PAHs-induced metabolic changes related to inflammation in childhood asthma

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Research Article

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Abstract

Background: Epidemiological studies have showed that PAHs may exert its adverse effects on childhood asthma. However, the underlying molecular mechanism remains to be fully elucidated. This study aimed to investigate this process in view of metabolic pathway, especially one carbon metabolism and tryptophan 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 concentrations were measured by GC-MS. One carbon-related metabolites and tryptophan 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 enrichment on *IL-17A* gene. Multivariable linear regression was performed to evaluate the associates between PAHs and one carbon metabolite and tryptophan metabolites and childhood asthma. HE staining in lung tissue, IgE and IL-17A in BALF, metabolic profiles in urine, and *AhR*, *iL-17a* and *Cyp1a1* gene expression were determined in PAHs-exposed mice.

Results: The asthmatic group presented significantly higher total serum IgE and IL-17A concentrations. Serum Fla was associated with childhood asthma (OR=1.380, 95%CI: 1.063-1.792). The asthmatic group displayed an increasing conversion from SAM to SAH and an 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 concentration and asthma. What's more, Fla had a positive effect on *LINE-1* DNA methylation (β=0.395, P=0.000) and H3K4 tri-methylation level in the *IL-17A* promoter region(β=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. The differential Trp metabolites, such as Trp, tryptamine, IA, IAA, Indole, IAld and IAAld indicated the asthmatic children had increased indole-AhR pathway. Mediation analysis failed to show a mediator effect of Trp metabolites in the association between PAHs and childhood asthma. Animal study confirmed that PAHs exposure increased methylation levels, and altered Trp metabolites -AhR-IL-17A axis, which may be influenced by gender.

Conclusion: PAHs disturbed one carbon metabolism to influence the methyl group refill of DNA methylation and histone methylation, and disturbed tryptophan metabolism to regulate Th17 cell differentiation, which may elevate serum IL-17A concentration in asthmatic children.

Highlights

- PAHs induced one carbon metabolic aspect of epigenetics in asthmatic children
- PAHs influenced tryptophan metabolic profile in asthmatic children
- PAHs may influence the expression of IL-17A by metabolic mechanism in asthmatic children

1 Introduction

Asthma is a complex and multiple etiological disease [Martinez,2007]. It is also a common chronic inflammation in children [Bousquet et al.,2000]. Asthma prevalence under age 18 years released in 2018 in United States was 7.5% [CDC,2018]. The third (2010–2011) national epidemiological survey in China showed that the prevalence of childhood asthma had been up to 3.02% [Chin J Pediatr,2013]. Although no latest official national statistics have been issued 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 [Li et al.,2020]. Epidemiological studies have demonstrated that exposure to air pollution have been linked to increasing asthma prevalence and asthma onset [Guarnieri and Balmes,2014; Orellano et al.,2017]. Especially, ambient polycyclic aromatic hydrocarbons (PAHs), which are traffic related air pollutants and main organic components of PM 2.5 and PM 10, have been known to contribute to the onset of asthma [Karimi et al.,2015; Liu et al.,2016]. In the process of PAHs biotransformation, a cascade of oxidative stress is triggered leading to cytotoxicity and DNA damage [Rossnerova et al.,2011; Wang et al.,2017; Zhang et al.,2020]. PAHs also stimulate inflammatory responses through increasing the production of IgE [Kepley et al.,2003].

Recently, there is growing evidence to demonstrate that PAHs exposure may alter global and gene-specific DNA methylation patterns [Herbstman et al.,2012]. 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 prenatal PAHs exposure to birth outcomes [Yang et al.,2018]. For specific gene, increased methylation of *FOXP3* gene was associated with chronic PAHs exposure leading to Treg dysfunction in atopic children [Hew et al.,2015]. DNA methylation is a biological process by which methyl groups (-CH_3) 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 [Anderson et al.,2012]. 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 [Lillycrop and Burdge,2012]. Histone methylation includes active H3K4me 1/2/3 and H3K36me3, and repressive H3K9me3 and H3K27me3 modification. 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.

In additon, tryptophan (Trp) metabolic abnormalities were found in asthmatic children [Licari et al.,2019]. Some tryptophan metabolites, such as kynurenine and indole, have been verified to be able to bind and activate the aryl hydrocarbon receptor (AhR). The activated Trp-AhR pathway can induce Th17 cell differentiation and regulate the expression of downstream cytokines such as IL-22 and IL-17, thereby regulating the immune homeostasis [Stevens et al.,2009]. Recent studies have demonstrated that Th17 cells and their signature cytokine IL-17 also play an important role in severe asthma [Ramakrishnan et al.,2019]. It is well known that PAHs such as B[a]P act as AhR ligand. After ligand binding activated AhR may upregulate its target genes such as cytochrome P450 1A1(CYP1A1) expression to mediate the carcinogenic effects. Here, we hypothesized that PAHs may also alter tryptophan metabolism, and aberrant tryptophan

metabolites acting on AhRs can induce Th17 cell differentiation and regulate IL-17 production to promote inflammation.

Therefore, in this paper, we used targeted metabolomics analysis to analyze the concentrations of metabolites in one-carbon metabolism and tryptophan metabolism in asthmatic children and sought to evaluate the effect of PAHs on childhood asthma from cell metabolism perspective. PAHs can disturb one-carbon metabolism to change global DNA methylation and histone methylation and induced *IL-17A* expression in asthmatic children. Although there was no medication effect of tryptophan metabolism between PAHs exposure and childhood asthma, animal study confirmed that PAHs can influence tryptophan metabolism to induce inflammation but should take the gender difference into account when analyzing data. Collectively, these data indicated that PAHs could disturb metabolic pathway to promote inflammation in asthma.

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 Animal and PAHs exposure

C57BL6 mice (Antpedia, Shanghai, China) were used in this study. Ten male and ten female mice (about 6-week-old, 16.0 ± 2.0g) were group housed in pathogen-free conditions in the animal facility at Nanjing Medical University. All experiments were approved by the Nanjing Medical University Institutional Animal Care and Use Committee (IACUC). Twenty mice were divided to two groups. The PAHs-exposed group included five males and five females, and was administrated with 50μg/mg PAHs mixture, i.n.,10μl/nasal. for 2 weeks. The control group included five males and five females with normal saline. The PAHs mixture was produced by our lab according to the proportional distribution of PAHs mixture that was measured in our previous work [Hu et al.,2020] as shown in **Table S1**. Urine specimen were collected for metabolomic analysis. Mice were gently restrained and decapitated. Bronchoalveolar lavage fluid (BALF) was collected by normal saline washing. Lung and colon tissues were collected for further analysis.

