PAHs Changed Epigenetics by One Carbon Metabolism in Childhood Asthma

Hao Wu

Nanjing Medical University

Yuling Bao

Children's Hospital of Nanjing Medical University

Jinye Hu

Nanjing Medical University

Hui Huang

Nanjing Medical University

Ping Jiang

Nanjing Medical University

Zhan Zhang

Nanjing Medical University

Lei Li

Nanjing Medical University

Qian Wu (✉ WUqian@njmu.edu.cn)

Nanjing Medical University <https://orcid.org/0000-0001-5155-2428>

Research

Keywords: PAHs, asthma, one carbon metabolism, DNA methylation, histone methylation

Posted Date: November 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1103501/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Background: Epigenetic mechanisms may play a role in which PAHs exert its adverse effects in childhood asthma. However, the underlying molecular mechanism remain to be fully elucidated. This study aimed to investigate this process in view of cellular metabolism, especially one carbon metabolism.

Methods: Fifty asthmatic children and fifty control subjects were recruited in this study. Serum IgE and IL-17A was detected by ELISA assay. Serum PAHs levels were measured by GC-MS. One carbon-related metabolites were determined by UPLC-Orbitrap-MS. Blood DNA methylation in long interspersed nucleotide element-1 (*LINE-1*) was analyzed by bisulfite sequencing PCR. ChIP assays were used to examine H3K4me3 modifications on *IL-17A* gene. Multivariable linear regression was performed to evaluate the associates between PAHs and DNA methylation and histone methylation mediated by one carbon metabolism.

Results: The asthmatic group presented significantly higher total serum IgE and IL-17A levels. Serum Fla was associated with childhood asthma. The asthmatic group displayed a significantly decreasing in SAM abundance and a smaller but corresponding decrease in SAH, which indicated the increasing conversion from SAM to SAH and the elevated capacity of methylation reactions. Fla had a great effect on one carbon metabolites, especially SAH, SAM and Ser, which exerted significant mediation effects between the Fla level and asthma. What's more, Fla had a positive effect on *LINE-1* DNA methylation ($\beta=0.395$, $P=0.000$) and H3K4 tri-methylation level in the *IL-17A* promoter region ($\beta=0.293$, $P=0.002$). We did find significant mediation effect between serum Fla and asthma by *LINE-1* DNA methylation and H3K4me3 level in the *IL-17A* promoter region.

Conclusion: PAHs disturbed one carbon metabolism to influence the methyl group refill of DNA methylation and histone methylation, which may elevate serum IL-17A level in asthmatic children.

Highlights

- PAHs induced one carbon metabolic aspect of epigenetics in asthmatic children
- PAHs increased global DNA methylation in asthmatic children
- PAHs increased the recruitment of H3K4me3 on *IL-17A* promoter in asthmatic children

1 Introduction

Asthma is a complex and multiple etiological disease [1]. It is also a common chronic inflammation in children [2]. Asthma prevalence under age 18 years released in 2018 in United States was 7.5% [3]. The third (2010-2011) national epidemiological survey in China showed that the prevalence of childhood asthma had been up to 3.02% [4]. Although no latest official national statistics have been conducted on the current prevalence of childhood asthma, a wide range of regional surveys have showed an increasing trend in different cities of China. The predicted prevalence of asthma in 2020 was estimated from 1.11% among rural girls aged 14 years to 10.27% among urban boys aged four years [5]. Exposure to air pollution have been linked to increasing asthma prevalence and asthma onset [6,7]. Especially, ambient polycyclic aromatic hydrocarbons (PAHs), which are traffic related air pollutants and organic components of PM 2.5 and PM 10, have been known to contribute to the onset of asthma [8,9]. In the process of PAHs biotransformation, a cascade of oxidative stress is triggered leading to cytotoxicity and DNA damage [10-12]. PAHs also stimulate inflammatory responses through increasing the production of IgE [13]. What's more, PAHs acting on aryl hydrocarbon receptors (AhRs) can affect regulatory T cell (Treg) function and induce Th17 cell differentiation [14].

In addition, there is growing evidence to demonstrate that PAHs exposure may alter global and gene-specific DNA methylation patterns [15]. For example, as global methylation indicators, long interspersed nuclear elements-1 (*LINE-1*) and short interspersed nuclear elements (*Alu*) was used to indicate the global methylation status and considered as intermediators of environmental exposure to future health risk [16]. For specific gene, increased methylation of *FOXP3* gene was associated with chronic PAHs exposure leading to Treg dysfunction in atopic children [17]. DNA methylation is a biological process by which

methyl groups (-CH₃) are added to cytosine residue to form 5-methylcytosine. Lack of methyl groups may result in hypomethylation. S-adenosylmethionine (SAM) is the major methyl donor, regeneration of which is dependent on folate cycle and methionine cycle, i.e. one-carbon metabolism [18]. Studies have shown the links between metabolites and epigenetic factors, as folate, choline, betaine, glycine and serine contributing to DNA methylation as methyl donors and co-factors, also to histone methylation [19]. Histone methylation includes active H3K4me 1/2/3 and H3K36me₃, and repressive H3K9me₃ and H3K27me₃ modification. H3K4me₃ is found in the promoter regions near transcription start site, which is associated with active gene transcription. One carbon metabolism, especially folate cycle and methionine cycle provide universal methyl group to refill epigenetics. While less is known about regulation of metabolic pathway by which PAHs exert its epigenetic effects in childhood asthma. Therefore, in this paper, we sought to evaluate the effect of PAHs on global DNA methylation and histone methylation by analyzing the levels of metabolites in one-carbon metabolism in asthmatic children.

