]	8.18 (2.64 – 25.31)[**]
≤ 5.03	>12.55		88/23	8.55 (3.07 – 23.81)[***]	8.09 (2.49 – 26.34)[**]
			Synergy index	1.18 (0.49 – 2.84)	0.96 (0.35 – 2.60)
			p interaction	0.6872	0.7160
5mdC (%)	Blood lead(μg/dL)				
>5.03	≤ 3.00		0/25	1.00[§,***]	1.00[§,***]
>5.03	>3.00		24/19		
≤ 5.03	≤ 3.00		32/19	3.00 (1.35 – 6.67)[**]	4.00 (1.53 – 10.49)[**]
≤ 5.03	>3.00		118/25	9.63 (4.77 – 19.47)[***]	9.51 (4.18 – 21.64)[***]
			p interaction	0.011	0.021
5mdC (%)	Total urinary arsenic(μg/L)	Blood lead(μg/L)	Children with /without developmental delay	Age-sex-adjusted ORs (95% CI)[a]	Multivariate ORs(95% CI)[c]
>5.03	≤ 12.55	≤ 3.00	0/16	1.00[§,***]	1.00[§,***]
>5.03	>12.55	≤ 3.00	9/16		
>5.03	> 12.55	>3.00	15/12	4.26 (1.33 – 13.66)[*]	3.46 (0.90 – 13.37)[+]
≤ 5.03	≤ 12.55	≤ 3.00	10/10	3.63 (1.07 – 12.33)[*]	7.87 (1.78 – 34.83)[**]
≤ 5.03	>12.55	≤ 3.00	74/20	12.48 (4.94 – 31.57)[***]	10.78 (3.64 – 31.87)[***]
≤ 5.03	> 12.55	>3.00	66/14	20.11 (7.18 – 56.35)[***]	18.82 (5.72 – 61.85)[***]

Abbreviations: *OR* Odds ratio, *CI* Confidence interval. 5mdC (%) = ratio of 5-methyl-2′deoxycytidine

$p_{\text{interaction}}$: *p* value for multiplicative interactions

[a] Adjusted for age, sex, and urinary creatinine

[b] Adjusted for age, sex, birth weight, maternal education level, and number of gestational weeks

[c] Adjusted for age, sex, birth weight, maternal education level, number of gestational weeks, and urinary creatinine

[+] $0.05 \leq p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

[§] for trend tests

or hypermethylation [52], which need further investigation. Using different techniques to represent global DNA methylation, whether the mechanism of 5mdC is similar to that of 5hmc and is critical for neurodevelopment and brain function [51], or whether it is like changes in methylation of specific genes [36] or influences the inflammatory process [17], the risk of DD is affected, which requires further exploration.

In utero exposure to arsenic and mercury has been found to interact to affect the epigenome by hypermethylating associated CpG regions, which has the potential to affect neurodevelopment and other child health outcomes [4]. A study identified that lead exposure resulted in diminished levels of global DNA methylation (specifically Line1 and Alu methylation), which contributed to lead-induced genotoxicity among workers exposed to

Table 4 Stepwise multiple logistic regression analysis

Variable	OR (95% CI)
Age	0.75 (0.66 – 0.86)***
Blood lead level (μg/dL)	1.39 (1.20 – 1.60)***
5mdC (%)	0.65 (0.52 – 0.81)***
Gestational weeks	0.83 (0.71 – 0.96)*
Sex	0.47 (0.23 – 0.94)*

Abbreviations: *OR* Odds ratio, *CI* Confidence interval. 5mdC (%) = ratio of 5-methyl-2'deoxycytidine

Variables included age, sex, blood lead level, total urinary arsenic concentration, gestational weeks, maternal education level, 5mdC (%) in the stepwise multiple logistic regression model

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

lead in China [49]. In a prospective birth cohort study, multiple regression analysis showed a positive association between relatively low cord blood lead levels (range 0.39 to 4.84 μg/dL) and the DNA methylation marker 5mdC [38]. Research on juvenile Nile tilapia found that long-term cadmium exposure reduced antioxidant capacity, caused oxidative damage to lipids and DNA, and reduced global DNA methylation levels, and it inhibited growth [25]. Arsenic and lead exposure leads to SAM deficiency and reduced DNA methyl transferases (DNMT) gene expression, resulting in global DNA hypomethylation [39, 40]. Pollutants such as arsenic, cadmium and lead may through these pathways affect DNA methylation of the leukocytes. However, this study did not find any correlations between blood lead and cadmium or total urinary arsenic levels and 5mdC (%). This suggests that perhaps the impacts of total urinary arsenic and blood lead concentrations on DD in preschool children are not directly influenced by global DNA methylation, specifically 5mdC (%).

