



Identification of multiple dysregulated metabolic pathways by GC-MS-based profiling of lung tissue in mice with PM_{2.5}-induced asthma

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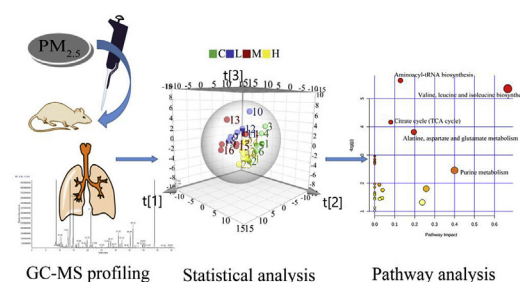
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HIGHLIGHTS

- PM_{2.5} induces asthma in mice.
- GC-MS analysis identified 13 biomarkers in PM_{2.5}-induced asthmatic mice.
- Screening identified four metabolic pathways involved in the pathogenesis of asthma.
- PM_{2.5} regulates amino acid and purine metabolism by disrupting the TCA cycle.

GRAPHICAL ABSTRACT



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ABSTRACT

The risk of development of asthma, a multi-faceted chronic disease, increases as a result of exposure to PM_{2.5}. However, the mechanism underlying asthma-related metabolic changes caused by PM_{2.5} exposure is unclear. Here, we investigated the major metabolic changes, metabolic pathways involved, and underlying molecular mechanisms in mice with PM_{2.5} exposure-induced asthma. Forty-eight adult female mice were randomly assigned to control (C), low concentration-PM_{2.5} exposure: 0.50 mg kg⁻¹ (L), medium concentration-PM_{2.5} exposure: 1.58 mg kg⁻¹ (M), and high concentration-PM_{2.5} exposure: 4.98 mg kg⁻¹ (H) groups. M and H groups presented significantly higher IL-4, IL-8, IL-1β, IL-5, IL-13, and OVA-specific IgE levels, and significantly lower IFN-γ levels, than the C group, as well as significantly increased eosinophil count and MUC5AC expression in the lung tissue. These findings indicate that exposure to medium and high concentrations of PM_{2.5} induced asthma in mice. Statistical analyses identified 13 asthma-related major metabolites, which were analyzed by gas chromatography-mass spectrometry (GC-MS). Meta Mapp Software revealed 4 major metabolic pathways. PM_{2.5}-induced ATP requirement and oxidative stress may perturb metabolic processes in asthma. The present findings increase our understanding of the toxic effect of PM_{2.5} in the development of asthma and identify potentially useful biomarkers.

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1. Introduction

Globally, exposure to polluted air is a major risk factor affecting human health. Fine particulate matter (PM_{2.5}) is a ubiquitous air pollutant and one of the major causes of respiratory disease. Asthma is a chronic disorder of the airways that is characterized by reversible airflow obstruction and airway inflammation, persistent airway hyperreactivity, and airway remodeling (And and Schwartz, 2002). WHO estimates that 235 million people currently suffer from asthma (2017). Environmental exposure is one of the biggest risk factors for this disease. Numerous studies have explored the role of asthma-related cytokine changes in the pathogenesis of asthma. Asthma is known to be caused by the tendency of helper T cells to differentiate into Th2 cells. The Th2 cytokines, IL-4, IL-5, and IL-13, are important factors responsible for inflammation and tissue damage caused by asthma, and play an important role in the synthesis of OVA-specific IgE and transition to eosinophils. The Th1 characteristic cytokine IFN- γ inhibits the differentiation of Th2 cells, reduces the synthesis and secretion of IL-4, and attenuates inflammatory cell infiltration, thereby improving cellular immunity and alleviating airway symptoms. More importantly, IL-8 induces Th2 differentiation and inhibits the differentiation of Th1 cells, thus promoting *in vivo* OVA-specific IgE production and eosinophil infiltration and exacerbating asthma symptoms (John et al., 1998; Tsuji et al., 2012; Li et al., 2014). However, studies of asthma-related cytokines did not clearly explain the changes in metabolites observed during lung tissue injury caused by PM_{2.5} exposure. Therefore, an incomplete understanding of the biological mechanisms and metabolic changes underlying the pathogenesis of asthma remains a major obstacle to the prevention and treatment of this disease. Metabolomics involves the study of all metabolites present in the body at a given time; the metabolites themselves reflect the environment surrounding the organism (Saude et al., 2011). In turn, this is related to the body's nutritional status, the role of environmental pollutants, as well as the influence of other external factors. At present, metabolomics has been used in the diagnosis and treatment of diseases through analysis of metabolite changes and clarification of metabolic pathways involved in pathogenesis, as well as the identification of related biomarkers. Additionally, metabolomics approaches have been used to study the metabolic response to various diseases. Metabolomics analysis of bronchoalveolar lavage fluid (BALF) collected from patients with cystic fibrosis has enabled the identification of key disease-related metabolic biomarkers, and has also provided novel insights into the disease (Wolak et al., 2009). Further, lung tissue-associated metabolites from animals exposed to silica dust (Wiklund et al., 2008), house dust (Ho et al., 2014b), or 1-nitronaphthalene (Azmi et al., 2005) have been analyzed using metabolomics to study lung toxicity. PM_{2.5}, which is the main component of atmospheric pollution, has a small particle size and is easy to carry heavy metals, polycyclic aromatic hydrocarbons, microorganisms, and other pollutants. On entry into the human body, PM_{2.5} causes damage to multiple organs. According to the 2016 China Environmental Quality Status Announcement, of the 338 prefecture-level and above cities in China, 254 cities (75.1%) had exceeded ambient air quality, and PM_{2.5} was the primary pollutant for 80.3% of the days of heavy pollution. To date, no studies using metabolomics technology to analyze PM_{2.5}-induced asthmatic disease have been reported.

The gas chromatography-mass spectrometry (GC-MS) method not only involves the use of a high-separation performance capillary column, but also has a high-sensitivity mass spectrometric detection capability and a stable commercial mass spectrometry database; the combination of the two is considered a useful metabolic profiling technique. GC-MS can analyze not only volatile substances, but also derivatized semi-volatiles and non-volatiles

substances. In this study, we used the metabolomics platform to explore the major asthma-related metabolite changes induced by PM_{2.5} exposure and clarified the associated metabolic pathways. Our findings provide valuable information for the prevention and treatment of asthma associated with metabolite changes.

2. Materials and methods

2.1. Collection and recovery of PM_{2.5}

The sampling point was located in Shanxi University (37°75'N, 112°57'E, 802 m above sea level, no obvious pollution sources around), Taiyuan City, Shanxi Province of China. PM_{2.5} were collected on quartz fiber filters (Φ 90 mm, PALLFLEX, USA) using PM air samplers (TH-150CIII, Wuhan, China) at a flow rate of 100 L min⁻¹. The quartz filter was pre-sampled in a 450 °C muffle furnace for 4 h (decontamination), weighed. Sampling was performed between November 2015 and November 2016, 23 h $\pm$ 0.5 h each day. PM_{2.5} was prepared by soaking with PM_{2.5} in ultrapure water for 10 min, followed by vortexing for 5 min and sonication for 30 min, and filtered through 8 layers of sterile gauze. Then, the PM_{2.5} suspension was processed with the vacuum freeze-drying technique. The dried samples were diluted with sterilized PBS solution and swirled for 10 min before using for animal experiments, and aqueous suspension was pooled and frozen at -20 °C.

All chemicals used for GC-MS analysis were of GC grade. Methoxyamine hydrochloride, N-Methyl-N (trimethylsilyl) tri-fluoroacetamide (MSTFA), 1% trimethylchlorosilane (TMCS), heptane, and pyridine were purchased from Sigma (St. Louis, USA). All reagents were used according to the instructions.

2.2. Animals and treatment groups

Female BALB/c mice, 6–8-week-old, weighing 18–22 g (Beijing, China) were bred under standard conditions (24 $\pm$ 2 °C, 50 $\pm$ 5% humidity, and 12 h:12 h light: dark cycle), with an air purifier. All mice were allowed to acclimatize for a week and then randomly divided into four groups of 12 mice each, as follows: Control (C group), low concentration-PM_{2.5} exposure (L group), medium concentration-PM_{2.5} exposure (M group), and high concentration-PM_{2.5} exposure (H group). Using the lung surface area-dose exchange algorithm (Gavett et al., 2003), we converted the human exposure PM_{2.5} concentration dose (50 μ g m⁻³; 159 μ g m⁻³; 500 μ g m⁻³) to the corresponding dose for mice (0.50 mg kg⁻¹, 1.58 mg kg⁻¹, 4.98 mg kg⁻¹). Mice were exposed to PM_{2.5} by intra-tracheal instillation on day 8 according to previously published procedures (Gavett et al., 2003; Rao et al., 2003; He et al., 2016). All mice were exposed at the same time every other day for seven consecutive times. Twenty-four hours after the final tracheal instillation, all mice were euthanized under isoflurane anesthesia. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Shanxi University and conformed to the Guide for Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee.