2 Methods And Materials

2.1 Subjects

The samples included 50 asthmatic subjects and 50 control subjects who were recruited from the Children's Hospital of Nanjing Medical University and the Affiliated hospital of Nanjing university of Traditional Chinese Medicine from 2020-2021, respectively. Asthmatic subjects were diagnosed by a clinician. The control subjects were with no history of inflammatory disease and atopy. The Nanjing Medical University Clinical Research Ethics Committee, Nanjing, China, reviewed and approved the protocols of this study. Written informed consent was obtained from the participants' parent for the use of samples in this study. Whole blood samples from each asthmatic or control subjects were collected. The samples were centrifuged at 3000 rpm for 10 min. The supernatant was stored at -20 °C until analysis. White blood cell DNA was isolated by using TIANamp Genomic DNA Kit (TIANGEN, DP304-03, China) following the instructions from the manufacturer. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Lymphocyte-Human Cell Separation Media (Cedarlane, Southern Ontario, Canada).

2.2 Total IgE and IL-17A analysis

The determination of total IgE and IL-17A levels in children serum were performed using Human IgE ELISA Kit (AMEKO, Shanghai, China) and Human IL-17A ELISA Kit (Cusabio, Wuhan, China) according to the manufacturer's instructions, respectively. The absorbance was measured at a wavelength of 450nm (Infinite M2000, Tecan Trading AG, Switzerland).

2.3 PAHs analysis by GC-MS

Briefly, 0.2 mL of the serum was spiked with internal standards D₁₀-Phe, D₁₂-Chr, Accustandard, New Haven, CT, USA. Then 0.5 mL of 6mol/L hydrochloric acid, 0.5 mL of isopropanol and 3mL of *n*-hexane/methyl tertiary butyl ether (v/v,1:1) were added and vortexed for 2 min. After centrifugation at 5000 rpm for 10 min, the organic phase was collected. The above extraction procedure was carried out for three times. The mixed extract was evaporated under gentle nitrogen, and then was redissolved in 100 µl of *n*-hexane and prepared for quantification.

The quantitative analysis of target PAH compounds in the serum, including fluorene (Flu), phenanthrene (Phe), anthracene (Ant), fluoranthene (Fla), pyrene (Pyr), benzo(a)anthracene (BaA), chrysene(Chr), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene(BkF), benzo(a)pyrene (BaP), indeno(1,2,3-cd)pyrene (InP), and dibenzo(a,h)anthracene (DBA), were performed using gas chromatograph mass spectrometer (TRACE 1310, Thermo Fisher Scientific, USA) with a chromatographic column (DB-5MS, 30 m, liquid film thickness 0.25 µm, internal diameter 0.25 mm). The program of the GC analysis was described in our previous work [20]. The quantification was performed using standard curves by a standard mixture solution (Supelco Company, 20 µg/mL). Isotopically labeled internal standards were used with the recoveries of 97.0% for D₁₀-Phe, and 123.7% for D₁₂-Chr. Human reference serum (RS10-100-4, BETHYL) was as blank. Low molecular weight PAH isomers (naphthalene, acenaphthylene, acenaphthene) had poor recoveries due to their high volatility. So, they were excluded in the analyte. Limits of detection (LODs) were defined as a signal-to-noise ratio of 3:1. If the concentration was below LOD, it was

reported as not detected (ND) and assigned a concentration of zero. INP and DBA were not well separated. Benzo[ghi]perylene was also excluded as the concentrations in a large proportion of samples were below LOD.

2.4 The analysis of one carbon metabolites by UPLC-MC

The serum was pretreated according to a previous study [21]. For metabolite quantitation, isotopically labeled one carbon metabolites including S-adenosylhomocysteine-d4(SAH-d4), L-Methionine-d4 (Met-d4) (Tornoto Research Chemicals, North York, Canada) were used as internal standards. In brief, 200µL of serum was added to 1mL of methanol containing 100µg/mL ascorbic acid, 100µg/mL citric acid and 1.5mg/mL dithiothreitol (DTT). The internal standard solution was spiked at 200ng/mL concentration. After vortexed for 2min and centrifuged at 13000rpm for 15min at 4°C, the supernatant was dried under nitrogen at room temperature. The residue was reconstituted with 100µL of methanol/water (3:1, v/v) containing 10µg/mL of ascorbic acid, citric acid, and DTT, and stored at -20 °C for further analysis. The calibration curve standards, 5-methyltetrahydrofolate (5-MT), serine (Ser), glycine (Gly), methionine (Met), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), homocysteine (Hcy), betaine (Betaine) (Sigma Aldrich, St. Louis, MO, USA), were prepared by spiking the internal standard solutions. Metabolites were quantified using UPLC Ultimate 3000 system (Dionex, Germering, Germany) with an Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The separation of the samples was performed on a Waters ACQUITY BEH-C18 column (2.1mm×100mm, 1.7µm) at a flow rate of 0.3 mL/min. Mobile phase A was water containing 20mM ammonium formate and 0.15% (v/v) formic acid, and mobile phase B was methanol containing 0.15% (v/v) formic acid. The column temperature was at 35±1°C. The injection volume of samples was 20µL. A linear gradient procedure was described in **Table S1**. The effluent was unsplit. Mass spectrometric analyses in the positive ion mode in full scan MS/SIM mode and the parameters were given in **Table S2**. The temperature of the turbo ion electrospray was set at 320 °C. The ion spray voltage was 3200V. Metabolite concentrations were calculated from their peak area ratios and the calibration curve. Surrogate standards were used with the recoveries of 74.04% for SAH-d4 and 93.91 for Met-d4. Human reference serum (RS10-100-4, BETHYL) was as blank. Limits of detection (LOD) were defined as a signal-to-noise ratio of 3:1. If the concentration was below LOD, it was reported as not detected (ND) and assigned a concentration of zero.