Most current studies focus on metal-induced changes in global DNA methylation that lead to neurotoxicity [11, 12, 32]. However, to the best of current knowledge, no studies investigating the interaction between metals and global DNA methylation on neurotoxicity have been identified. We noted that elevated levels of blood lead and total urinary arsenic, whether individually or in conjunction with 5mdC (%), were linked to a higher OR of DD in preschool children. Remarkably, we observed a significant multiplicative interaction between elevated blood lead concentration and decreased 5mdC (%), intensifying the risk of DD in this age group. It is conceivable that Pb^{2+} ions might displace Ca^{2+} ions efficiently, facilitating their passage through the blood–brain barrier and subsequent accumulation in brain cells [15]. This accumulation appears to impact various aspects of neural function, including neuronal signaling, myelination, and glial cell activity [31, 45]. Lead seems to disrupt calcium release from mitochondria, resulting in the generation of reactive oxygen species (ROS) and subsequent impairment of mitochondrial function [2], thereby indirectly influencing nervous system functionality [31, 45]. ROS-mediated oxidative stress has been associated with global hypomethylation, and the DNA oxidation structure called 8-hydroxy-2'-deoxyguanosine (8-OHdG) might contribute to DNA hypomethylation by obstructing the methylation process of nearby cytosine bases [50]. Blood lead levels alone could directly impact the central nervous system, or they may interact significantly with lower levels of 5mdC (%) (global DNA hypomethylation), potentially leading to higher risk of DD [44]. However, a thorough investigation is necessary to elucidate the precise underlying mechanism.

Our study possesses certain limitations. First, as this was a case–control study, it was not feasible to establish a temporal association between metal concentrations, global DNA methylation, and DD. The lack of information on other potential factors that may affect methylation, such as colds, illnesses, or diet in children, was also a limitation. Additionally, the sample size was small, leading to limited statistical power, particularly in examining joint effects across multiple levels. In addition, due to the small number of samples, if classified according to different DD categories or different degrees of DD, the number of samples in each category would be smaller. Therefore, we used unclassified DD for data analysis. Moreover, there was an inadequate number of healthy children to match for age and sex with those experiencing DD. While we used various variables to mitigate confounding effects, future research endeavors will aim to include a larger pool of healthy children for a more robust stratified analysis. At present, the correlation between DNA methylation and disease is unclear. There are currently very few studies on 5mdC and disease. We observed that a decrease in 5mdC (%) was associated with DD risk. The mechanism needs to be further explored and confirmed in the future.

Conclusions

Our study was groundbreaking in identifying significant negative associations between the global DNA methylation marker 5mdC (%) and the likelihood of DD in preschool children. Moreover, our exploratory findings revealed that low levels of 5mdC (%) exhibited a significant multiplicative interaction with high blood lead levels, exacerbating DD risk in a dose–response fashion.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12940-024-01151-6.

Supplementary Material 1.

Acknowledgements
We appreciate the assistance with study subject recruitment provided by Drs. Ming-I Lin, Shu-Chi Mu, Tseng-Chen Sung, Cheng Hui Lin, and Ling-Jen Wang of the Department of Pediatrics, Shin Kong Wu Ho-Su Memorial Hospital. The present study was supported by grants from the Ministry of Science and Technology (MOST 107-2314-B-038-073, MOST 108-2314-B-038 -089, MOST 109-2314-B-038-081, MOST 109-2314-B-038-067, MOST 110-2314-B-038-054, MOST 111-2314-B-038-052, MOST 111-2314-B-002-240-MY3, and NSTC 112-2314-B-038-094).

Authors' contributions
Mei-Chieh Chen, Chih-Yin Wu, Ying-Chin Lin, and Horng-Sheng Shiue partly contributed to the conception and design of the work. Ru-Lan Hsieh, Ming-I Lin, and Shu-Chi Mu recruited the study subjects. Hui-Ling Lee has done the experiment. Ya-Li Huang contributed to the statistical analysis and analyzed the data. Yuu-Hueih Hsu wrote the manuscript; Yu-Mei Hsueh performed the study design and executed the whole research plan. All authors reviewed the manuscript.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
No actual or potential competing financial interests.