2.3 Total IgE and IL-17 analysis

The determination of total IgE and IL-17 concentrations were performed using Human/Mouse IgE ELISA Kit (AMEKO, Shanghai, China) and Human IL-17A/ Mouse IL-17 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).

2.4 PAHs analysis by GC-MS

Briefly, 0.2 mL of the serum was spiked with internal standards(D_{10}-Phe, D_{12}-Chr, Accustandard, New Haven, CT, USA). Then 0.5 mL of 6mol/L hydrochloric acid, 0.5 mL of isopropanol and 3mLof *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 repeatedly 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 [Hu et al.,2020]. 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 D_{10}-Phe, and 123.7% for D_{12}-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 exclude as the concentrations in a large proportion of samples were below LOD.

2.5 The analysis of one carbon metabolites by UPLC-MS

The serum was pretreated according to Wang's study [Wang et al.,2008]. 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℃, 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 ℃ 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 S2**. The effluent was unsplitted. Mass spectrometric analyses in the positive ion mode in full scan MS/SIM mode and the parameters were given in **Table S3**. 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.

2.6 The analysis of tryptophan metabolites by UPLC-MS

Tryptophan and its metabolites, including L-Tryptophan (Trp), L-kynurenine (Kyn), 2-picolinic acid (PIC), quinolinic acid (QUI), indole acrylic acid (IA), indole-3-propionic acid (IPA), and tryptamine from Aladdin Biochemical Technology Co., Ltd (Shanghai, China);5-Hydroxyindoleacetic acid (5-HIAA), indoleacetaldehyde (IAAld), indoleacetic acid (IAA) and serotonin (5-hydroxytryptamine, 5-HT) hydrochloride from Sigma Aldrich (MO, USA); 5-Hydroxy-L tryptophan (5-HTP) from MAYA-Reagent (Jiaxing, China); Indole-3-aldehyde (IAld) from TCI (Shanghai) Development Co., Ltd., were determined in this study. An isotopically labeled Trp metabolite, tryptophan-d5(Trp-d5) (Toronto Research Chemicals, Toronto, Canada), was used as internal standards. In brief, 50μL of serum was added to 150μL of methanol. The internal standard solution was spiked at 50ng/ml concentration. After vortexed and centrifuged at 13000 rpm for 15 min at 4℃, the supernatant was 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 Hypersile C18 column (100 mm × 2.1 mm, 1.9 μm) at a flow rate of 0.3 mL/min. Mobile phase A was water containing 0.1% (v/v) formic acid, and mobile phase B was acetonitrile containing 0.1% (v/v) formic acid. The column temperature was at 40°C. The injection volume of samples was 10μL. A linear gradient procedure was described in **Table S4**. Mass spectrometric analyses in the positive ion mode in full scan MS/SIM mode and the parameters were given in **Table S5**. The effluent was unsplitted. The temperature of the turbo ion electrospray was set at 300°C. The ion spray voltage was 3500V. Surrogate standard was used with the recovery of 95.31% for Trp-d5. Human reference serum (RS10-100-4, BETHYL) was as blank.

2.7 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'-TTATTAGGGAGTGTTAGATAGTGGG-3' for forward; 5'-CCTCTAAACCAAATATAAAATATAATCTC – 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%.

2.8 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 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 H_2O) 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℃ 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℃ microcentrifuge to pellet the nuclei. Add the working stock of Enzymatic Shearing Cocktail (200U/ml) and incubate at 37℃ 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℃ 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 for human IL-17A were as follows: 5'-CTAGTTCTCATCACTCTCTACTCCC-3' (forward) and 5'-ATTGAATTTAACAATTCTTTTGTTG-3' (reverse), -738bp to -512 bp from transcription start site [Liu et al.,2015], and β-*actin* was used as an internal reference [Wu et al.,2019]. 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.

2.9 Metabolic profile analysis based on UPLC-Orbitrap-MS

The analyses of metabolomics profile for urine were performed on a UPLC Ultimate 3000 system (Dionex, Germering, Germany), coupled to an Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray source (HESI) at the resolution of 7×10^5 in both positive and negative mode. The detail was described in our previous work [Hu et al.,2021].

2.10 Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from frozen tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. Genes were measured by qRT-PCR according to our previous work

[Xu et al.,2021]. The primers were *AhR*: 5'-TTGGTTGTGATGCCAAAGGGC-3'for forward; 5'-CATGCGGATGTGGGATTCTGC-3' for reverse [Yang et al.,2020]; *Il-17a*: 5'-CAGACTACCTCAACCGTTCCAC – 3' for forward; 5'-TCCAGCTTTCCCTCCGCATTGA-3' for reverse (www.origene.com); *Cyp1a1*: 5'-GGGTTTGACACAGTCACAACT-3'for forward; 5'-GGGACGAAGGATGAATGCCG-3' for reverse[Yang et al.,2020]. *Gapdh* was used as an internal control: 5'-AAGAAGGTGGTGAAGCAGG-3' for forward, and 5'-GAAGGTGGAAGAGTGGGAGT-3' for reverse.

2.11 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*0.05. The association between the PAHs concentrations 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 tryptophan metabolites. A redundancy analysis (RDA) was performed to determine the multivariate relationship between PAHs and sample distribution and one carbon metabolites and by R package. In addition, mediation analysis for the association between PAHs and asthma mediated by intermediates was implemented considering one carbon metabolites and tryptophan metabolites as mediators with reference to our previous work [Hu et al.,2021].

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. **Figure. 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 concentrations.