2.3. Sample collection and cell counts

In accordance with previously used methods (Schedel et al., 2016; Huang et al., 2018), cardiac puncture was performed on anesthetized mice at 24 h after the final challenge; the collected blood was incubated for 45 min prior to serum collection (1500 rpm, 15 min, 4 °C) (Luan et al., 2015), and the serum was used for subsequent measurement of OVA-IgE levels. Bronchial alveolar lavage was followed by blood sampling from the heart and ligation of the right lung. Ice-cold PBS (4 $\times$ 200 μ L) was instilled into the

airways and the mean volume retrieved was 90% of the amount instilled (700–750 μL), followed by centrifugation (1000 rpm, 10 min, 4 °C). The cell pellet was resuspended in 1.0 mL of 10% fetal bovine serum in PBS, and the total number of cells was determined using a hemocytometer. The cell-free supernatant was used for subsequent measurement of inflammatory cytokine levels. The lung tissue was rapidly removed and rinsed several times with 0.1 M ice-cold PBS (pH = 7.4). A small part of the tissue fixed in formalin for 24 h at RT (20 °C) was used for subsequent immunohistochemistry. The remaining tissue was frozen in liquid nitrogen and stored at –80 °C until further analysis.

2.4. Inflammatory cell and multiplex cytokine profile analysis

The interleukin (IL)-4, IL-1 β , IL-5, IL-8, IL-13, and interferon (IFN)- γ levels in the BALF were measured using an enzyme-linked immunosorbent assay (ELISA) (XiTang Biotechnology Co., Ltd., Shanghai). OVA-specific IgE antibodies in serum were measured using a Mouse OVA-Specific IgE ELISA kit (BioLegend, Cat. No. 439807, San Diego, CA).

2.5. Pathological evaluation

The tissue was removed from formalin, dehydrated in graded ethanol solutions, and embedded in paraffin. For histopathological examinations (HE), sections (5–6 μm -thick) were deparaffinized with xylene, stained with hematoxylin and eosin, and observed by light microscopy. The data were quantified as the average number of eosinophils present per square millimeter of the peribronchial area: (number of EOS cells in the peribronchial region)/(area of the peribronchial region). For immunohistochemistry, 4- μm -thick paraffin sections layered on silane-coated slides were used for immunohistochemistry with primary mouse antibodies against MUC5AC (Abcam Biotechnology, England). The negative control sections were incubated with PBS instead of the primary antibody. The stained sections were observed by light microscopy. The following formula was used to assess the extent of mucin production in bronchial airways: mucus index = (area of MUC5AC staining $\times$ mean intensity of MUC5AC staining)/all superficial areas of bronchial epithelial cells. Image analysis of digital photomicrographs (magnification 400 $\times$) was performed with the Image-Pro Plus 6.0 (Silver Spring, MD, USA) analysis system. The final values were the results of an average of 15 bronchioles (150–200 μm internal diameter) randomly chosen by an investigator blinded to animal group assignments (Zaynagetdinov et al., 2010).

2.6. GC-MS metabolomics analysis

The lung tissue samples were prepared by weighing 0.1 g of the lung tissue in an EP tube, adding 0.2 mL of ultrapure water, and homogenizing on ice for 30 s until no tissue debris were visible. Acetonitrile and methanol (1:1 mixture, 0.6 mL) was then added to the sample and vortex mixed for 1 min followed by ice bath ultrasound treatment for 15 min. After centrifugation (11,000 rpm, 15 min, 4 °C) the supernatant from the sample (around 150 μL) was used for subsequent GC-MS assays (Surowiec et al., 2016). This solution was freeze-dried in vacuum and derivatized with 50 μL methoxyamine (15 mg mL⁻¹ in pyridine, at 70 °C for 1 h) followed by 50 μL MSTFA (containing 1% TMC, v/v, at 40 °C for 90 min), and finally, 50 μL of n-heptane (containing 0.1 mg mL⁻¹ n-tetracosane). The samples were stored at –20 °C before GC-MS analysis. Next, splitless injection of 1.0 μL of the derivatized supernatant was performed with a Thermo Fisher DSQ Series. Then, chromatography was performed on a DB-5MS capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μm film thickness; Agilent J&W Scientific, Folsom, CA). The

inlet temperature was set at 280 °C, helium was used as the carrier gas at a constant flow rate of 1.0 mL min⁻¹; the column temperature was initially maintained at 80 °C for 2 min, followed by a ramp to 140 °C at 10 °C min⁻¹, held for 1 min, and then increased to 240 °C at 4 °C min⁻¹, held for 2 min. Finally, the post-run temperature was increased to 300 °C at 5 °C min⁻¹ and maintained for 3 min; the solvent delay was set as 8 min. The transfer line temperature was set at 280 °C, and the ion source temperature was 230 °C (Ho et al., 2014a). The mass spectrometer was operated in the electron impact (EI) mode (70 eV) (Araujo et al., 2018). Data acquisition was performed in the full scan mode from m/z 50 to 650. The GC-MS total ion chromatogram was pretreated and combined with the retention time of normal alkane to calculate the retention index of the metabolite. Metabolites were analyzed and determined by Automated Mass Deconvolution and Identification System (AMDIS) library searches, combining retention times and retention indices for each metabolite. To ensure the accuracy of the data, the matching between the value of the test compound and that in the database was >80%. The quality control (QC) samples were prepared by mixing equal volumes of lung tissue obtained from 24 samples before sample preparation as they were aliquoted for analysis. A quality assessment strategy based on the periodic analysis of QC samples together with the real samples was employed in this study. A threshold of 30% was set for the relative standard deviation values of metabolites in the QC samples, which is accepted as standard in the assessment of repeatability in metabolomics data sets. After pre-processing screened metabolites (normalization involves calibration of the internal standard in each sample, followed by calculation of the correction coefficient, and then calibration each substance in the sample according to the correction coefficient) (Zeng et al., 2016). Finally, the main metabolites showing a difference were screened by principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). (Ho et al., 2014c).

2.7. Data analysis

SIMCA14.0 software was used for PCA of the integral data obtained by processing. The OPLS-DA method was used to determine the differences between the metabolites in the samples. Heatmap analysis was performed with Metaboanalyst 3.0 (Montréal, QC, Canada), a web-based tool for pathway analysis and visualization metabolomics. Pathway mapping was analyzed with Meta Mapp Software (Mishra et al., 2017). Differential metabolites of PM_{2.5}-induced asthmatic mice were further identified and validated by searching online databases, including the Kyoto Encyclopedia of Genes and Genomes (KEGG). Origin was used for statistical analysis of cytokines and metabolites.

3. Results

3.1. Body and organ-to-body weight

The growth of the mice was recorded every seven days. During the experimental period, the mean body weight gain of mice from the L group did not differ from that of those in the C group. However, the body weight of mice in the M and H groups was significantly lower than that of mice in the C group (M vs. C, $p < 0.05$; H vs. C, $p < 0.01$) (Table S3). After the end of the exposure period, the lung-to-body ratio of mice changed most significantly (L vs. C, $p < 0.01$; M vs. C, $p < 0.001$; H vs. C, $p < 0.001$) (Fig. S1); this was followed by changes in the organ-to-body ratio of the kidney, heart, brain, spleen, liver and stomach, in the order indicated (Fig. S1).

3.2. Impact of PM_{2.5} exposure on lung histology

In order to examine the inflammatory lesions in pulmonary tissue in response to PM_{2.5} exposure, the lung tissue sections of each group were subjected to hematoxylin and eosin staining and observed by TEM (Fig. S2). Mice in the C group maintained intact terminal bronchioles and alveolar epithelia, and showed no inflammation (Fig. S2A). After low concentration-PM_{2.5} exposure, slight infiltration of inflammatory cells in the peribronchiolar region was observed and the small blood vessels were found to be expanded (Fig. S2B). Inflammatory cell influx into the peribronchiolar regions and airspaces occurred in the M group (Fig. S2C). In the lung tissue of the H group, alveolar septum thickening and fibrosis were more pronounced than in the M group, and the alveolar contraction and number of goblet cells around the peribronchiolar region were higher than in the C group (Fig. S2D). PM_{2.5} exposure tended to exacerbate pulmonary eosinophil infiltration in a dose-dependent manner. Notably, widespread eosinophil-rich inflammation was observed in the H group. Thus, M and H groups showed higher infiltration of inflammatory cells than the C group.