Received: 10 September 2024 Accepted: 24 December 2024
Published online: 16 January 2025

References

1. Barr DB, Wilder LC, Caudill SP, Gonzalez AJ, Needham LL, Pirkle JL. Urinary creatinine concentrations in the U.S. population: implications for urinary biologic monitoring measurements. Environ Health Perspect. 2005;113:192–200.
2. Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol. 2004;287:C817–33.
3. Browne MJ, Turnbull JF, McKay EL, Adams RL, Burdon RH. The sequence specificity of a mammalian DNA methylase. Nucleic Acids Res. 1977;4:1039–45.
4. Cardenas A, Koestler DC, Houseman EA, Jackson BP, Kile ML, Karagas MR, Marsit CJ. Differential DNA methylation in umbilical cord blood of infants exposed to mercury and arsenic in utero. Epigenetics. 2015;10:508–15.
5. Chen CY, Liu CY, Su WC, Huang SL, Lin KM. Factors associated with the diagnosis of neurodevelopmental disorders: a population-based longitudinal study. Pediatrics. 2007;119:e435–43.
6. Chen HJ, Hsin-Ju KM, Li ST, Chiu NC, Hung KL. Prevalence of preschool children developmental disabilities in northeastern Taiwan - Screening with Taipei City Developmental Screening Checklist for Preschoolers, 2nd Version. J Formos Med Assoc. 2020;119:1174–9.
7. Choi J, Chang JY, Hong J, Shin S, Park JS & Oh S. Low-Level Toxic Metal Exposure in Healthy Weaning-Age Infants: Association with Growth, Dietary Intake, and Iron Deficiency. Int J Environ Res Public Health. 2017;14.
8. Chung CJ, Chang CH, Liou SH, Liu CS, Liu HJ, Hsu LC, Chen JS, Lee HL. Relationships among DNA hypomethylation, Cd, and Pb exposure and risk of cigarette smoking-related urothelial carcinoma. Toxicol Appl Pharmacol. 2017;316:107–13.
9. Cicero CE, Mostile G, Vasta R, Rapisarda V, Signorelli SS, Ferrante M, Zappia M, Nicoletti A. Metals andneurodegenerative diseases. A systematic review Environ Res. 2017;159:82–94.
10. Department of Statistics M.o.t.I. (2010) Reporting No. of Early Intervention Services for Developmentally-delayed Children. Department of Statistics, Ministry of the Interiors,Tapei, Taiwan.
11. Dou JF, Farooqui Z, Faulk CD, Barks AK, Jones T, Dolinoy DC, Bakulski KM. (2019) Perinatal Lead (Pb) Exposure and Cortical Neuron-Specific DNA Methylation in Male Mice. Genes (Basel) 10.
12. Du X, Tian M, Wang X, Zhang J, Huang Q, Liu L, Shen H. Cortex and hippocampus DNA epigenetic response to a long-term arsenic exposure via drinking water. Environ Pollut. 2018;234:590–600.
13. Fiore M, Barone R, Copat C, Grasso A, Cristaldi A, Rizzo R & Ferrante M. Metal and essential element levels in hair and association with autism severity. J Trace Elem Med Biol. 2020;57:126409.
14. Flora SJ. Arsenic-induced oxidative stress and its reversibility. Free Radic Biol Med. 2011;51:257–81.