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

3.2 Serum PAH concentrations were associated with childhood asthma

As shown in **Figure.1**, the distribution of twelve types of PAHs was similar in the asthmatic group and the control. The concentration of 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 about 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 [Singh et al.,2008]. 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 urinary1-hydroxypyrene (1-OHPyr) concentrations were associated with childhood asthma [Hu et al.,2021]. Internal exposure to PAHs has been assessed commonly by urinary 1-OHPyr as a general biomarker [Hansen et al.,2008]. However, environmental exposure to PAHs may be understatedly assessed only based on exposure biomarkers such as urinary OH-PAHs metabolite concentrations [Yang et al.,2021]. 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 [Huang et al.,2021]. 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 [Yang et al.,2021;WHO,2010]. And there is mounting evidence of unmetabolized PAHs as a biomarker to reflect body burden [WHO,2010; Sexton et al.,2011], which can directly represent the actual

exposure concentrations 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 [Craemer et al.,2016].

Table 2
Serum PAH concentrations 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)	*P* 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, * $P < 0.05$

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 [Mentch et al.,2016] (**Figure.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 concentration and childhood asthma [Hu et al.,2021]. Urinary 1-OHPyr concentration was associated with 7-methylguanine which could reflect global DNA methylation in asthmatic children [Wishnok et al.,1993]. So, we compared the concentrations of one carbon metabolites between two groups (Table 3, **Figure.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 **(Figure.2C**). What's more, the methionine concentrations 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 [Roy et al.,2020]. 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, **Figure.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 **(Figure.2E)**. We used differential one carbon metabolites to perform mediation analysis to assess the association between the PAHs concentration and asthma mediated by metabolites. The directed acyclic graph (DAG) showed significant mediation effects between the Fla concentration and asthma by SAH, SAM and Ser **(Figure.2F, Table S6)**.

Table 3
The serum concentrations 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				

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[Lisanti et al.,2013]. The distributions of *LINE-1* DNA methylation were presented in **Fig. 3A.** 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 (β = 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 **(Figure.3B)**. In contrast to our work, current evidence showed that prenatal urinary 2-OHNa and 1-hydroxyphenanthrene were associated with lower *Alu* and *LINE-1* methylation [Yang et al.,2018]. 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 [Yang et al.,2012]. Interestingly, global DNA hypermethylation levels were associated with asthma severity by assessing the percentage of 5-methylcytosine [Chan et al.,2017]. 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 [Wang and Liu,2008]. For example, elevated concentrations of IL-17A have been found in the sputum and in bronchoalveolar lavage fluid of patients with asthma[Molet et al.,2001]. What' more, Milovanovic *et al* demonstrated that IL-17A secretion promoted IgE production [Milovanovic et al.,2010]. So, we collected the serum from both groups and performed ELISA to detect the concentration of IL-17A. **Figure.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 concentration 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.* [Kohli et al.,2012] 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. 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(**Figure.3C**). Interestingly, Fla could upregulate H3K4 tri-methylation level in the *IL-17A* promoter region and associated with children asthma (β = 0.293, P = 0.002) (**Figure.3D**). By ChIP-qPCR results we demonstrated that PAHs could promote secretion of IL-17A through changes in histone methylation levels. In a previous study, PAH might affect, through their effects on the aryl hydrocarbon receptor, IL-17 production in asthma [Plé C et al.,2015]. Our data suggested 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.

3.5 PAHs changed the expression of tryptophan metabolites in asthmatic children

Tryptophan is an essential amino acid in all animal, metabolism of which is involved in the regulation of immunity, neuronal function and intestinal homeostasis [Martin et al.,2020]. Trp metabolism includes three major pathways, i.e. the kynurenine pathway, the serotonin (5-hydroxytryptamine [5-HT]) production pathway and a series of molecules, including AhR ligands by microbiota [Zelante et al.,2013]. In this present study, we detected Trp metabolic profile to find out key metabolites associated with PAHs-related childhood asthma (Table 4). In **Figure.4A**, the concentrations of Trp, tryptamine, IA, IAA, and Indole were significantly higher in asthmatic group, and that of IAld and IAAld were relatively lower, which is consistent with Licari's study [Licari et al.,2019]. The differential Trp metabolites was mainly enriched in AhR ligands derived from microbiota, such as tryptamine, IA, IAA, IAld and Indole, which indicated the asthmatic children had increased indole-AhR pathway. There was no change in the concentration of the metabolites in kynurenine pathway and 5-HT pathway. Reports have shown that circulating Trp concentrations were increased in germ-free mice [El Aidy et al.,2012]. In this present study, the circulating Trp concentration was also increased.

Lactobacillus, *Clostridium* and others can directly utilize Trp. *Lactobacillus* can metabolize Trp to IAld, which can active AhR anti-inflammatory responses [Zelante et al.,2013]. However, the concentration of IAld in asthmatic children in this study was decreased. What's more, the microbial diversity was decreased in asthmatic children, especially *Lactobacillus* in our previous work [Hu et al.,2021]. These data suggested he microbiota-AhR axis can influence host metabolism. Although there was some correlation between PAHs and Trp metabolites (**Figure.4B**), it was also showed that Trp metabolites has direct effects on IL-17A production or childhood asthma, but no mediation effects between PAHs concentration and asthma by Trp metabolites (**Table S7 and S8**). AhR acting a crucial transcription factor is involved in Th17 cells differentiation [Veldhoen et al.,2008]. IL-17A is secreted from Th17 cells. In agreement with the present studies, increased IL-17A concentration was found in serum of asthmatic children in our study. However, there were controversial results that some AhR such as TCDD induced IL-17A generation in mice and inhibited that in humans [Veldhoen et al.,2008;Ramirez et al.,2010], and this discrepancy may be due to the different ligands and the types of AhR-expressed cells. Additional studies are needed to further explore the effects of PAHs on Trp metabolites, AhR, and IL-17A profiles.