2.5 Bisulfite sequencing PCR

DNA methylation status of the *LINE-1* (X58075.1) was detected by bisulfite genomic sequencing PCR amplification (BSP). In *silico* analyses and detailed databases searches were used to predict the 5'-CpG islands in *LINE-1* gene. For *LINE-1*, BSPCR primers were designed to amplify a CpG-rich region spanning from 113bp to 357 bp from the transcription start site, which contains 15 CpG sites, and the full length is 275bp. BSPCR primer sequences were 5'-TTATTAGGGAGTGTAGATAGTGGG-3' for forward; 5'-CCTCTAAACCAATATAAAATATAATCTC -3' for reverse. 200 ng of genomic DNA was used for bisulfite treatment using the EZDNA Methylation™ Kit (Zymo Research, CA, USA). The bisulfite treated DNA was amplified with methylation specific primers using GoTaq Green Master Mix (Promega, WI, USA) and optimized PCR condition (95°C for 10 min, 35 cycles of 95°C for 30 sec, 52.6°C for 1 min and 72°C for 2 min, followed by an extension at 72°C for 10 minutes. PCR products were purified by Gel Extraction Kit (E.Z.N.A., USA) and subcloned into pMD 19-T Vector (TaKaRa, Japan). Ten clones from each sample were sequenced (TsingKe Biological Technology, China) to obtain direct measures of DNA methylation at each CpG site in the promoter region. Sequencing data was analyzed with the DNAMAN to examine the methylation status. The percentage of DNA methylation was calculated with the formula: methylated CG / (methylated CG+ unmethylated CG) *100%.

2.6 ChIP-qPCR assay for H3K4me3 enrichments

Chromatin immunoprecipitation (ChIP) was conducted with a ChIP-IT Express Enzymatic (Active Motif) according to the manufacturer's protocol. Homogenate from zebrafish larvae was fixed with 1% formaldehyde for 10 min at room temperature to cross-link proteins and DNA, and then add Glycine Stop-Fix Solution (1ml 10× Glycine Buffer, 1ml 10× PBS and 8ml distilled H₂O) rocking at room temperature for 5 minutes to stop cross-linking. After washing with 1× PBS at room temperature three times, the tissue was pelleted by centrifugation for 10 min at 2,500 rpm at 4°C then resuspended in ice-cold Lysis Buffer supplemented with protease inhibitor cocktail and 100mM PMSF. Transfer the cells to an ice-cold dounce homogenizer. Dounce on ice with 10 strokes to aid in nuclei release and centrifuge for 10 min at 5,000 rpm in a 4°C microcentrifuge to pellet

the nuclei. Add the working stock of Enzymatic Shearing Cocktail (200U/ml) and incubate at 37°C for 15 minutes and add ice-cold 0.5M EDTA to stop the reaction. Centrifuge for 10 min at 15,000 rpm in a 4°C microcentrifuge. The supernatants were immunoprecipitated with H3K4me3 (1:50, #9751; Cell Signaling Technology) antibody with rotation after taking out part of as input DNA, which was followed by incubation with protein G magnetic beads for 4 h at 4 °C. The anti-IgG (1:1000, #3900; Cell Signaling Technology) was used as negative control. Protein G magnetic beads antibody/chromatin complexes were collected, washed, and eluted. Then, cross-links were reversed, and DNA was purified and analyzed via real-time PCR. The ChIP qPCR primer sequences were as follows: 5'-CTAGTTCTCATCACTCTCTACTCCC-3' (forward) and 5'-ATTGAATTTAACAATTCTTTTGTTG-3' (reverse), -738bp to -512 bp from transcription start site [22], and β -actin was used as an internal reference [23]. The levels of bound DNA sequences were then calculated using the percent input method ($2^{-[Ct(ChIP) - Ct(Input)]} \times 100$) by calculating the qPCR signal relative to the input sample.

2.7 Statistical analyses

The differences between asthmatic children and the control were analyzed using student's *t* test by GraphPad Prism 8 software (GraphPad Software, La Jolla, CA). Differences were considered statistically significant at $P \leq 0.05$. The association between the PAHs level and asthma was determined with logistic regression. Age and gender were considered as covariates in the regression model. Pearson correlation was used to assess the associations between PAHs and one carbon metabolites and methylation. A redundancy analysis (RDA) was performed to determine the multivariate relationship between PAHs and sample distribution and one carbon metabolites by R package. In addition, mediation analysis for the association between PAHs and asthma mediated by intermediates was implemented considering one carbon metabolites and DNA methylation and histone methylation as mediators with reference to our previous work [24].

3 Results And Discussion

3.1 The characteristics of the subject in this study.

In this study, the characteristics of all subjects were shown in **Table 1**. the ratio of males to females was 31:19 in the asthmatic group, and 33:17 in the control. The average age was 3.15 ± 2.28 years in the asthmatic group, and 5.78 ± 3.36 years in the control. The blood was collected before medication. **Fig. S1** showed the mean of total IgE concentrations was 292.74 ± 168.37 IU/mL (ranged from 31.92-716.67 IU/mL) in the asthmatic group, and 147.37 ± 79.71 IU/mL (ranged from 13.33-308.75 IU/mL) in the control, respectively. The asthmatic group presented significantly higher total serum IgE levels.