3.3. Effects of PM_{2.5} exposure on MUC5AC protein expression

Immunohistochemistry and light microscopy were performed to observe the expression and distribution of MUC5AC protein in the lungs of mice exposed to PM_{2.5}. (Fig. S3). As shown in the PM_{2.5} exposure groups, MUC5AC was mainly distributed in the bronchiolar epithelium and cytoplasm with no expression in the basolateral and alveolar regions. The intensity of parenchymal staining for MUC5AC was semi-quantitatively analyzed using a visual analog scale. There was no significant difference in MUC5AC expression between the L and C groups (Fig. S4). The ratio of the M group to C group was significantly increased to 5.35-fold ($p < 0.01$) and that of the H group to the C group was significantly increased to 6.22-fold ($p < 0.01$) (Fig. S4).

3.4. PM_{2.5} enhanced serum OVA-specific IgE

The serum OVA-specific IgE content was measured in mice exposed to different concentrations of PM_{2.5}, and the stimulation of antigen-specific IgE in serum in response to PM_{2.5} was determined (Fig. S5). There was no significant difference in OVA-specific IgE expression between the L and C groups. Compared with the C group, the M group significantly increased by 15.34-fold ($p < 0.001$), and the H group significantly increased by 24.46-fold

($p < 0.001$).

3.5. Effects of PM_{2.5} exposure on the inflammatory cytokine levels in BALF

To analyze the influence of PM_{2.5} on lung inflammation, the expression of inflammatory cytokines and chemokines was analyzed in BALF from mice exposed to various concentrations of PM_{2.5} (Fig. 1). Inflammatory cytokines such as the Th1-related cytokine IFN- γ ; Th2-related cytokines IL-4, IL-13, and IL-5; and chemokines such as IL-8 and IL-1 β in BALF displayed different response patterns. IL-4 showed a significant increase upon exposure to high concentration-PM_{2.5} and a small increase upon exposure to medium concentration-PM_{2.5} compared with that in the control, whereas the low concentration-PM_{2.5} showed no effect on IL-4 expression. Exposure of mice to high concentration-PM_{2.5} induced a significant increase in IL-13 BALF levels, and a small increase upon exposure to medium concentration-PM_{2.5} compared with the control. IL-5 levels showed a significant increase following exposure to low concentration-PM_{2.5}, medium concentration-PM_{2.5}, and high concentration-PM_{2.5}. High concentration-PM_{2.5} strongly augmented the expression of IL-1 β and IL-8 compared with that in the control, whereas medium concentration-PM_{2.5} strongly enhanced the expression of IL-1 β , but elicited a small increase in that of IL-8 ($p < 0.05$). Exposure to high concentration-PM_{2.5} and medium concentration-PM_{2.5} elicited a striking reduction in IFN- γ levels compared with those in the control, whereas the level of IFN- γ in the H group was slightly lower than in the M group. However, the decrease in IFN- γ caused by low concentration-PM_{2.5} was not significant compared with that in the C group (Fig. 1).

3.6. Pulmonary exposure to PM_{2.5} alters the lung metabolome

Metabolites were identified using the AMDIS mass spectrometry database. Deconvolution parameters are shown in Table S1. Database matching resulted in the retrieval of 96 substances. The match indicates the degree of similarity between the mass spectrum of the detected substance and the mass spectrum of the standard substance in the library. Based on a match of >900 and a probability of >80%, a total of 57 metabolites were detected. During GC-MS analysis, the PCA score plot of QC samples showed no drift (Fig. S6), confirming the repeatability and stability of the instrument and the usability of the data for subsequent statistical analysis. The nonparametric univariate method, Mann-Whitney-Wilcoxon test, was applied to measure the significance of each

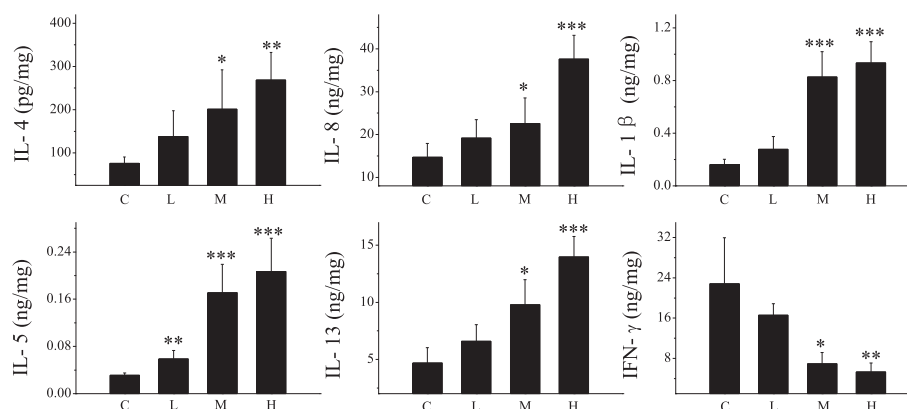


Fig. 1. Effects of various concentrations of PM_{2.5} on IL-4, IL-13, IL-5, IL-8, IL-1 β , and IFN- γ secretion in BALF of mice. The values are expressed as the mean $\pm$ SEM, $n = 12$ per group. *, **, and *** p -values less than 0.05, 0.01, and 0.001, respectively, versus the C group.

peak in the different groups, with results adjusted for multiple testing using false discovery rates (FDR) correction. The models of OPLS-DA show a clear distinction between the groups and improved result prediction (L group from C group: $R^2X_{cum} = 0.867$, $R^2Y_{cum} = 0.993$, $Q^2_{cum} = 0.967$, and CV-ANOVA: $p = 0.000221$), (M group from C group: $R^2X = 0.894$, $R^2Y_{cum} = 0.990$, $Q^2_{cum} = 0.960$, and CV-ANOVA: $p = 0.000397$), and (H group from C group: $R^2X = 0.866$, $R^2Y_{cum} = 0.996$, $Q^2_{cum} = 0.986$, and CV-ANOVA: $p = 0.000018$). The OPLS-DA scores (Fig. 2 L-1, M-1, H-1) showed that the exposure groups and the C group were dispersed in two different regions. The R^2 and Q^2 values of the original OPLS-DA models were higher than the randomly classified permutation distribution, showing that the three OPLS-DA models are valid (Fig. 2 L-2, M-2, H-2). The S-plot of OPLS-DA indicates the size of the loading ($p[1]$), shows the size of its correlation, and represents the contribution size and stabilization of candidate metabolites (Fig. 2 L-3, M-3, H-3).

VIP is a variable load evaluation parameter. Using $VIP > 1.0$ as the threshold to select the multivariate statistical difference variables (Wiklund et al., 2008), it was found that there were differences in metabolite metabolism between the L, M, and H groups compared with the normal group. Specifically, changes in aspartic acid, butanoic acid, glucose, hexadecanoic acid, idose, inosine, isoleucine, malic acid, octadecanoic acid, octadecenoic acid, serine, uric acid, and valine content were observed (Table S1), reflecting differences in the body's physiological and metabolic status. In order to verify the multidimensional statistical results, the obtained differential metabolites were analyzed by *t*-test analyses. Metabolites that ultimately met $VIP > 1$ and $p < 0.05$ were considered to be the final differential metabolites. Thirty-five metabolites were chosen by the Shared and Unique Structures-plot (SUS-plot) scores. SUS plots were constructed with the two OPLS-DA models using the C group as a common reference. This plot combines the values of $p(\text{corr})$ (correlation) from the two models into one 2-D plot. As the SUS-plot displays correlation, it should be scaled between -1 and 1 for both the axes. As the C group constituted a common reference for the comparison of the low-concentration- $PM_{2.5}$ exposure and mid-concentration- $PM_{2.5}$ exposure, two OPLS-DA models were computed to distinguish the metabolic patterns of (i) control and low concentration- $PM_{2.5}$ exposure (C vs. L, model 1) and (ii) control and medium concentration- $PM_{2.5}$ exposure (C vs. M, model 2), $M3.p(\text{corr}) \geq 0.5$, $M2.p(\text{corr}) \geq 0.5$ represent metabolites with highly significant responses. Conversely, $M3.p(\text{corr}) < 0.5$, $M2.p(\text{corr}) < 0.5$ represent metabolites with non-significant responses (Fig. 3A). Two OPLS-DA models were computed to distinguish the metabolic patterns of (i) control and medium concentration- $PM_{2.5}$ exposure (C vs. M, model 1) and (ii) control and high concentration- $PM_{2.5}$ exposure (C vs. H, model 2), $M4.p(\text{corr}) \geq 0.5$, $M3.p(\text{corr}) \geq 0.5$ represent metabolites with high responses, $M4.p(\text{corr}) < 0.5$, $M3.p(\text{corr}) < 0.5$ represent metabolites with non-significant responses (Fig. 3B). To better observe the changes in metabolites and the relationship between the metabolites in each group, the above 35 metabolites were analyzed by hierarchical clustering, in which 13 metabolites showed a decreasing trend and 22 metabolites showed an increasing trend, (*t*-test, $p < 0.5$) (Fig. 3C).