Table 4
The serum concentrations of tryptophan metabolites of subjects in this study

PAHs	Limit of Detection(ng/mL)	Min ~ Max value(ng/mL)	Mean ± SD(ng/mL)	Median(ng/mL)
PIC	7.5	84.55-369.543	143.301 ± 44.590	132.231
QUI	10	176.768-453.643	234.826 ± 55.912	217.376
5-HTP	16.67	232.695-1391.512	677.133 ± 256.877	639.341
5-HT	15	52.115-464.017	126.754 ± 79.644	99.651
Kyn	3.75	328.37-1681.422	838.017 ± 251.845	792.212
IA	0.33	9138.89-36006.643	18308.153 ± 6081.868	17380.28
Trp	0.33	9154.257-36424.142	18414.979 ± 6134.758	17391.55
Tryptamine	1.67	ND-96.628	31.223 ± 20.247	28.04
5-HIAA	0.67	3.2-112.644	33.185 ± 20.85	27.204
IAld	0.3	0.996–16.412	6.755 ± 3.489	6.62
IAA	3.75	145.464-9663.26	1229.170 ± 1178.662	1039.154
Indole	75	ND-623.324	209.647 ± 155.352	161.96
IAAld	15	ND-150.284	37.865 ± 24.783	39.544
IPA	2	ND-532.54	142.565 ± 139.532	90.558
ND: not detected				

3.6 PAHs induced Il-17A production by altering tryptophan metabolism in mice

To validate whether PAHs have the effects on tryptophan metabolism and inducing IL-17A production or not, PAHs mix were exposed on mice. We selected more than four rings PAHs due to more stable and more toxic. The results showed that lung tissue was infiltrated by lymphocytes in PAHs -exposed group (**Figure.5**). The concentration of IgE in BALF of PAHs-exposed group was higher than that in the control (**Figure.6A)**. Interestingly, after stratified by gender, the concentration of Il-17 in BALF of male PAHs-exposed group was higher than that in the control (**Figure. 6B**). In lung tissue, the expression of *Ahr* was upregulated in PAHs-exposed group. *Il-17a* gene was downregulated in PAHs-exposed group. Another AhR target genes *Cyp1a1* was upregulated in female PAHs-exposed group (**Figure. 6C, 6D and 6E**). In gut tissues, the expression of *Ahr* was upregulated only in female PAHs-exposed group while *Il-17a* genes was upregulated in both female and male PAHs-exposed groups. *Cyp1a1* was only upregulated in male PAHs-exposed group (**Figure. 6F, 6G and 6H**). Here we found that the transcription of *Il-17a* was inhibited in lung tissue and induced in gut tissue, while the concentration of IL-17 in BALF was increased in male PAHs-exposed group. There were different AhR-induced responses in lung and gut tissue, respectively. We speculated that gut-derived IL-17A can influence inflammation in lung through gut-lung axis. Reports have shown that tetrachlorodibenzo-p-dioxin (TCDD)-active AhR mediated immunosuppressive effects in human [Kerkvliet,2009], while controversial results have shown that endogenous AhR ligand 6-formylindolo[3,2-b] carbazole (FICZ) induced IL-17A production in mice [Quintana et al.,2008]. Other than TCDD, polycyclic aromatic hydrocarbons are rapidly metabolized by AhR-inducible enzymes that may produce a different type of effects on the immune system. What's more, active AhR may induce the expressions of AhR target genes, such as IL-6, which can initiate Th17 cell differentiation and promote IL-17 production [Xu and Cao,2010]. But excessive immune reactions lead to inhibit anti-immune responses in the microenvironment and ultimately result in uncontrolled and promoted immunity response. Th17 cell differentiation may be modulated by AhR through transcriptional changes. Although the mechanisms mediating AhR-Th17 axis still remain controversial in human and mice studies, cytokine milieu may be contributed to induce Th17 cell differentiation [Veldhoen et al.,2008]. Besides of as direct ligands of AhR, PAHs may also influence AhR-IL-17A by disturbing tryptophan metabolism. Metabolic profiling was applied to compare the global metabolites in mice with or without PAHs treatment. The differential metabolites partly shaped the gender difference **(Figure. 7A**). It was also found that the differential metabolites were mainly enriched in tryptophan metabolism, such as indoleacetic acid (IAA), indoxyl sulfate, kynurenic acid (Kyn), indolelactic acid, 5-hydroxyIndoleacetic acid(5-HIAA), indole-3-carboxylic acid, and indoleacetaldehyde (IAAld), most of which are AhR-ligands, such as IAA, Kyn and IAAld (**Figure. 7B**). IAA and indoxyl sulfate were lower in both PAHs-exposed groups; Kyn and indolelactic acid were higher in male PAHs-exposed group; 5-HIAA, indole-3-carboxylic acid, and IAAld were higher in female PAHs-exposed group. In addition, acetyl-methionine,1-methyladeosine, and 7-methylguanine were higher in PAHs-exposed group. These animal results supported that PAHs altered tryptophan metabolism and disturbed IL-17A production.

4 Conclusion

In this study, we assessed the effects of PAHs exposure on asthmatic children from the perspective of one carbon metabolism and tryptophan metabolism. Our data gave the evidence that PAHs exposure induced DNA methylation and histone methylation by disturbing one carbon metabolism, and altered Try-AhR pathway to disturb IL-17A production, which could help in identifying the underlying mechanisms of PAHs exposure-related asthma. However, there are limitations in the present study. We found gender differences in the effects of PAHs on inflammation in animal study. Due to the small sample size and low proportion of female subjects in asthmatic children, we did not find the effects of gender on PAHs toxicity. In addition, microbiota-mediated immunodulation have been associated with the etiology of asthma and microbiota can also modulate Trp metabolism. Therefore, we should investigate the effects of PAHs-related microbiota on Trp metabolism and childhood asthma in future work.

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
L-Tryptophan
Trp
L-kynurenine
Kyn
2-picolinic acid
PIC
quinolinic acid
QUI
indole acrylic acid
IA
indole-3-propionic acid
IPA

5-Hydroxyindoleacetic acid
5-HIAA
indoleacetaldehyde
IAAld
indoleacetic acid
IAA
5-hydroxytryptamine
5-HT
5-Hydroxy-L tryptophan
5-HTP
Indole-3-aldehyde
IAld
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

The Nanjing Medical University Clinical Research Ethics Committee, Nanjing, China, reviewed and approved the protocols of this study.

Consent to participate

Written informed consent was obtained from the participants' parents for the use of samples in this study.

Consent to publish

Its publication has been approved by all co-authors

Author contribution

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

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Competing Interests

There are no conflicts to declare.