15. Florea AM, Taban J, Varghese E, Alost BT, Moreno S, Büsselberg D. (2013) Lead (Pb2+) neurotoxicity: Ion-mimicry with calcium (Ca2+) impairs synaptic transmission. Journal of Local and Global Health Science 4.
16. Garcia-Ortiz MV, de la Torre-Aguilar MJ, Morales-Ruiz T, Gomez-Fernandez A, Flores-Rojas K, Gil-Campos M, Martin-Borreguero P, Ariza RR, Roldan-Arjona T, Perez-Navero JL. Analysis of global and local DNA methylation patterns in blood samples of patients with autism spectrum disorder. Front Pediatr. 2021;9:685310.
17. Gonzalez-Jaramillo V, Portilla-Fernandez E, Glisic M, Voortman T, Ghanbari M, Bramer W, Chowdhury R, Nijsten T, Dehghan A, Franco OH, Nano J. Epigenetics and inflammatory markers: a systematic review of the current evidence. Int J Inflam. 2019;2019:6273680.
18. Gurugubelli KR, Ballambattu VB, Bobby Z. (2021) Global DNA Methylation in Cord Blood and Neurodevelopmental Outcome at 18 Months of Age among Intrauterine Growth Restricted and Appropriate for Gestational Age Infants. J.Trop.Pediatr. 67.
19. Hitchler MJ, Domann FE. An epigenetic perspective on the free radical theory of development. Free Radic Biol Med. 2007;43:1023–36.
20. Hosmer DW, Lemeshow S. Confidence interval estimation of interaction. Epidemiology. 1992;3:452–6.
21. Hsieh RL, Hsueh YM, Huang HY, Lin MI, Tseng WC, Lee WC. (2013) Quality of life and impact of children with unclassified developmental delays. J.Paediatr.Child Health 49, E116-E21.
22. Hsieh RL, Huang YL, Shiue HS, Huang SR, Lin MI, Mu SC, Chung CJ, Hsueh YM. Arsenic methylation capacity and developmental delay in preschool children in Taiwan. Int J Hyg Environ Health. 2014;217:678–86.
23. Hsueh YM, Huang YL, Huang CC, Wu WL, Chen HM, Yang MH, Lue LC, Chen CJ. Urinary levels of inorganic and organic arsenic metabolites among residents in an arseniasis-hyperendemic area in Taiwan. J Toxicol Environ Health A. 1998;54:431–44.
24. Hsueh YM, Lee CY, Chien SN, Chen WJ, Shiue HS, Huang SR, Lin MI, Mu SC, Hsieh RL. Association of blood heavy metals with developmental delays and health status in children. Sci Rep. 2017;7:43608.
25. Hu F, Yin L, Dong F, Zheng M, Zhao Y, Fu S, Zhang W, Chen X. Effects of long-term cadmium exposure on growth, antioxidant defense and DNA methylation in juvenile Nile tilapia (Oreochromis niloticus). Aquat Toxicol. 2021;241:106014.
26. Ijomone OM, Ijomone OK, Iroegbu JD, Ifenatuoha CW, Olung NF, Aschner M. Epigenetic influence ofenvironmentally neurotoxic metals. Neurotoxicology. 2020;81:51–65.
27. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science. 2011;333:1300–3.
28. Kriaucionis S, Heintz N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science. 2009;324:929–30.
29. Lin CY, Lee HL, Hwang YT, Huang PC, Wang C, Sung FC, Wu C, Su TC. Urinary heavy metals, DNA methylation, and subclinical atherosclerosis. Ecotoxicol Environ Saf. 2020;204:111039.