The identified markers shown in Fig. 3 were entered into the KEGG (<http://www.metaboanalyst.ca/>) database to construct and analyze metabolic pathways. The cutoff for the metabolic pathway effects was set at 0.10, above which values were selected as potential target pathways. These metabolites represent key metabolic pathways involving the tricarboxylic acid (TCA cycle); purine metabolic pathway; valine, leucine, and isoleucine biosynthesis pathway; and alanine, aspartate, and glutamate metabolism pathway (Fig. 4).

3.7. Lung tissue of cytokine, chemokine and its associations with metabolites

The data for 6 cytokines (IL-4, IL-5, IL-13, IL-1 β , IL-8 and IFN- γ) in the BALF of four group were summarized in Fig. 1. Pearson correlation analysis (Table 1.) was used to identify potential links between metabolites and cytokines IL-4, IL-5, IL-13, IL-1 β , and IL-8 were determined to correlate negatively with malic acid, these cytokines were also statistically linked positively to aspartic acid, butanoic acid, glucose, hexadecanoic acid, inosine, isoleucine, serine, uric acid and valine levels. However, cytokine IFN- γ was opposite.

4. Discussion

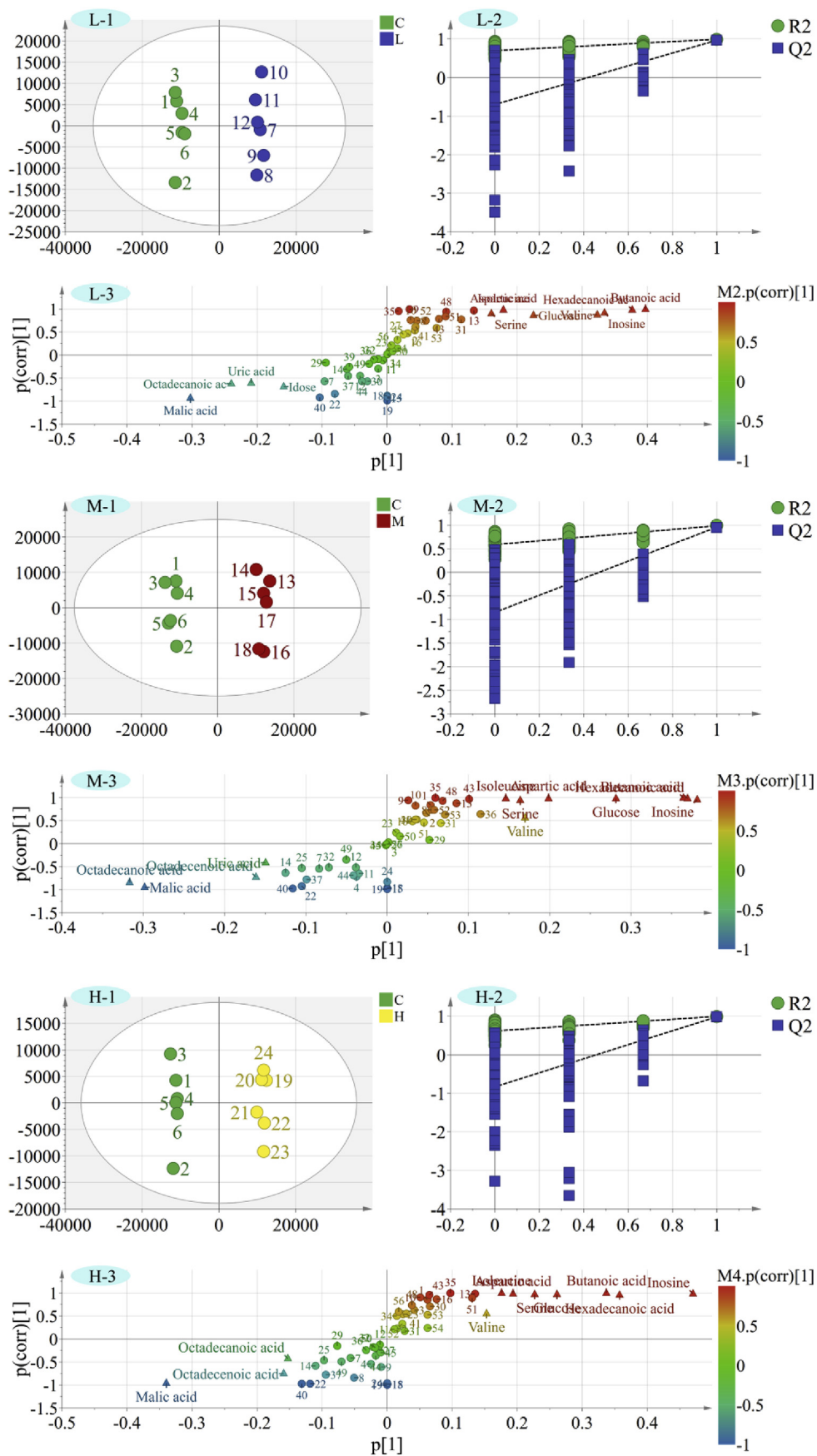
4.1. $PM_{2.5}$ -induced Th1/Th2 immunity imbalance

In this study, compared with that in the C group, Th2 cytokines IL-4, IL-5, and IL-13 and chemokines IL-8 and IL-1 β were significantly increased in the BALF of the M and H groups, and OVA-specific IgE in serum was also significantly increased as high concentrations of $PM_{2.5}$ promote Th cell differentiation into Th2, upregulate the expression of Th2 cytokines IL-4, IL-5, and IL-13, and activate inflammatory cells (Fig. 1). Th2 cells are known to activate inflammatory processes after allergen challenge, resulting in eosinophil infiltration and abundant mucus secretion in asthmatic mice (Fig. S2, S3). In the pathogenesis of asthma, eosinophils serve as terminal effector cells and active regulators of the Th2 immune response. Eosinophils have been demonstrated to induce T cell proliferation and promote effector T cell recruitment to the lung in response to allergen challenge (Jacobsen et al., 2015). Medium and high concentrations of $PM_{2.5}$ inhibited the expression of IFN- γ (secreted by Th1 cells) in lungs. As IFN- γ did not suppress cellular immunological responses, OVA-specific IgE levels were elevated in the serum of mice (Fig. S5). Th1/Th2 imbalance and OVA-specific IgE expression are important markers of asthma, further confirming that medium and high concentrations of $PM_{2.5}$ cause asthma in mice. It is noteworthy that, although exposure to low concentrations of $PM_{2.5}$ did not induce asthma in mice, cytokines (IL-4, IL-13 and IFN- γ) and chemokines (IL-8 and IL-1 β) were affected to some extent, although not significantly.

Here, we studied the effects of exposure to various concentrations of $PM_{2.5}$ on the metabolites in the lung tissue of mice using GC-MS combined techniques. Multivariate statistical analysis was used to screen 13 different metabolites that could be used as markers of $PM_{2.5}$ and potential biomarkers for the induction of acute asthma. Through the Meta Mapp Software pathway analysis platform impact values and $-\log(p)$, four major metabolic pathways were screened for the distribution of these 13 differential metabolites (Fig. 5).

4.2. The tricarboxylic acid cycle

The TCA cycle is the metabolic pathway for the three major nutrients (sugars, lipids, and amino acids), and the most effective way to oxidize sugar or other substances to obtain energy. The first step in the TCA is the conversion of glucose or fatty acids to acetyl-CoA, followed by the condensation of acetyl-CoA with oxaloacetic acid to citric acid, dehydrogenation of citric acid and decarboxylation to α -ketoglutarate; α -ketoglutarate is then dehydrogenated and decarboxylated to produce malic acid. Malic acid produces oxaloacetate under the action of malate dehydrogenase. Repeated reuse of oxaloacetate results in consumption of acetyl-CoA (Luo et al., 2007; Adams et al., 2009; Olsvik et al., 2017; Springsteen et al., 2018). The experimental results show that the malic acid