Availability of data and material

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

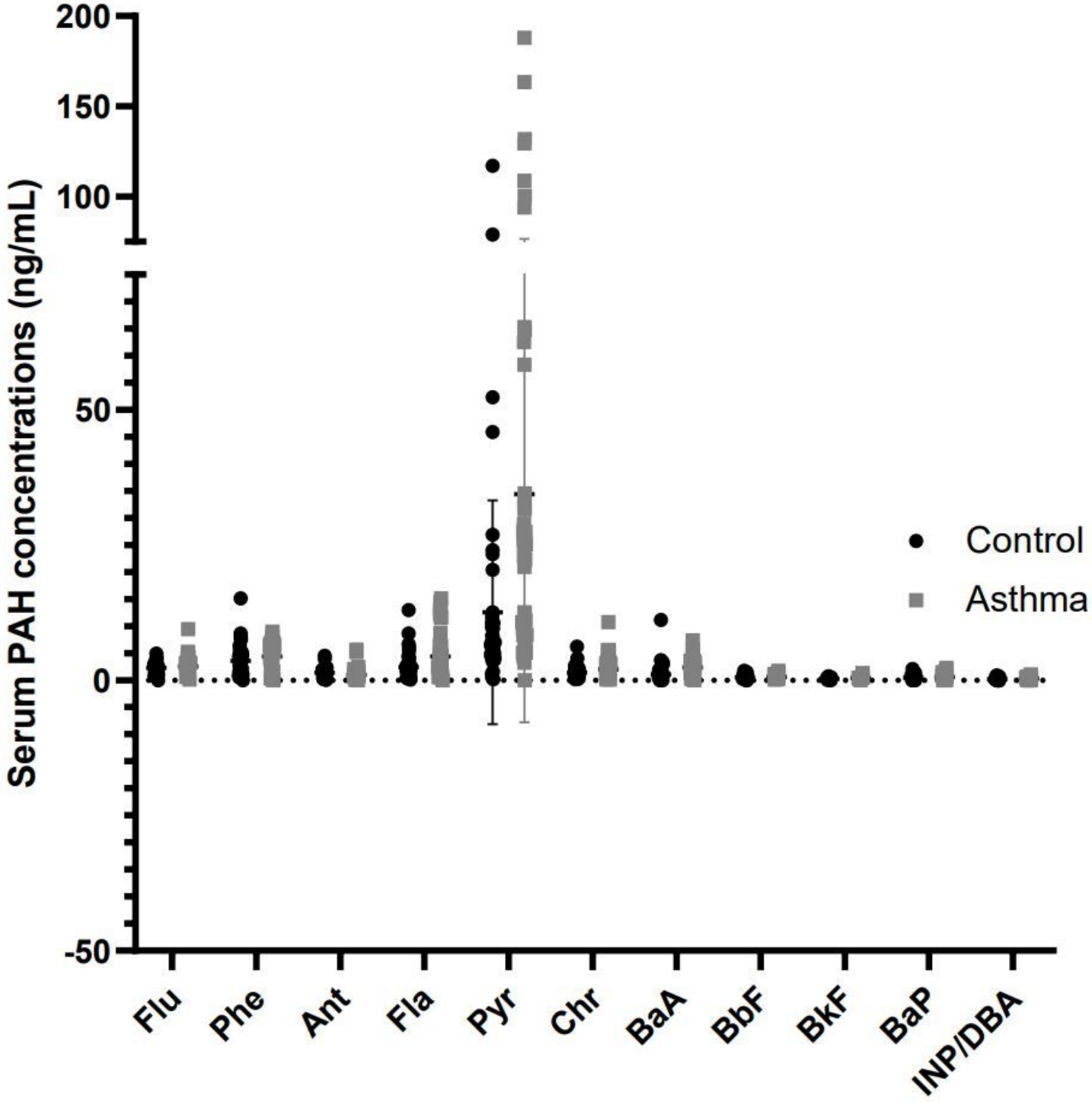

Figure 1

Legend not included with this version.