30. Liou SH, Wu WT, Liao HY, Chen CY, Tsai CY, Jung WT, Lee HL. Global DNA methylation and oxidative stress biomarkers in workers exposed to metal oxide nanoparticles. J Hazard Mater. 2017;331:329–35.
31. Mason LH, Harp JP, Han DY. Pb neurotoxicity: neuropsychological effects of lead toxicity. Biomed Res Int. 2014;2014:840547.
32. Mei Z, Liu G, Zhao B, He Z, Gu S. Emerging roles of epigenetics in lead-induced neurotoxicity. Environ Int. 2023;181:108253.
33. Mooney MA, Ryabinin P, Wilmot B, Bhatt P, Mill J, Nigg JT. Large epigenome-wide association study of childhood ADHD identifies peripheral DNA methylation associated with disease and polygenic risk burden. Transl Psychiatry. 2020;10:8.
34. Muller D, Grevet EH, Figueira da Silva NA, Bandeira CE, Barbosa E, Vitola ES, Charao MF, Linden R, Rohde LA, Ramos JKN, da Silva BS, Rovaris DL, Bau CHD. Global DNA methylation changes in adults with attention deficit-hyperactivity disorder and its comorbidity with bipolar disorder: links with polygenic scores. Mol Psychiatry. 2022;27:2485–91.
35. Naujokas MF, Anderson B, Ahsan H, Aposhian HV, Graziano JH, Thompson C, Suk WA. The broad scope of health effects from chronic arsenic exposure: update on a worldwide public health problem. Environ Health Perspect. 2013;121:295–302.
36. Neumann A, Walton E, Alemany S, Cecil C, Gonzalez JR, Jima DD, Lahti J, Tuominen ST, Barker ED, Binder E, Caramaschi D, Carracedo A, Czamara D, Evandt J, Felix JF, Fuemmeler BF, Gutzkow KB, Hoyo C, Julvez J, Kajantie E, Laivuori H, Maguire R, Maitre L, Murphy SK, Murcia M, Villa PM, Sharp G, Sunyer J, Raikkonen K, Bakermans-Kranenburg M, IJzendoorn MV, Guxens M, Relton CL, Tiemeier H. Association between DNA methylation and ADHD symptoms from birth to school age: a prospective meta-analysis. Transl Psychiatry. 2020;10:398.
37. Niedzwiecki MM, Hall MN, Liu X, Oka J, Harper KN, Slavkovich V, Ilievski V, Levy D, van Geen A, Mey JL, Alam S, Siddique AB, Parvez F, Graziano JH, Gamble MV. A dose-response study of arsenic exposure and global methylation of peripheral blood mononuclear cell DNA in Bangladeshi adults. Environ Health Perspect. 2013;121:1306–12.
38. Okamoto Y, Iwai-Shimada M, Nakai K, Tatsuta N, Mori Y, Aoki A, Kojima N, Takada T, Satoh H, Jinno H. Global DNA methylation in cord blood as a biomarker for prenatal lead and antimony exposures. Toxics. 2022;10(4):157.
39. Reichard JF, Schnekenburger M, Puga A. Long term low-dose arsenic exposure induces loss of DNA methylation. Biochem Biophys Res Commun. 2007;352:188–92.
40. Ruiz-Hernandez A, Kuo CC, Rentero-Garrido P, Tang WY, Redon J, Ordovas JM, Navas-Acien A, Tellez-Plaza M. Environmental chemicals and DNA methylation in adults: a systematic review of the epidemiologic evidence. Clin Epigenetics. 2015;7:55.
41. Sanchez OF, Lee J, Yu King Hing N, Kim SE, Freeman JL, Yuan C. Lead (Pb) exposure reduces global DNA methylation level by non-competitive inhibition and alteration of dnmt expression. Metallomics. 2017;9:149–60.
42. Sawcer S, Hellenthal G, Pirinen M, Spencer CC, Patsopoulos NA, Moutsianas L, Dilthey A, Su Z, Freeman C, Hunt SE, Edkins S, Gray E, Booth DR, Potter SC, Goris A, Band G, Oturai AB, Strange A, Saarela J, Bellenguez C, Fontaine B, Gillman M, Hemmer B, Gwilliam R, Zipp F, Jayakumar A, Martin R, Leslie S, Hawkins S, Giannoulatou E, D'Alfonso S, Blackburn H, Martinelli BF, Liddle J, Harbo HF, Perez ML, Spurkland A, Waller MJ, Mycko MP, Ricketts M, Comabella M, Hammond N, Kockum I, McCann OT, Ban M, Whittaker P, Kemppinen A, Weston P, Hawkins C, Widaa S, Zajicek J, Dronov S, Robertson N, Bumpstead SJ, Barcellos LF, Ravindrarajah R, Abraham R, Alfredsson L, Ardlie K, Aubin C, Baker A, Baker K, Baranzini SE, Bergamaschi L, Bergamaschi R, Bernstein A, Berthele A, Boggild M, Bradfield JP, Brassat D, Broadley SA, Buck