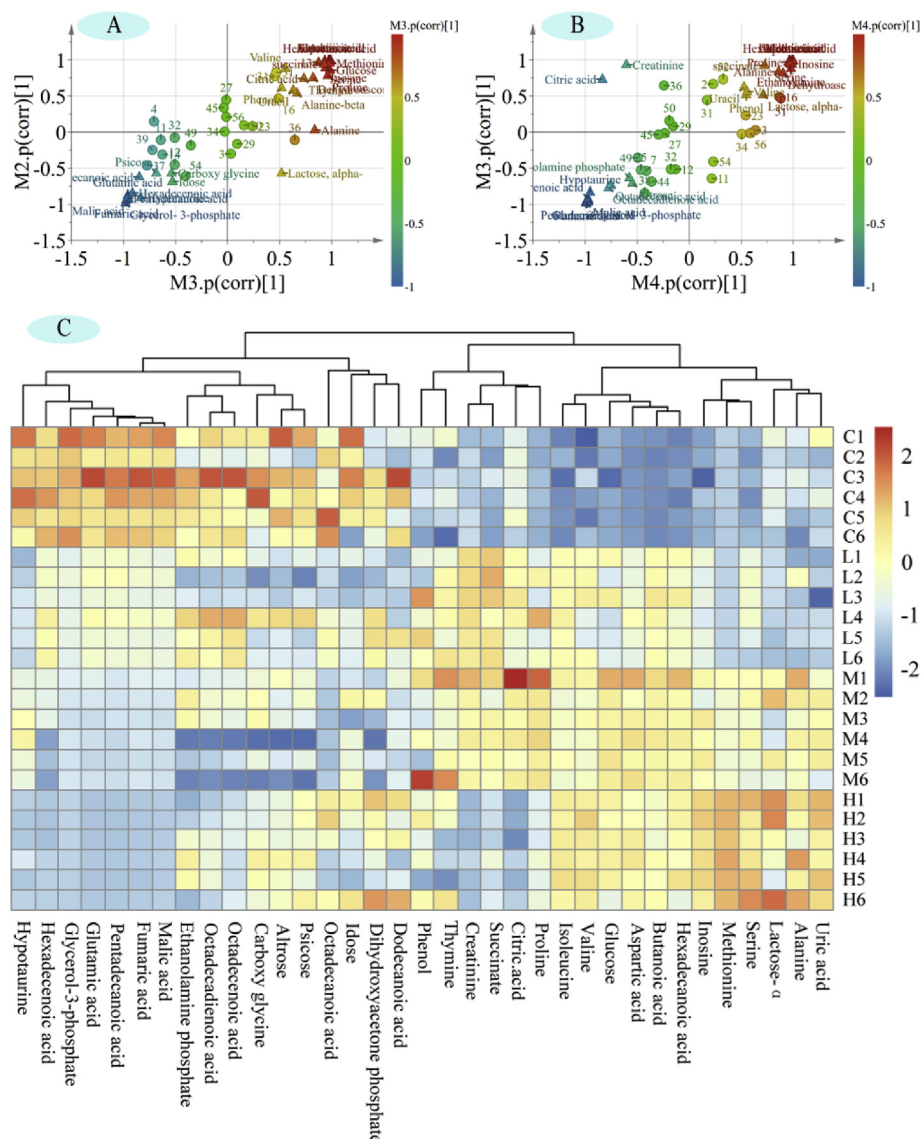


Fig. 3. Screening and variation characteristics of various metabolites in lung tissue exposed to PM_{2.5} at different concentrations. (A) SUS-plot for mice subjected to medium-concentration PM_{2.5} exposure and low-concentration PM_{2.5} exposure. Circle: $M3.p(corr) < 0.5$, $M2.p(corr) < 0.5$. Triangle: $M3.p(corr) \geq 0.5$, $M2.p(corr) \geq 0.5$. (B) SUS-plot for mice subjected to high concentration-PM_{2.5} exposure and medium concentration-PM_{2.5} exposure. Circle: $M4.p(corr) < 0.5$, $M3.p(corr) < 0.5$. Triangle: $M4.p(corr) \geq 0.5$, $M3.p(corr) \geq 0.5$. (C) Heat map, the data set was screened using variable importance for the projection (VIP) values > 1 and $|p(corr)| \geq 0.5$, in the lung metabolites.

and fumaric acid content decreased with increasing PM_{2.5} concentration, whereas the content of citric acid increased with exposure to PM_{2.5}. OVA-specific IgE induces smooth muscle contraction and increases vascular contractility, which requires large amounts of energy. Thus, consumption of high amounts of energy caused accelerated oxidative activity in the tricarboxylic acid cycle, which coincided with the increase in OVA-specific IgE. Similar reports on changes in citric acid and malic acid have been published (Seo et al., 2017). However, unlike other findings, we detected no other TCA intermediates (oxaloacetate, cis-aconitate,

and 2-oxoglutarate) possibly because of the different organ studied. The significant increase in glucose is also different from that in previous reports, possibly because of the difference in PM_{2.5} exposure time. This experiment only caused acute asthma in mice. In the early stage of acute asthma, the body self-regulates the production of a large amount of glucose to meet metabolic needs. More importantly, we also found a large amount of aspartate enrichment in the M and L groups. It has been reported in related literature that oxaloacetate can also be converted to aspartic acid when TCA is interfered by foreign substances (Tang et al., 2007;

Fig. 2. OPLS-DA analyses of the metabolite profile. The L series, M series, and H series show the OPLS-DA analysis of the lung tissue compared with the C group at low, mid, and high concentrations of PM_{2.5}, respectively. L-1, M-1, and H-1 represent OPLS-DA scores. L-2, M-2, and H-2 represent OPLS-DA model test chart, R^2 and Q^2 generated at any one time on the left end were less than the right end in 200 ordering experiments. The regression line of Q^2 intersected with the vertical axis and was below the zero point, and the slopes of the two regression lines are larger and are the rightmost ones. The small distance between the two points indicates that the predictive ability of the original model is greater than the ability to predict the y variable randomly at any one time, i.e., the model is valid, and a subsequent search for differential metabolites can be continued. L-3, M-3, and H-3 OPLS-DA S-plots obtained for the respective pairwise comparison with the variables colored according to their VIP value; metabolites showing differences (represented by triangles) were determined by a combined analysis of S-plot maps and Variable Importance on Projection (VIP) (VIP > 1).

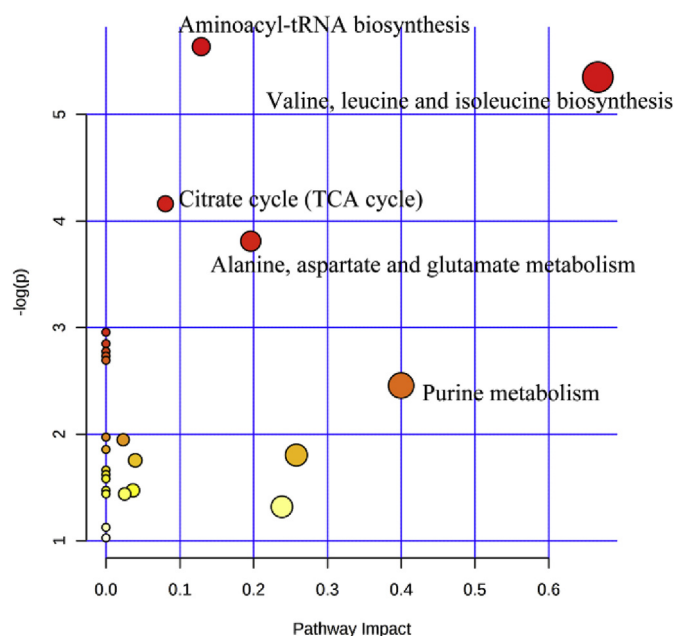


Fig. 4. Pathway analysis of the identified metabolites affected in the four groups; the abscissa of the pathway impact analysis in the figure indicates the importance of metabolic pathways calculated by topological analysis; the ordinate $-\log(P)$ indicates the significance level of the metabolic pathway enrichment analysis. In this study, metabolic pathway effects were set at 0.10, wherein the metabolic pathways influence values above this value. Values of $p < 0.05$ indicate a potential target metabolic pathway.

Table 1
A correlation of statistical associations of metabolites to cytokines.

	IL-4	IL-5	IL-13	IL-1 β	IFN- γ	IL-8
Aspartic acid	0.81**	0.78**	0.72*	0.78**	-0.76*	0.59**
Butanoic acid	0.63*	0.56	0.52	0.54*	-0.65*	0.4
Glucose	0.72*	0.69*	0.61	0.7**	-0.75	0.47*
Hexadecanoic acid	0.675*	0.62	0.61	0.63*	-0.72	0.45
Idose	-0.28	-0.21	-0.21	-0.23	0.07	-0.13
Inosine	0.9**	0.82**	0.83**	0.82**	-0.76*	0.71**
Isoleucine	0.76**	0.64	0.68*	0.62*	-0.69	0.63
Malic acid	-0.85**	-0.75**	-0.73**	-0.75*	0.71*	-0.62**
Octadecanoic acid	-0.29	-0.33	-0.18	-0.32	0.57	-0.16
Octadecenoic acid	-0.54	-0.53	-0.41	-0.53	0.51	-0.27
Serine	0.84**	0.78**	0.87**	0.73**	-0.75**	0.83*
Uric acid	0.7*	0.77**	0.76**	0.76*	-0.69*	0.68**
Valine	0.8**	0.67*	0.75**	0.65*	-0.66	0.63

Moderate correlations are values of -0.5 to -0.3 (negative correlations) or 0.3 to 0.5 ; strong correlations are values of -1.0 to -0.5 (negative correlations) or 0.5 to 1.0 .

*, $p < 0.05$. **, $p < 0.01$.