3.2 Serum PAH levels were associated with childhood asthma

As shown in **Fig.1**, the distribution of twelve types of PAHs was similar in the asthmatic group and the control. The PAHs listed in the order was Pyr > Phe > Flu > Fla > Chr > BaA. Serum Pyr showed the highest proportion and the highest detection frequency. There are some reports on the concentrations of PAHs in children serum. For example, Singh *et al.* reported values ranged from 1.05 ng/mL to 160.6 ng/mL (25th-75th percentile) in the distributions of nine types of PAH concentrations in the blood of children in Lucknow, India [25]. Our data showed the concentration of the total PAHs ranged from 8.15 ng/mL to 209.99 ng/mL, and 17.74 ng/mL to 44.49 ng/mL (25th-75th percentile) in the serum of children in Nanjing, China. **Table 2** listed the compounds sought in this analysis, detection rate, and the summary statistics for all subjects. After adjusted for sex and age, a logistic regression model showed that Fla, Pyr, BaA, and INP/DBA were associated with childhood asthma shown in **Table 2**. In our previous work, we demonstrated that urinary 1-hydroxypyrene (1-OHPyr) levels were associated with childhood asthma [24]. Internal exposure to PAHs has been assessed commonly by urinary 1-OHPyr as a general biomarker [26]. However, environmental exposure to PAHs may be understatedly assessed only based on exposure biomarkers such as urinary OH-PAHs metabolite levels [27]. High molecular weight PAHs composed of four or more rings (e.g., fluoranthene, pyrene, chrysene, benzo[a]pyrene, dibenz[a,h]anthracene) are dominant components of particle matter and easily taken up by inhalation [28]. In developing countries children are exposed to multiple sources of PAHs including heating and cooking from biomass fuel, and industrial coal-burning and traffic emission, and in these situations the inhalation of particle-bound PAHs is at least as important a route of exposure as dietary exposure [29,30]. And there is mounting evidence of unmetabolized PAHs as a

biomarker to reflect body burden [30,31], which can directly represent the actual exposure levels of the environment. Based on toxicokinetics, PAHs may result in relatively more toxicity through inhalation than after dietary exposure since inhalation avoid the hepatic first-pass effect [32].

3.3 PAHs changed the expression of one carbon metabolites in asthmatic children

One carbon unit from the folate cycle and betaine metabolism is used to form methionine. The methionine intermediate SAM functions as substrates for DNA methylation and histone methylation [33] (**Fig.2A**). In our previous work, it was found that 7-methylguanine as PAHs-related intermediate showed a mediation effect on the association between urinary 1-OHPyr levels and childhood asthma [24]. Urinary 1-OHPyr level was associated with 7-methylguanine which could reflect global DNA methylation in asthmatic children [34]. So, we compared the concentrations of one carbon metabolites between two groups (**Table 3, Fig.2B**). The asthmatic group displayed a significantly decreasing in SAM abundance and a smaller but corresponding decrease in SAH, which indicated the increasing conversion from SAM to SAH and the elevated capacity of methylation reactions (**Fig.2C**). What's more, the methionine levels in the asthmatic group were higher than that in the control. Roy *et al* reported that methionine restriction can reduce histone H3K4 methylation at the promoter regions of key genes involved in Th17 cell proliferation and cytokine production [35]. Therefore, metabolic changes-induced by PAHs has profound effects on epigenetics, especially methylation. Then a redundancy analysis (RDA) was performed to determine the multivariate relationship between the environmental variable-PAHs and sample distribution and one carbon metabolites by R package. RDA-analysis revealed two groups of all subjects, each characterized by a specific set of PAHs-one carbon parameters (groups are indicated on the diagram, **Fig.2D**). The effect of PAHs on one carbon metabolites in different groups in the RDA diagram is mainly characterized by the length of PAH variables and by the cosine value of the angle. The metabolites-PAHs correlation was 0.64 for RDA axis 1 and 0.23 for axis 2. Fla had a great effect on one carbon metabolites between two groups. There was a significant negative correlation between Fla and one carbon metabolites (**Fig.2E**). We used differential one carbon metabolites to perform mediation analysis to assess the association between the PAHs level and asthma mediated by metabolites. The directed acyclic graph (DAG) showed significant mediation effects between the Fla level and asthma by SAH, SAM and Ser (**Fig.2F, Table S3**).

3.4 PAHs changed epigenetic pattern in asthmatic children

To study epigenetic alterations induced by PAHs, firstly, we examined the methylation levels of long interspersed nuclear elements-1 (*LINE-1*) promoter, which comprise approximately 17% of the human genome and is used to assess global DNA methylation[36]. The distributions of *LINE-1* DNA methylation were presented in **Fig 3 A**. The geometric mean for *LINE-1* methylation were 17.26 % in the control, and 34.19 % in asthmatic group, respectively, which indicated elevated global DNA methylation in asthmatic group. Fla had a positive effect on *LINE-1* methylation ($\beta=0.395$, $P=0.000$). There were no any significant associations of other PAHs with global DNA methylation. Because Fla was associated with childhood asthma and *LINE-1* methylation, we conducted the mediation analysis to assess global DNA methylation could be mediator of the association between PAHs and asthma. We did find significant mediation effect between serum Fla and asthma by *LINE-1* methylation (**Fig.3B**). In contrast to our work, current evidence showed that prenatal urinary 2-OHNa and 1-hydroxyphenanthrene were associated with lower *A/u* and *LINE-1* methylation [37]. On the other hand, studies have shown a positive association between gene-specific hypermethylation and PAHs exposure. For example, CpG Site-specific hypermethylation of *p16^{INK4a}* was found in peripheral blood lymphocytes of PAH-exposed workers by BSP sequencing [38]. Interestingly, global DNA hypermethylation levels were associated with asthma severity by assessing the percentage of 5-methylcytosine [39]. Perhaps these discrepancies in the literature about the correlation between global DNA methylation and PAHs exposure can be attributed to the fact that the resource of PAHs exposure and the representative indices of global DNA methylation were differentially assessed.