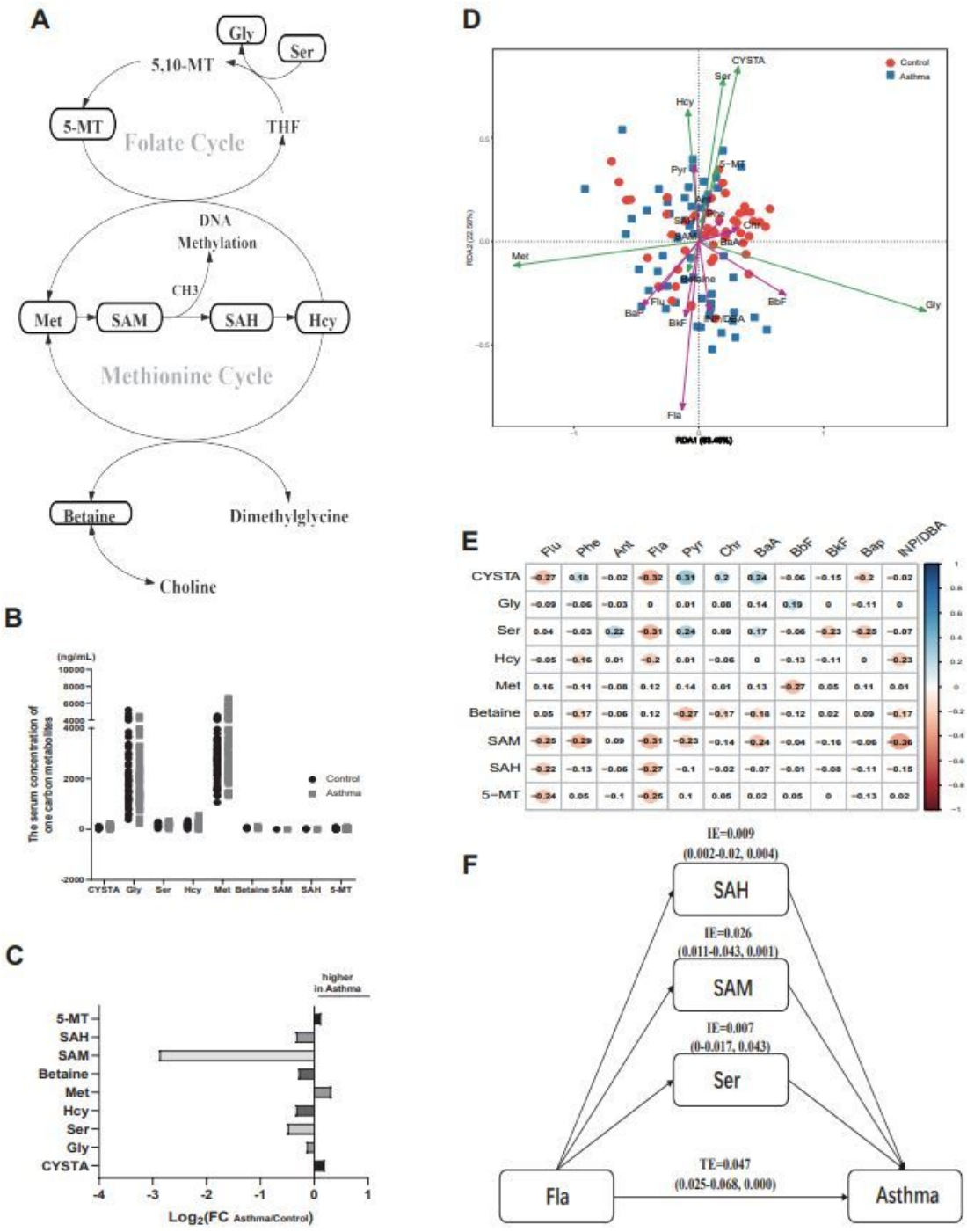

Figure 2

Legend not included with this version.

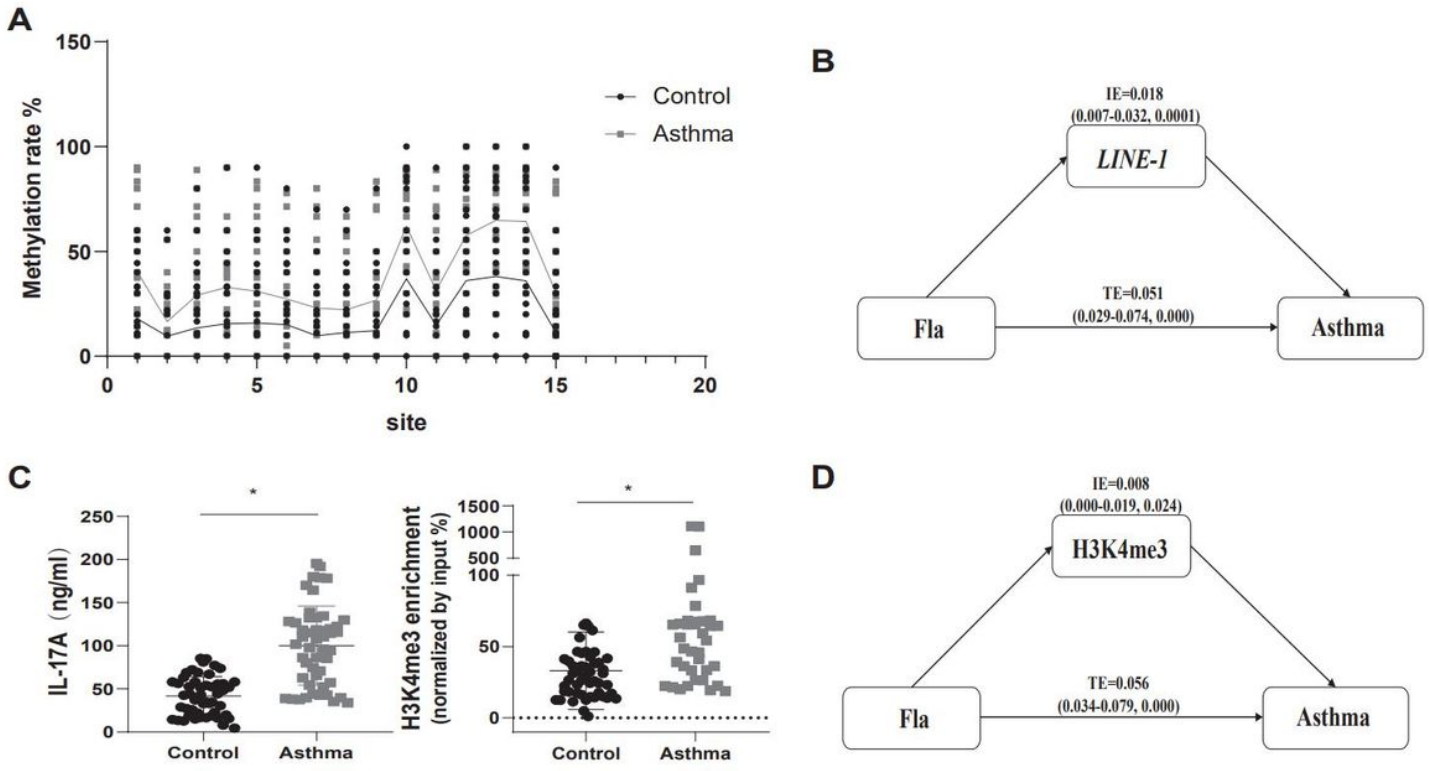

Figure 3

Legend not included with this version.