Badoud et al., 2014). This further proves that PM_{2.5} exposure stimulates the body to accelerate TCA metabolism.

4.3. Purine metabolic pathway

The second pathway identified was the purine metabolic pathway. Adenosine produced by the tricarboxylic acid cycle is converted to inosine by adenosine deaminase (Jin et al., 1997). Inosine, a key metabolite of purine metabolism, is oxidized and decomposed by hypoxanthine and xanthine to generate uric acid. Uric acid is the final oxidation product of purine metabolism (Ashihara et al., 2001). In the M and L groups, inosine and uric acid in the lungs of mice showed a significant upward trend suggesting that PM_{2.5} stimulates the expression of oxidase in xanthine

metabolism, thereby accelerating purine metabolism. Furthermore, related studies show that inosine is closely related to inflammatory reactions (Junqueira et al., 2017). More importantly, inflammatory cells are reported to induce ATP decomposition and accelerate adenosine formation (Yu et al., 2016). Thus, uric acid and inosine can be used as metabolic markers for PM_{2.5}-induced asthma. However, oxidases that serve as markers of purine metabolism are not known and need to be studied further.

4.4. The valine, leucine, and isoleucine catabolic pathway

The valine, leucine, and isoleucine catabolic pathway was the third pathway identified. This pathway, which is also known as branched-chain amino acid (BCAA) metabolism, involves valine, leucine, and isoleucine (Marchesini et al., 2003; Zhang et al., 2018). With the participation of branched chain aminotransferase (BCAT), BCAAs are converted to branched alpha-keto acids via the removal of their amino groups (leucine is converted to alpha-ketoisocaproate, proline to alpha-ketoisovalerate, and isoleucine to alpha-keto-beta-methylvalerate). Subsequently, these branched alpha-keto acids are decarboxylated by branched alpha-ketoacid dehydrogenase (BCKD); these BCAA metabolites are then broken down through a series of enzymatic reactions into final products (acetyl-CoA is derived from leucine, succinyl-CoA is derived from valine, and isoleucine is finally degraded to acetyl-CoA and succinyl-CoA), which finally enters the TCA cycle (Kimball and Jefferson, 2006; Ho et al., 2016). Groups M and L showed an enrichment in branched chain amino acids. This may be attributed to the following two reasons: First, in the M and L groups, the mice developed acute asthma; a large amount of ATP is required for smooth muscle contraction, which accelerates the TCA cycle, and the enrichment of BCAAs is thus an emergency for the mouse body reaction. Second, when the BCAAs are broken down to their corresponding products, the required enzymes are changed and cannot perform normal decomposition of amino acids. Consistent with these results, accelerated metabolism of BCAAs is reported to cause weight loss (Newgard, 2012). Therefore, the enzymes required for the metabolism of BCAAs need to be studied further.

4.5. The alanine, aspartate, and glutamate metabolic pathway

The alanine, aspartate, and glutamate metabolism pathway was the fourth pathway to be identified in our results. Oxaloacetate is an essential substance in the TCA cycle; however, it can also be directly converted to aspartate, which is ubiquitous in biosynthesis. Aspartate is a synthetic precursor of purine, pyrimidine bases, and fumaric acid in metabolic pathways (Sookoian and Pirola, 2012; Wang et al., 2013). Fumaric acid can inhibit the occurrence of allergic reactions. It has strong and long-lasting anti-allergic effects and inhibits the release of histamine from mast cells, eosinophils, and neutrophils (Nilsson et al., 2002). In this experiment, aspartic acid levels were elevated with an increase in PM_{2.5} and decrease in fumaric acid. Fumaric acid is also known to be associated with acute asthmatic inflammation, which was induced by PM_{2.5} in this study. Thus, this is the first report to show that the disease mechanism underlying the induction of asthma by PM_{2.5} exposure involves acceleration of the alanine, aspartate, and glutamate metabolic pathways.

In this study, 13 metabolic markers and four metabolic pathways were found to be involved, which provide a reference for the clinical treatment of acute asthma induced by PM_{2.5}. The foundation for the study of relationships between genes has been laid. Metabolites are end products downstream of proteins and genes. Future studies should aim to use the metabolomic analysis of PM_{2.5}-induced changes in asthmatic mice described here to clarify

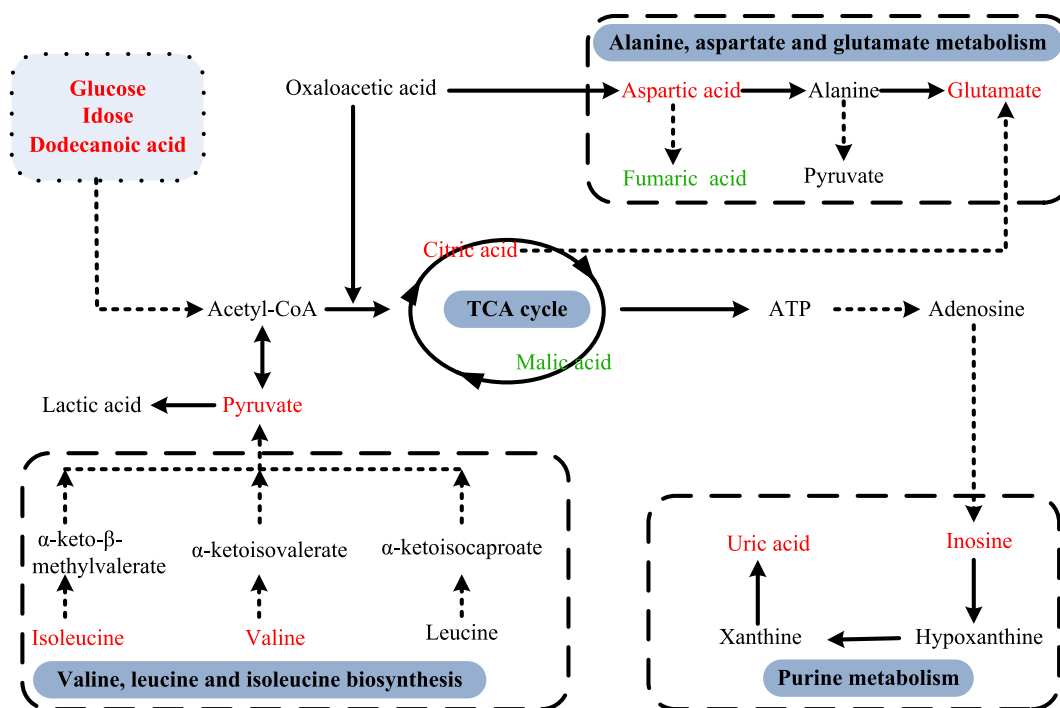


Fig. 5. The constructed metabolic network for PM_{2.5}-induced asthma; the metabolic pathways are shown in blue. Metabolites whose levels were increased with increasing concentration of PM_{2.5}, as determined by GC-MS, are shown in red. Metabolites whose levels decreased with increasing PM_{2.5} exposure, as indicated by GC-MS, are shown in green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the intrinsic relationship between the final metabolites and related genes and provide a basis for disease treatment.

5. Conclusion

The results in our study have shown that medium and high concentrations of PM_{2.5} induce acute asthma in mice. Based on GC-MS analysis of total metabolites in the lung tissue of mice, 13 metabolic variants with high VIP and $p < 0.05$ were screened as potential markers of acute asthma induced by PM_{2.5} exposure. Further, a comprehensive metabolic profile of acute asthma induced by PM_{2.5} was proposed. The spectrum involved four major metabolic pathways. These results provide a means to assess and treat asthma by examining marker metabolites associated with acute asthma induced by PM_{2.5} exposure, thus providing a reasonable basis for the understanding of PM_{2.5}-induced acute asthma and the underlying metabolic processes at the molecular level.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

<https://doi.org/10.1016/j.chemosphere.2018.12.092>.