In addition, there is evidence that IL-17A markedly contribute to the immune imbalance in asthma [40]. For example, elevated levels of IL-17A have been found in the sputum and in bronchoalveolar lavage fluid of patients with asthma[41]. What' more, Milovanovic *et al* demonstrated that IL-17A secretion promoted IgE production [42]. So, we collected the serum from both groups and performed ELISA to detect the concentration of IL-17A. **Fig.3C** showed the mean of IL-17A concentrations of

100.20±45.99 pg/mL (ranged from 33.83-195.23 pg/mL) in the asthmatic group, and 40.92±22.41 pg/mL (ranged from 4.56-85.35 pg/mL) in the control, respectively. The results showed that high level of IL-17A was detected in asthmatic group when compared with the control. Research has reported some genes that are associated with PAHs-induced methylation and immune dysfunction. For example, Kohli *et al.* [43] reported that tobacco smoke (in which PAHs are critical constituents) exposure was associated with hypermethylation of the promoter region for *IFN-gamma* in T effector cells and *Foxp3* in regulatory T cells. PAH might affect, through their effects on the aryl hydrocarbon receptor, IL-17 production in asthma [44]. In the present study, because PAHs can increase methylation capacity by interfering one carbon metabolism including SAM-dependent histone modifications, we hypothesized that H3K4 tri-methylation level (a histone modification associated with active transcription in chromatin structure) may be induced by PAHs exposure. To determine whether PAHs could regulate the production of IL-17A through epigenetic changes, ChIP assay was performed using antibodies against tri-methylated Lys4 of H3(**Fig.3C**). Interestingly, Fla could upregulate H3K4 tri-methylation level in the *IL-17A* promoter region and associated with children asthma ($\beta=0.293$, $P=0.002$) (**Fig.3D**). By ChIP-qPCR results we demonstrated that PAHs could promote secretion of IL-17A through changes in histone methylation levels. The data suggest that PAHs might contribute to increased IL-17A production through mechanisms involving not only AhR-dependent but also independent pathways, epigenetics. In addition, we report here that one carbon metabolism forms a function link between metabolic and epigenetic reprogramming to regulate gene expression. Epigenetic modification is induced by the environment with one carbon metabolites function as key substrates for DNA methylation and histone methylation.

Conclusion

In this study, we assessed the effects of PAHs exposure on epigenetics in view of one carbon metabolism in asthmatic children. The evidence was proved that PAHs exposure induced DNA methylation and histone methylation by disturbing one carbon metabolism. Our data could help in identifying the underlying mechanisms of global and gene-specific methylation-induced by PAHs exposure.

Abbreviations

polycyclic aromatic hydrocarbons: (PAHs); aryl hydrocarbon receptors (AhRs); regulatory T cell: Treg; long interspersed nucleotide element-1: *LINE-1*; short interspersed nuclear elements: *Alu*; S-adenosylmethionine: SAM; Peripheral blood mononuclear cells: PBMC; gas chromatograph mass spectrometer: GC-MS; fluorene: Flu; phenanthrene: Phe; anthracene: Ant; fluoranthene: Fla; pyrene: Pyr; benzo(a)anthracene: BaA; chrysene: Chr; benzo(b)fluoranthene: BbF; benzo(k)fluoranthene: BkF; benzo(a)pyrene: BaP; indeno(1,2,3-cd)pyrene: InP; dibenzo(a,h)anthracene: DBA; ultra-performance liquid chromatography-Orbitrap-mass spectrometry: UPLC-Orbitrap-MS; 5-methyltetrahydrofolate: 5-MT, serine: Ser; glycine: Gly; methionine: Met; S-adenosylhomocysteine: SAH; homocysteine: Hcy; betaine: Betaine; dithiothreitol: DTT; Limits of detection: LODs; bisulfite genomic sequencing PCR: BSP; chromatin immunoprecipitation: ChIP; redundancy analysis: RDA; 1-hydroxypyrene: 1-OHPyr; Odds ratio: OR; confidence interval: CI; directed acyclic graph: DAG; indirect effect: IE; total effect: DE

Declarations

Ethical approval and consent to participate

The Nanjing Medical University Clinical Research Ethics Committee, Nanjing, China, reviewed and approved the protocols of this study. Written informed consent was obtained from the participants' parents for the use of samples in this study.

Consent for publication

Consent for publication was obtained from the participants' parents.

Availability of data and material

Not applicable

Competing Interests

There are no conflicts to declare.

Funding

This work was supported by the National Natural Science Foundation of China (82073630 and 81728018); Natural Science Foundation of Jiangsu Province (BK20161571), Natural Science Foundation of the Higher Education Institution of Jiangsu Province (16KJA330002). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contribution

Conceptualization, Lei Li and Qian Wu; Formal analysis, Hao Wu, Jinye Hu, Yuling Bao, Hui huang, Ping Jiang, and Zhang Zhan; Funding acquisition, Qian Wu; Investigation, Hao Wu and Jinye Hu; Resources, Yuling Bao, Lei Li and Qian Wu; Writing – original draft, Qian Wu.