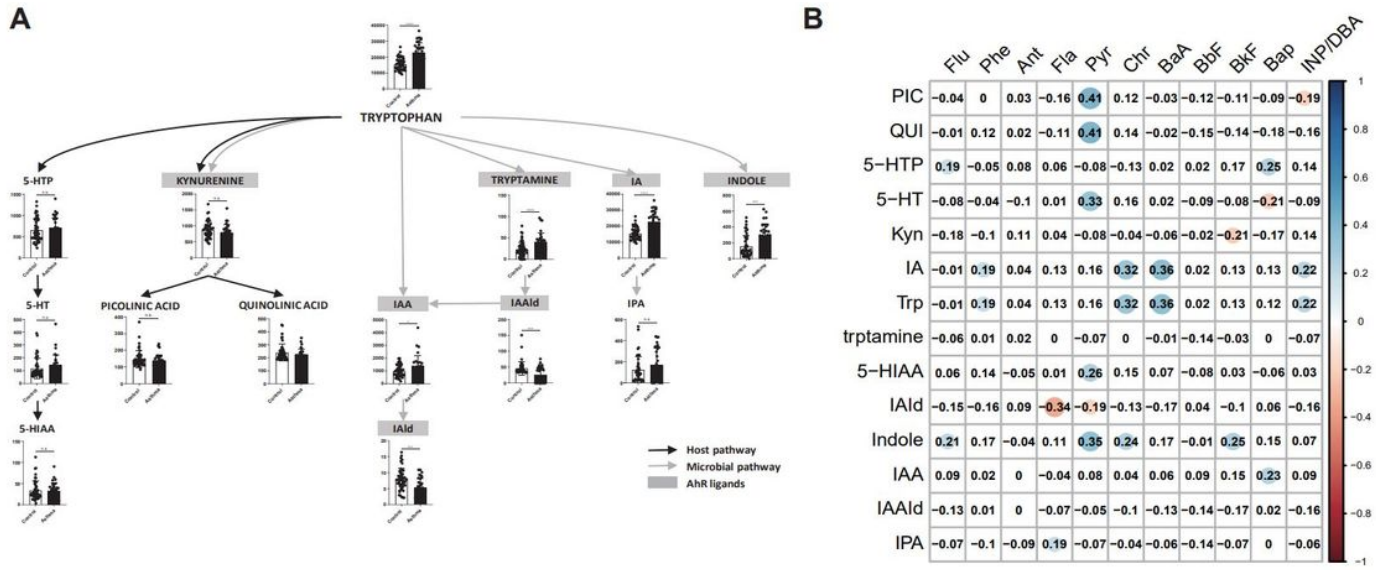

Figure 4

Legend not included with this version.

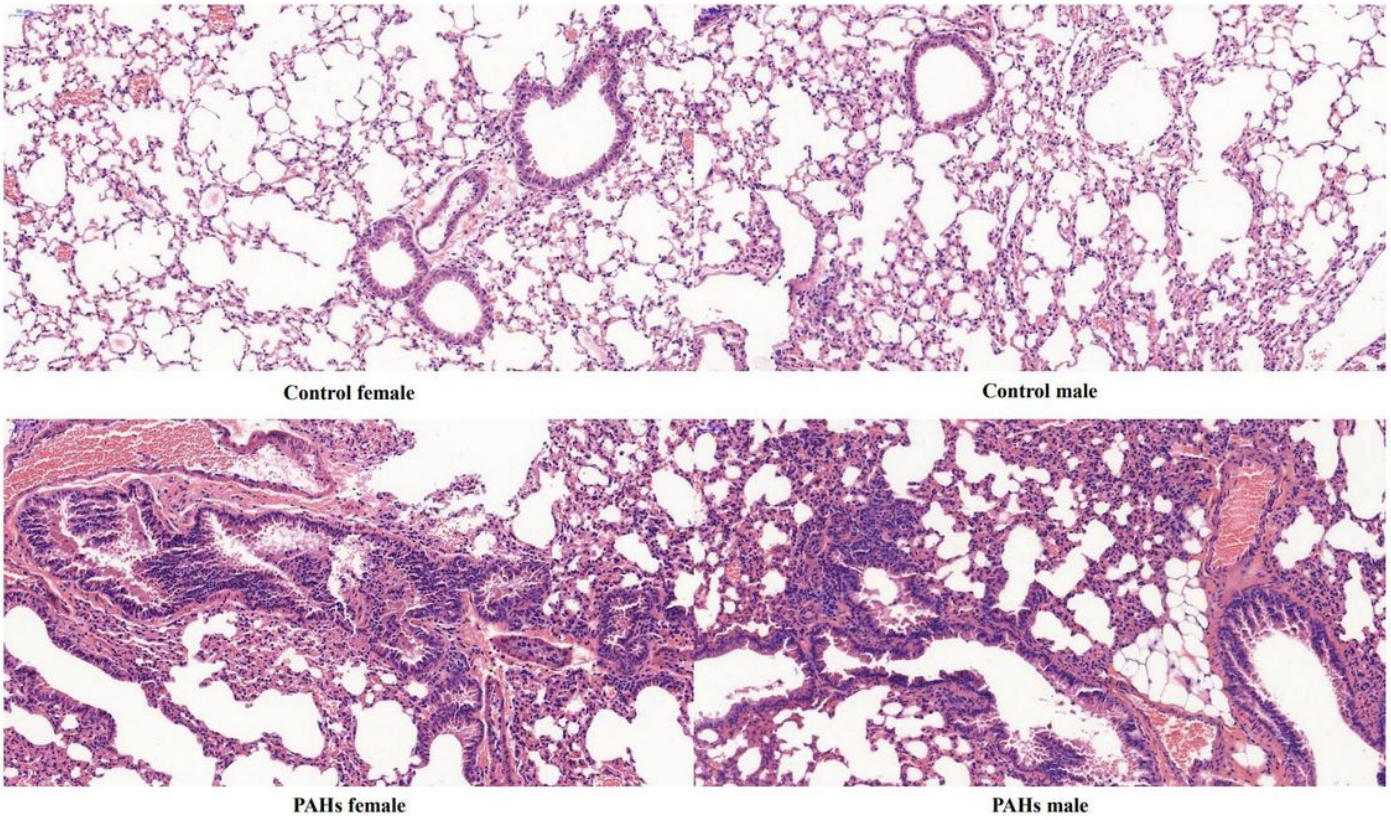

Figure 5

Legend not included with this version.