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1 **Supplementary Material for**

2

3 **Identification of Multiple Dysregulated Metabolic Pathways by GC-MS-based**

4 **Profiling of Lung Tissue in Mice with PM_{2.5}-induced Asthma**

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21 **Supporting Information**

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26 **Fig. S1. Organ coefficients.**

27 **Fig. S2. Effects of PM_{2.5} on inflammatory cell recruitment to lung tissue.**

28 **Fig. S3. MUC5AC expression in lung tissue by Immunohistochemistry.**

29 **Fig. S4. MUC5AC protein expression in the lungs.**

30 **Fig. S5. Expression of OVA-specific IgE in serum in different PM_{2.5} groups.**

31 **Fig. S6. PCA plot and PCA plot for QC samples.**

32 **Fig. S7. The changes in these 13 metabolites.**

33 **Table S1. Deconvolution parameters of GC-MS.**

34 **Table S2. Changes in body weight during exposure to PM_{2.5} in mice**

35 **Table S3. Identification and selection of differentially expressed metabolites.**

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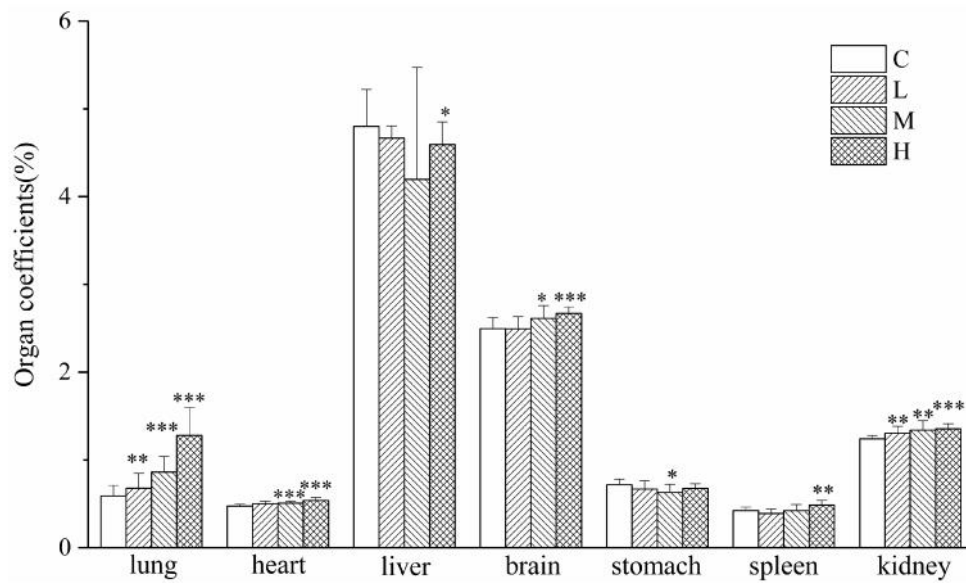
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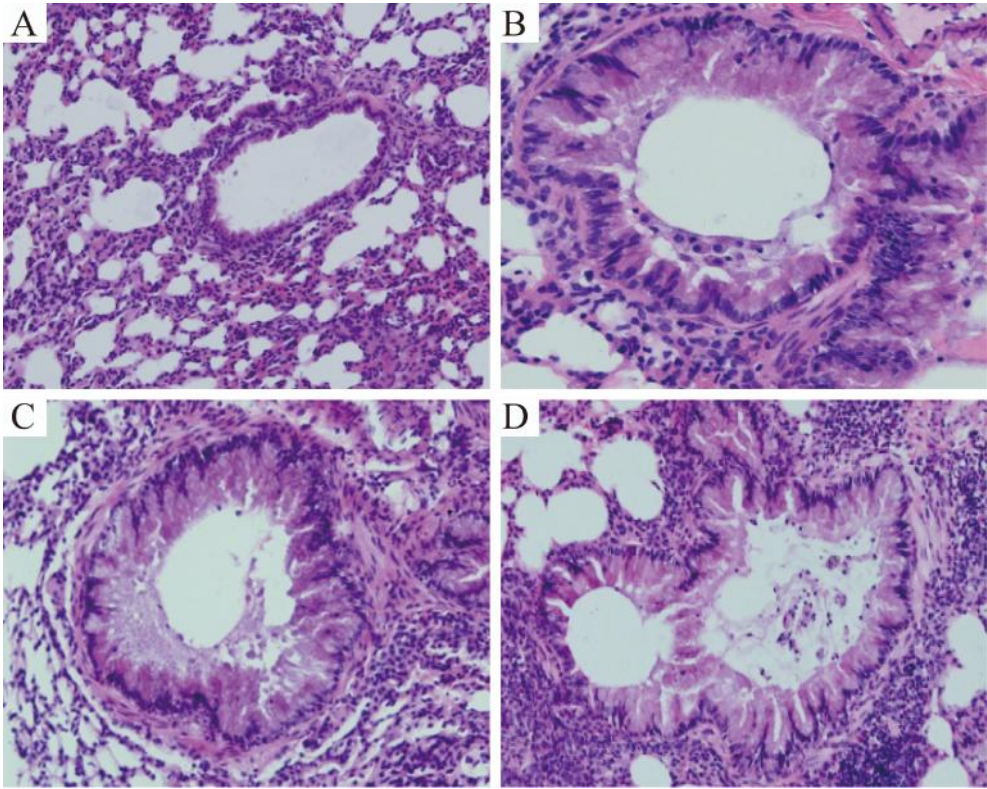
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44 **Fig. S1. Organ coefficients.**



46 Fig. S1. Organ coefficients. Mice were anesthetized and sacrificed for harvesting
 47 organs at 24 h after the final challenge. The data are expressed as the means $\pm$ SEM, n
 48 = 12 per group. *, **, ***P-values less than 0.05, 0.01, and 0.001, respectively,
 49 compared with the C group.

60 **Fig. S2. Effects of PM_{2.5} on inflammatory cell recruitment to lung tissue.**



61
62 Fig. S2. Effects of PM_{2.5} on inflammatory cell recruitment to lung tissue. HE staining
63 of mouse lung tissue to observe inflammation in bronchioles and alveoli
64 (magnification 400×). A: C group, B: L group, C: M group, D: H group.

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Fig. S3. MUC5AC expression in lung tissue by Immunohistochemistry.

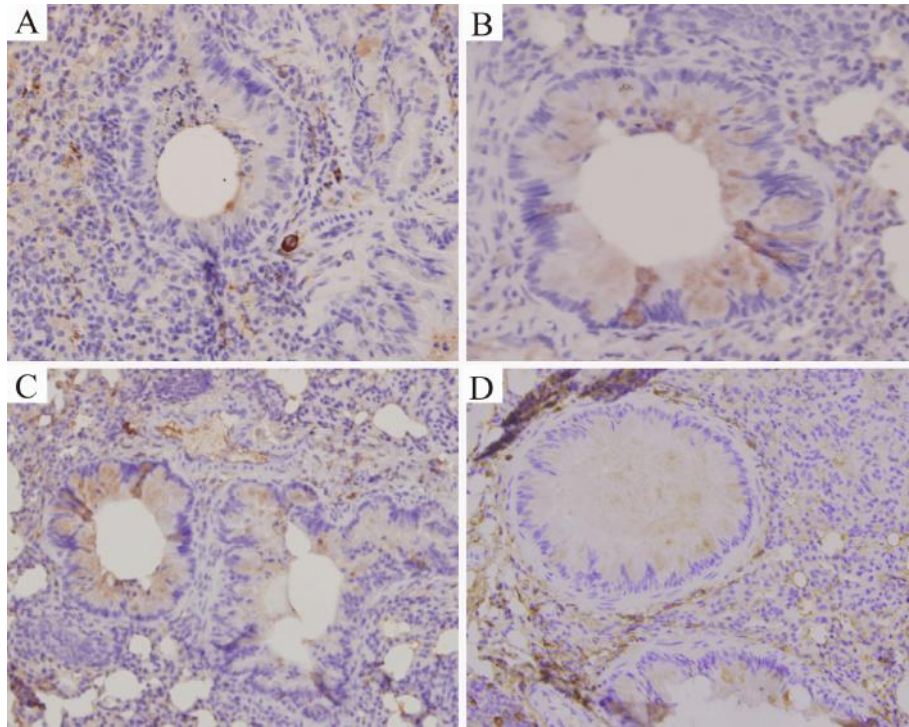
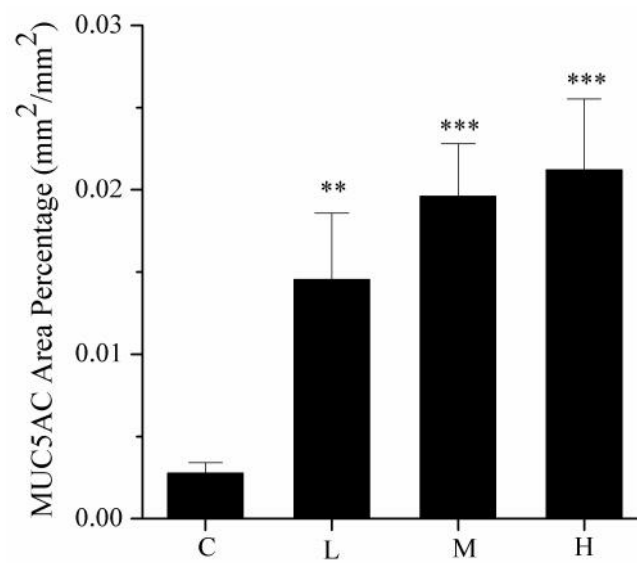


Fig. S3. Immunohistochemistry was used to observe MUC5AC expression in lung tissue. MUC5AC is mainly distributed in the bronchiolar epithelium and entosarc. Differential regulation of MUC5AC expression in lung tissue after exposure to different concentrations of PM_{2.5} in mice. All the images are shown at 400× magnification. A: C group, B: L group, C: M group, D: H group.