Acknowledgement

Not applicable

References

1. Martinez FD. Genes, environments, development and asthma: a reappraisal. *Eur Respir J*. 2007, 29(1):179-184.
2. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med*. 2000,161(5):1720-1745.
1. Summary Health Statistics Tables for U.S. Children: National Health Interview Survey, 2018, <https://www.cdc.gov/nchs/fastats/asthma.htm>.
2. [Third nationwide survey of childhood asthma in urban areas of China]. National cooperation group on childhood asthma. *Chin J Pediatr*. 2013,51(10):729-735. (in Chinese)
3. Li X, Song P, Zhu Y, Lei H, Chan KY, Campbell H, Theodoratou E, Rudan I. Global Health Epidemiology Research Group (GHERG). The disease burden of childhood asthma in China: a systematic review and meta-analysis. *J Glob Health*. 2020,10(1):010801.
4. Guarnieri M, Balmes JR. Outdoor air pollution and asthma. *Lancet*. 2014,383(9928):1581-1592.
5. Orellano P, Quaranta N, Reynoso J, Balbi B, Vasquez J. Effect of outdoor air pollution on asthma exacerbations in children and adults: Systematic review and multilevel meta-analysis. *PLoS One*. 2017,12(3):e0174050.
6. Karimi P, Peters KO, Bidad K, Strickland PT. Polycyclic aromatic hydrocarbons and childhood asthma. *Eur J Epidemiol*. 2015,30(2):91-101.
7. Liu H, Xu C, Jiang ZY, Gu A. Association of polycyclic aromatic hydrocarbons and asthma among children 6-19 years: NHANES 2001-2008 and NHANES 2011-2012. *Respir Med*. 2016;110:20-27.

8. Rossnerova A, Spatova M, Rossner P Jr, Novakova Z, Solansky I, Sram RJ. Factors affecting the frequency of micronuclei in asthmatic and healthy children from Ostrava. *Mutat Res*. 2011, 708(1-2):44-49.
9. Wang IJ, Karmaus WJ, Yang CC. Polycyclic aromatic hydrocarbons exposure, oxidative stress, and asthma in children. *Int Arch Occup Environ Health*. 2017, 90(3):297-303.
10. Zhang H, Han Y, Qiu X, Wang Y, Li W, Liu J, Chen X, Li R, Xu F, Chen W, Yang Q, Fang Y, Fan Y, Wang J, Zhang H, Zhu T. Association of internal exposure to polycyclic aromatic hydrocarbons with inflammation and oxidative stress in prediabetic and healthy individuals. *Chemosphere*. 2020;253:126748.
11. Kepley CL, Lauer FT, Oliver JM, Burchiel SW. Environmental polycyclic aromatic hydrocarbons, benzo(a) pyrene (BaP) and BaP-quinones, enhance IgE-mediated histamine release and IL-4 production in human basophils. *Clin Immunol*. 2003;107(1):10-19.
12. Stevens EA, Mezrich JD, Bradfield CA. The aryl hydrocarbon receptor: a perspective on potential roles in the immune system. *Immunology*. 2009;127(3):299-311.
13. Herbstman JB, Tang D, Zhu D, Qu L, Sjödin A, Li Z, Camann D, Perera FP. Prenatal exposure to polycyclic aromatic hydrocarbons, benzo[a]pyrene-DNA adducts, and genomic DNA methylation in cord blood. *Environ Health Perspect*. 2012;120(5):733-738.
14. Yang P, Gong YJ, Cao WC, Wang RX, Wang YX, Liu C, Chen YJ, Huang LL, Ai SH, Lu WQ, Zeng Q. Prenatal urinary polycyclic aromatic hydrocarbon metabolites, global DNA methylation in cord blood, and birth outcomes: A cohort study in China. *Environ Pollut*. 2018, 234:396-405.
15. Hew KM, Walker AI, Kohli A, Garcia M, Syed A, McDonald-Hyman C, Noth EM, Mann JK, Pratt B, Balmes J, Hammond SK, Eisen EA, Nadeau KC. Childhood exposure to ambient polycyclic aromatic hydrocarbons is linked to epigenetic modifications and impaired systemic immunity in T cells. *Clin Exp Allergy*. 2015;45(1):238-248.
16. Anderson OS, Sant KE, Dolinoy DC. Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. *J Nutr Biochem*. 2012;23(8):853-859.
17. Lillycrop KA, Burdge GC. Epigenetic mechanisms linking early nutrition to long term health. *Best Pract Res Clin Endocrinol Metab*. 2012;26(5):667-676.
18. Hu J, Bao Y, Zhu Y, Osman R, Shen M, Zhang Z, Wang L, Cao S, Li L, Wu Q. The Preliminary Study on the Association Between PAHs and Air Pollutants and Microbiota Diversity. *Arch Environ Contam Toxicol*. 2020;79(3):321-332.
19. Wang Y, Zhang HY, Liang QL, Yang HH, Wang YM, Liu QF, Hu P, Zheng XY, Song XM, Chen G, Zhang T, Wu JX, Luo GA. Simultaneous quantification of 11 pivotal metabolites in neural tube defects by HPLC-electrospray tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2008;863(1):94-100.
20. Liu Y, Liao J, Zhao M, Wu H, Yung S, Chan TM, Yoshimura A, Lu Q. Increased expression of TLR2 in CD4(+) T cells from SLE patients enhances immune reactivity and promotes IL-17 expression through histone modifications. *Eur J Immunol*. 2015;45(9):2683-93.
21. Wu Q, Odwin-Dacosta S, Cao S, Yager JD, Tang WY. Estrogen down regulates COMT transcription via promoter DNA methylation in human breast cancer cells. *Toxicol Appl Pharmacol*. 2019;367:12-22.
22. Hu J, Bao Y, Huang H, Zhang Z, Chen F, Li L, Wu Q. The preliminary investigation of potential response biomarkers to PAHs exposure on childhood asthma. *J Expo Sci Environ Epidemiol*. 2021 May 10. doi: 10.1038/s41370-021-00334-4. Epub ahead of print.
23. Singh VK, Patel DK, Ram S, Mathur N, Siddiqui MKJ, Behari JR. Blood levels of polycyclic aromatic hydrocarbons in children of Lucknow. *India Arch Environ Contam Toxicol*. 2008; 54:348-354.
24. Hansen AM, Mathiesen L, Pedersen M, Knudsen LE. Urinary 1-hydroxypyrene (1-HP) in environmental and occupational studies—a review. *Int J Hyg Environ Health*. 2008;211(5-6):471-503.
25. Yang Z, Guo C, Li Q, Zhong Y, Ma S, Zhou J, Li X, Huang R, Yu Y. Human health risks estimations from polycyclic aromatic hydrocarbons in serum and their hydroxylated metabolites in paired urine samples. *Environ Pollut*. 2021;290:117975.