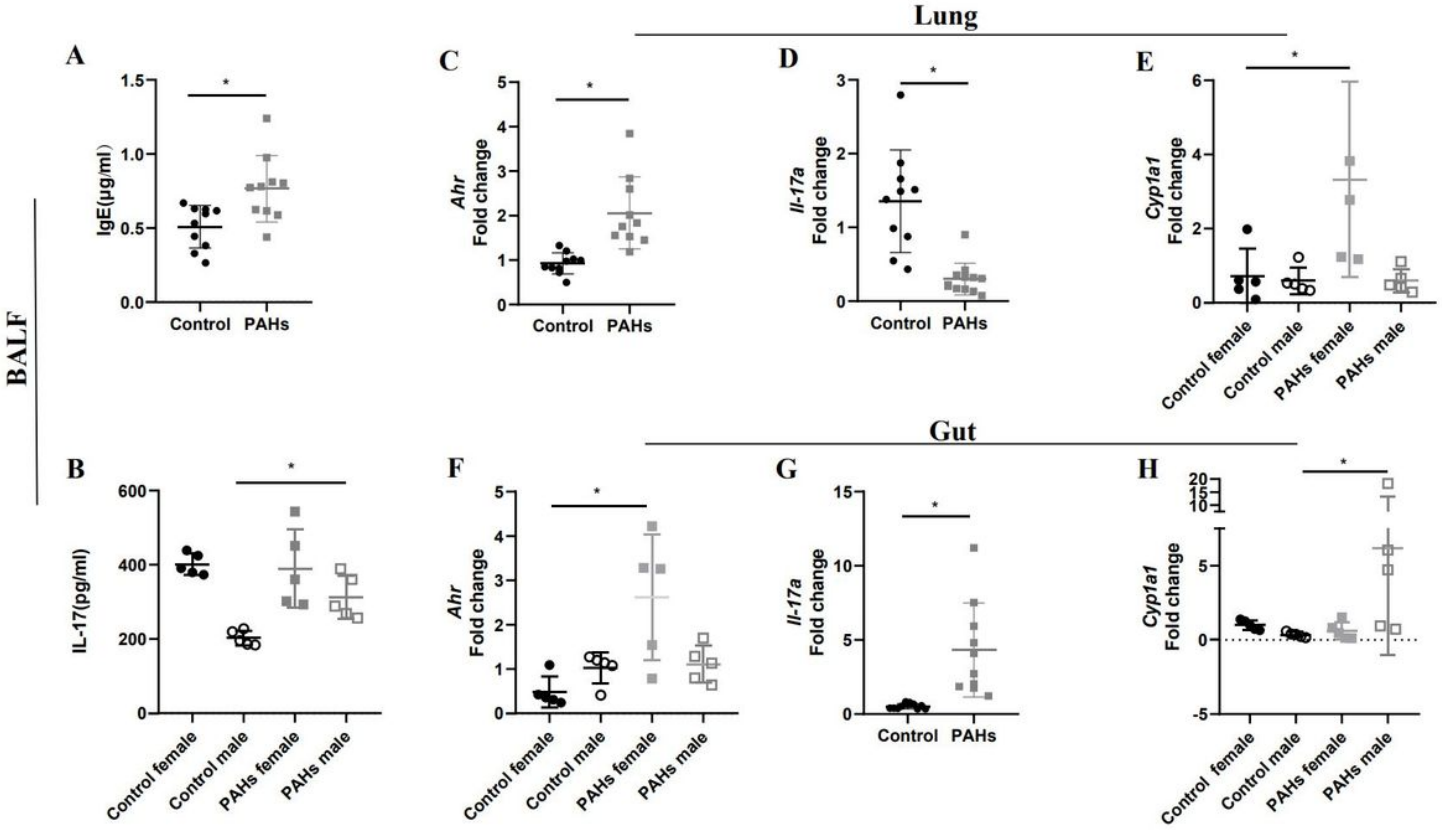

Figure 6

Legend not included with this version.

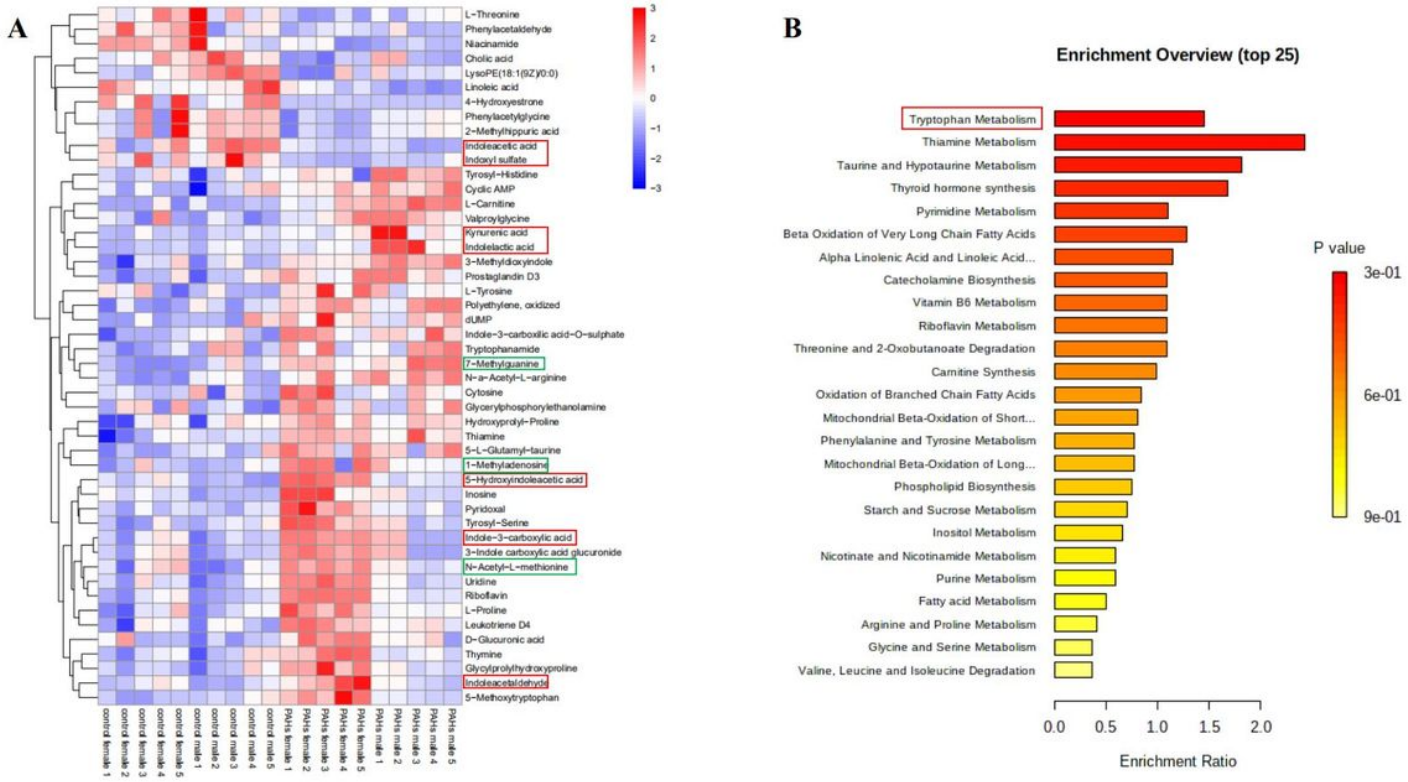

Figure 7

Legend not included with this version.

Supplementary Files

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

- SupplementaryMaterial.docx