88 **Fig. S4. MUC5AC protein expression in the lungs.**



89
90 Fig. S4. Effects of PM_{2.5} exposure on MUC5AC protein expression in the lungs. The
91 values are expressed as the mean $\pm$ SEM, n = 6 per group. *, **, ***P-values less
92 than 0.05, 0.01, and 0.001, respectively, compared with the C group.

Fig. S5. Expression of OVA-specific IgE in serum in different PM_{2.5} groups.

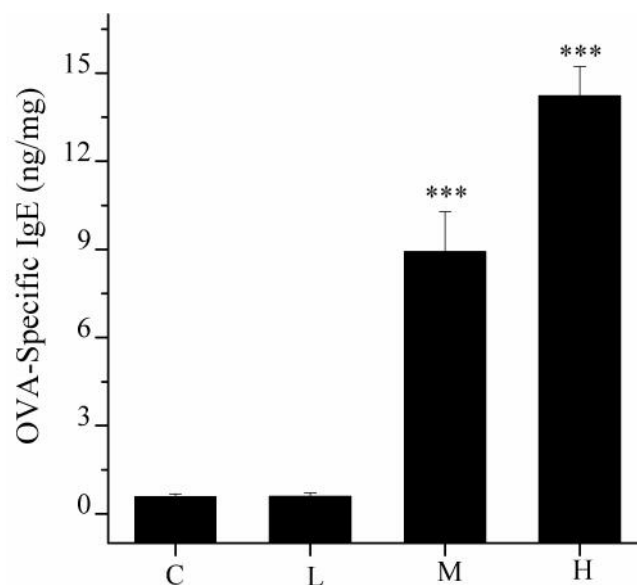


Fig. S5. Expression of OVA-specific IgE in serum after exposure to different concentrations of PM_{2.5}. Measurements were obtained using the mouse OVA-specific IgE ELISA kit and values were relative to the C group. The values are expressed as the mean $\pm$ SEM, n = 12 per group. ***P-values less than 0.001, compared with the C group.

Fig. S6. PCA plot and PCA plot for QC samples

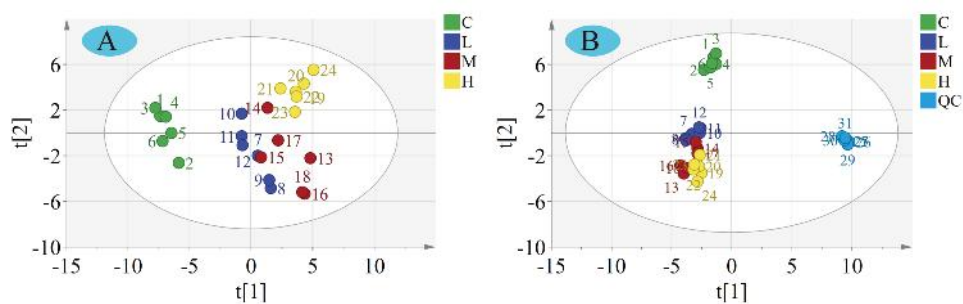


Fig. S6. 2D PCA score plot. (A) PCA score plot showing a separation of C group, L group, M group, and H group. (B) PCA score plot of QC samples showed no drift.

Fig. S7. The changes in these 13 metabolites.

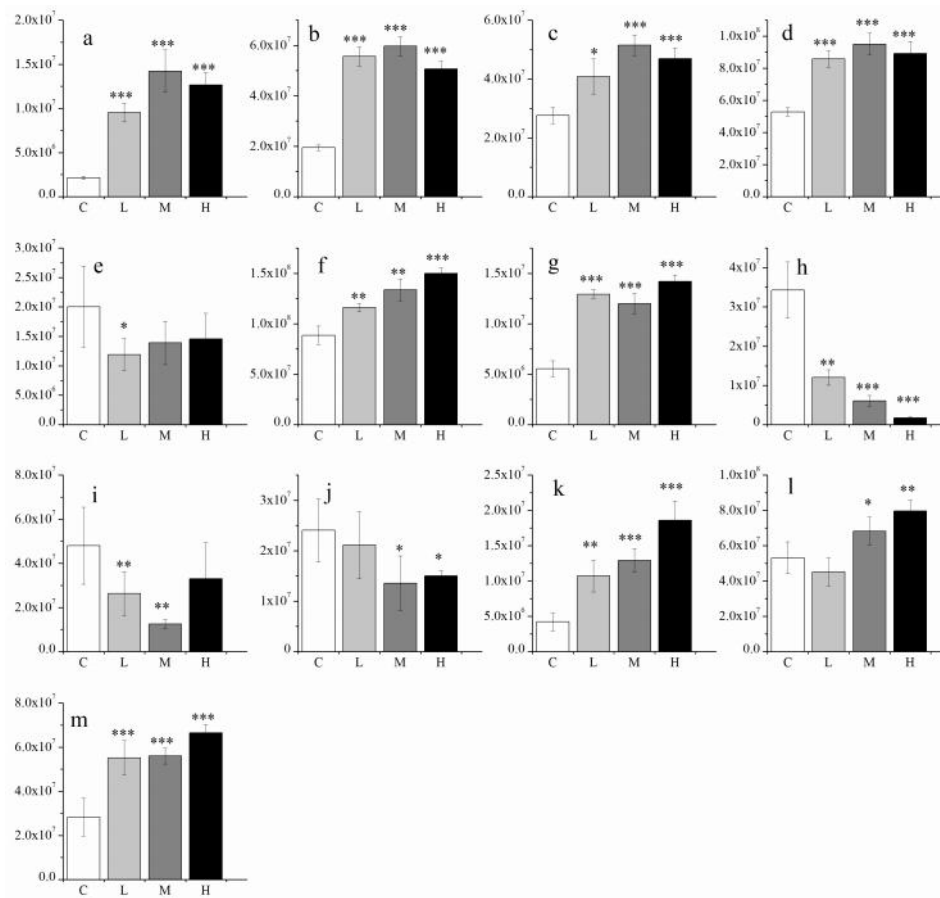


Fig. S7. Metabolites. a: Aspartic acid; b: Butanoic acid; c: Glucose; d: Hexadecanoic acid; e: Idose; f: Inosine; g: Isoleucine; h: Malic acid; i: Octadecanoic acid; j: Octadecenoic acid; k: Serine; l: Uric acid; m: Valine.

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Table S1. Deconvolution parameters of GC-MS

AMDIS Deconvolution Parameters	
Threshold	Low
m/z	50-650
Component width	12
Adjacent peak subtraction	1
Deconvolution Resolution	medium
Deconvolution Sensitivity	medium
Shape requirements	medium
Minimum match factor	60
Library	GMD-MS20111121-VAR5-ALK-MSL

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Table S2. Changes in body weight resulting from exposure to PM_{2.5} in mice

Group	8 th day (g)	15 th day (g)	22 th day (g)
C	19.88 ± 0.71	20.24 ± 0.76	20.76 ± 0.87
L	19.58 ± 0.55	20.03 ± 0.64	20.59 ± 0.79
M	19.82 ± 0.56	20.04 ± 0.42	19.91 ± 0.39*
H	19.73 ± 0.64	19.94 ± 0.47	19.71 ± 0.43**

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Table S3. Identification and selection of differentially expressed metabolites.

Metabolites	Low	Medium	High
	concentration-PM _{2.5}	concentration-PM _{2.5}	concentration-PM _{2.5}
	exposure vs.	exposure vs.	exposure vs. Control
	Control VIP	Control VIP	VIP
Aspartic acid	1.30 ^{***}	1.44 ^{***}	1.41 ^{***}
Butanoic acid	2.89 ^{***}	2.65 ^{***}	2.45 ^{***}
Glucose	1.64 [*]	2.05 ^{***}	1.90 ^{***}
Hexadecanoic acid	2.75 ^{***}	2.69 ^{***}	2.60 ^{***}
Idose	1.16 [*]	0.76	0.71
Inosine	2.43 ^{**}	2.78 ^{**}	3.43 ^{***}
Isoleucine	1.30 ^{***}	1.07 ^{***}	1.28 ^{***}
Malic acid	2.20 ^{**}	2.17 ^{***}	2.47 ^{***}
Octadecanoic acid	1.75 ^{**}	2.31 ^{**}	1.11
Octadecenoic acid	0.42	1.18 [*]	1.16 [*]
Serine	1.17 ^{**}	1.19 ^{***}	1.65 ^{***}
Uric acid	1.52 [*]	1.09 [*]	0.45
Valine	2.36 ^{***}	1.23 ^{**}	1.12 ^{**}

VIP was obtained from the OPLS-DA model with a threshold of 1.0. *, **, and

128 *** p -values less than 0.05, 0.01, and 0.001 by respectively, compared with the C
129 group using t -test. $n = 6$ per group.

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