26. Huang Y, Wang J, Fu N, Zhang S, Du W, Chen Y, Wang Z, Qi M, Wang W, Zhong Q, Duan Y, Shen G, Tao S. Inhalation exposure to size-segregated fine particles and particulate PAHs for the population burning biomass fuels in the Eastern Tibetan Plateau area. *Ecotoxicol Environ Saf.* 2021;211:111959.
27. Yang L, Zhang H, Zhang X, Xing W, Wang Y, Bai P, Zhang L, Hayakawa K, Toriba A, Tang N. Exposure to Atmospheric Particulate Matter-Bound Polycyclic Aromatic Hydrocarbons and Their Health Effects: A Review. *Int J Environ Res Public Health.* 2021;18(4):2177.
28. WHO Guidelines for Indoor Air Quality: Selected Pollutants. Geneva: World Health Organization; 2010. PMID: 23741784.
29. Sexton K, Salinas JJ, McDonald TJ, Gowen RMZ, Miller RP, McCormick JB, Fisher-Hoch SP. Polycyclic aromatic hydrocarbons in maternal and umbilical cord blood from pregnant hispanic women living in brownsville, Texas. *Int. J. Environ. Res. Public Health* 2011, 8: 3365e3379.
30. Craemer S, Croes K, van Larebeke N, Sioen I, Schoeters G, Loots I, Nawrot T, Nelen V, Campo L, Fustinoni S, Baeyens W. Investigating unmetabolized polycyclic aromatic hydrocarbons in adolescents' urine as biomarkers of environmental exposure. *Chemosphere* 2016;155:48e56.
31. Mentch SJ, Locasale JW. One-carbon metabolism and epigenetics: understanding the specificity. *Ann N Y Acad Sci.* 2016;1363(1):91-98.
32. Wishnok JS, Tannenbaum SR, Stillwell WG, Glogowski JA, Leaf CD. Urinary markers for exposures to alkylating or nitrosating agents. *Environ Health Perspect.* 1993;99:155-159.
33. Roy DG, Chen J, Mamane V, Ma EH, Muhire BM, Sheldon RD, Shorstova T, Koning R, Johnson RM, Esaulova E, Williams KS, Hayes S, Steadman M, Samborska B, Swain A, Daigneault A, Chubukov V, Roddy TP, Foulkes W, Pospisilik JA, Bourgeois-Daigneault MC, Artyomov MN, Witcher M, Krawczyk CM, Larochelle C, Jones RG. Methionine Metabolism Shapes T Helper Cell Responses through Regulation of Epigenetic Reprogramming. *Cell Metab.* 2020, 31(2):250-266.e9.
34. Lisanti S, Omar WA, Tomaszewski B, De Prins S, Jacobs G, Koppen G, Mathers JC, Langie SA. Comparison of methods for quantification of global DNA methylation in human cells and tissues. *PLoS One.* 2013;8(11):e79044.
35. Yang P, Gong YJ, Cao WC, Wang RX, Wang YX, Liu C, Chen YJ, Huang LL, Ai SH, Lu WQ, Zeng Q. Prenatal urinary polycyclic aromatic hydrocarbon metabolites, global DNA methylation in cord blood, and birth outcomes: A cohort study in China. *Environ Pollut.* 2018, 234:396-405.
36. Yang P, Ma J, Zhang B, Duan H, He Z, Zeng J, Zeng X, Li D, Wang Q, Xiao Y, Liu C, Xiao Q, Chen L, Zhu X, Xing X, Li Z, Zhang S, Zhang Z, Ma L, Wang E, Zhuang Z, Zheng Y, Chen W. CpG site-specific hypermethylation of p16^{INK4a} in peripheral blood lymphocytes of PAH-exposed workers. *Cancer Epidemiol Biomarkers Prev.* 2012;21(1):182-90.
37. Chan MA, Ciaccio CE, Gigliotti NM, Rezaiekhalthigh M, Siedlik JA, Kennedy K, Barnes CS. DNA methylation levels associated with race and childhood asthma severity. *J Asthma.* 2017 Oct;54(8):825-832. doi: 10.1080/02770903.2016.1265126. Epub 2016 Dec 8. PMID: 27929694.
38. Wang YH, Liu YJ. The IL-17 cytokine family and their role in allergic inflammation. *Curr Opin Immunol.* 2008;20(6):697-702.
39. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Pagé N, Olivenstein R, Elias J, Chakir J (September 2001). IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *The Journal of Allergy and Clinical Immunology.* 108 (3): 430-438.
40. Milovanovic M, Drozdenko G, Weise C, Babina M, Worm M. Interleukin-17A promotes IgE production in human B cells. *J Invest Dermatol.* 2010;130(11):2621-2628.
41. Kohli A, Garcia MA, Miller RL, Maher C, Humblet O, Hammond SK, Nadeau K. Secondhand smoke in combination with ambient air pollution exposure is associated with increased CpG methylation and decreased expression of IFN- γ in T effector cells and Foxp3 in T regulatory cells in children. *Clin Epigenetics.* 2012;4(1):17.
42. Plé C, Fan Y, Ait Yahia S, Vorng H, Everaere L, Chenivresse C, Balsamelli J, Azzaoui I, de Nadai P, Wallaert B, Lazennec G, Tsicopoulos A. Polycyclic aromatic hydrocarbons reciprocally regulate IL-22 and IL-17 cytokines in peripheral blood mononuclear cells from both healthy and asthmatic subjects. *PLoS One.* 2015;10(4):e0122372.