D, Butzkueven H, Capra R, Carroll WM, Cavalla P, Celius EG, Cepok S, Chiavacci R, Clerget-Darpoux F, Clysters K, Comi G, Cossburn M, Cournu-Rebeix I, Cox MB, Cozen W, Cree BA, Cross AH, Cusi D, Daly MJ, Davis E, de Bakker PI, Debouverie M, D'Hooghe MB, Dixon K, Dobosi R, Dubois B, Ellinghaus D, Elovaara I, Esposito F, Fontenille C, Foote S, Franke A, Galimberti D, Ghezzi A, Glessner J, Gomez R, Gout O, Graham C, Grant SF, Guerini FR, Hakonarson H, Hall P, Hamsten A, Hartung HP, Heard RN, Heath S, Hobart J, Hoshi M, Infante-Duarte C, Ingram G, Ingram W, Islam T, Jagodic M, Kabesch M, Kermode AG, Kilpatrick TJ, Kim C, Klopp N, Koivisto K, Larsson M, Lathrop M, Lechner-Scott JS, Leone MA, Leppa V, Liljedahl U, Bomfim IL, Lincoln RR, Link J, Liu J, Lorentzen AR, Lupoli S, Macciardi F, Mack T, Marriott M, Martinelli V, Mason D, McCauley JL, Mentch F, Mero IL, Mihalova T, Montalban X, Mottershead J, Myhr KM, Naldi P, Ollier W, Page A, Palotie A, Pelletier J, Piccio L, Pickersgill T, Piehl F, Pobywajlo S, Quach HL, Ramsay PP, Reunanen M, Reynolds R, Rioux JD, Rodegher M, Roesner S, Rubio JP, Ruckert IM, Salvetti M, Salvi E, Santaniello A, Schaefer CA, Schreiber S, Schulze C, Scott RJ, Sellebjerg F, Selmaj KW, Sexton D, Shen L, Simms-Acuna B, Skidmore S, Sleiman PM, Smestad C, Sorensen PS, Sondergaard HB, Stankovich J, Strange RC, Sulonen AM, Sundqvist E, Syvanen AC, Taddeo F, Taylor B, Blackwell JM, Tienari P, Bramon E, Tourbah A, Brown MA, Tronczynska E, Casas JP, Tubridy N, Corvin A, Vickery J, Jankowski J, Villoslada P, Markus HS, Wang K, Mathew CG, Wason J, Palmer CN, Wichmann HE, Plomin R, Willoughby E, Rautanen A, Winkelmann J, Wittig M, Trembath RC, Yaouanq J, Viswanathan AC, Zhang H, Wood NW, Zuvich R, Deloukas P, Langford C, Duncanson A, Oksenberg JR, Pericak-Vance MA, Haines JL, Olsson T, Hillert J, Ivinson AJ, De Jager PL, Peltonen L, Stewart GJ, Hafler DA, Hauser SL, McVean G, Donnelly P, Compston A. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature. 2011;476:214–9.
43. Senut MC, Sen A, Cingolani P, Shaik A, Land SJ, Ruden DM. Lead exposure disrupts global DNA methylation in human embryonic stem cells and alters their neuronal differentiation. Toxicol Sci. 2014;139:142–61.
44. Shirvani-Farsani Z, Maloum Z, Bagheri-Hosseinabadi Z, Vilor-Tejedor N, Sadeghi I. DNA methylation signature as a biomarker of major neuropsychiatric disorders. J Psychiatr Res. 2021;141:34–49.
45. Simons TJ. Cellular interactions between lead and calcium. Br Med Bull. 1986;42:431–4.
46. Song L, James SR, Kazim L, Karpf AR. Specific method for the determination of genomic DNA methylation by liquid chromatography-electrospray ionization tandem mass spectrometry. Anal Chem. 2005;77:504–10.
47. Tsang SY, Ahmad T, Mat FW, Zhao C, Xiao S, Xia K, Xue H. Variation of global DNA methylation levels with age and in autistic children. Hum Genomics. 2016;10:31.
48. Von Walden F, Fernandez-Gonzalo R, Pingel J, McCarthy J, Stål P, Pontén E. Epigenetic marks at the Ribosomal DNA promoter in skeletal muscle are negatively associated with degree of impairment in cerebral palsy. Front Pediatr. 2020;8:236.
49. Wang T, Meng Y, Tu Y, Zhang G, Wang K, Gong S, Zhang Y, Wang T, Li A, Christiani DC, Au W, Xia ZL. Associations between DNA methylation and genotoxicity among lead-exposed workers in China. Environ Pollut. 2023;316:120528.
50. Wu Q, Ni X. ROS-mediated DNA methylation pattern alterations in carcinogenesis. Curr Drug Targets. 2015;16:13–9.
51. Yang Q, Cao Q, Yu Y, Lai X, Feng J, Li X, Jiang Y, Sun Y, Zhou ZW, Li X. (2023) Epigenetic and transcriptional landscapes during cerebral cortex development in a microcephaly mouse model. J Genet Genomics.
52. Younesian S, Yousefi AM, Momeny M, Ghaffari SH, Bashash D. The DNA Methylation in Neurological Diseases. Cells. 2022;11:3439.

Publisher's Note