Tables

Table 1 The characteristics of all subjects

Variables	Values
Sex	Number (Control/Asthma)
Male	64 (31:33)
Female	36 (19:17)
Age	
0-1	16(2:14)
2-5	54(25:29)
>6	30(23:7)
IgE	Mean±SD (IU/mL)
Control	147.37±79.71
Asthma	292.74±168.37

Table 2 Serum PAH levels of subjects in this study

PAHs	Detection rate	Limit of Detection(ng/mL)	Min-Max (ng/mL)	Mean±SD (ng/mL)	Median (ng/mL)	OR (95%CI)	<i>P</i> value
Flu	99%	0.049	ND-9.438	2.434±1.412	2.327	1.002(0.535, 1.877)	0.994
Phe	99%	0.029	ND-15.120	3.988±2.683	3.851	1.041(0.802, 1.350)	0.763
Ant	97%	0.014	ND-5.654	1.202±1.031	0.871	0.675 (0.357, 1.276)	0.226
Fla	99%	0.054	ND-15.134	3.468±3.608	2.125	1.380 (1.063, 1.792)	0.016*
Pyr	99%	0.013	ND-188.043	23.921±35.201	9.756	1.033 (1.000, 1.067)	0.047*
Chr	99%	0.035	ND~10.709	1.725±1.843	1.028	0.498(0.237, 1.047)	0.066
BaA	92%	0.012	ND~11.156	1.719±1.790	0.984	2.266 (1.018, 5.042)	0.045*
BbF	91%	0.017	ND~1.771	0.545±0.440	0.445	0.941(0.171, 5.157)	0.944
BkF	90%	0.006	ND~1.353	0.313±0.228	0.279	3.891 (0.062, 246.174)	0.521
BaP	91%	0.011	ND~2.315	0.516±0.467	0.428	1.634(0.272, 9.813)	0.591
INP/DBA	84%	0.004	ND~1.063	0.260±0.238	0.217	32.835 (1.432, 753.007)	0.029*

ND: not detected; OR: Odds ratio; CI: confidence interval, * *P*<0.05

Table 3 The serum concentration of one carbon metabolites of subjects in this study

PAHs	Limit of Detection(ng/mL)	Min~Max value(ng/mL)	Mean±SD(ng/mL)	Median(ng/mL)
5-MT	2.0	ND-95.076	22.648±20.435	15.05117
SAH	0.5	ND-25.930	10.245±4.948	10.941
SAM	0.5	ND-0.741	0.179±0.200	0.144
Betaine	1.0	2.563-117.121	34.3651±17.644	31.832
Met	3.0	1065.416-6634.955	3077.496±1212.843	2848.938
Hcy	10.0	2.718-569.948	114.0546±97.510	95.346
Ser	10.0	17.764-334.8852	114.6048±83.428	82.450
His	3.0	45.512-214.226	85.992±35.489	73.125
Gly	1.0	225.477-5208.570	2128.707±1108.04	1985.819
CYSTA	25.0	6.322-213.732	59.242±52.372	36.519

ND: not detected

Supplementary

Supplemental Figure S1 is not available with this version

Figures

Figure 1

The distribution of serum PAHs in all subjects

Figure 2

The difference of one carbon metabolites induced by PAHs between two groups (A) Schema of one carbon-related metabolism. (B) The concentration of one carbon metabolites in two groups. (C) The mean fold-change of the abundance of each metabolite between the two samples. (D) Redundancy analysis (RDA) plot summarizing variation in metabolites across subject properties and PAHs levels. Circles and rectangle represent different groups; pink and green arrows illustrate PAHs and metabolites, respectively. Eigenvalues for the first two were presented as RDA1 and RDA2. The quadrant of the arrow indicates the orientation of correlation between PAHs and the sorting axis (RDA1 and RDA2). The length of the arrow line between the arrow and the origin represents the importance of correlation between an PAH and metabolites distribution. The longer the arrow line, the greater the correlation, and vice versa. The angle between the arrow line and the sorting axis represents the correlation between an PAH and the sorting axis. The smaller the angle, the higher the correlation, and vice versa. (E) Pearson correlation coefficients between PAHs and one carbon metabolites. Correlation matrix: the orange and blue dots correspond to negative and positive correlations, respectively. $P < 0.05$. (F) Mediation effects between serum Fla and asthma mediated by one carbon metabolites levels. The figure presents one carbon metabolites levels in blood as mediator, the estimate of the indirect effect (IE), and the estimate of the total effect (DE), The mediation model was adjusted for sex and age.

Figure 3

PAHs influenced DNA methylation and histone methylation in asthmatic children. (A) Methylation levels of 15 individual CpG sites in the promoter region of LINE-1 gene. (B) Mediation effects between serum Fla and asthma mediated by global DNA methylation levels. The figure presents LINE-1 methylation levels in blood as mediator, the estimate of the indirect effect (IE), and the estimate of the total effect (DE). The mediation model was adjusted for sex and age. (C) Serum IL-17A levels and ChIP analysis of chromatin extracts were performed to evaluate the enrichment of H3K4me3 in the promoter of IL-17A gene. Data are presented relative to input DNA prepared from untreated chromatin. * $P < 0.05$, Student's t-test. (D) Mediation effects between serum Fla and asthma mediated by H3K4me3 levels in the promoter of IL-17A gene.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementaryfiles.docx](#)