



Cite this: *Mol. BioSyst.*, 2017, 13, 1172

Metabolic profiling of asthma in mice and the interventional effects of SPA using liquid chromatography and Q-TOF mass spectrometry†

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Asthma is a chronic inflammatory lung disease that leads to 250 000 deaths annually. There is a need to better understand asthma by identifying new pathogenic molecules. We conducted a liquid-chromatography time-of-flight mass spectrometry (LC-Q-TOF-MS)-based metabolomics study to test for asthma and investigate the interventional mechanisms of surfactant protein A (SPA) in OVA-induced asthma mice. The results revealed that asthma disturbed 32 metabolites in 9 metabolic pathways. After SPA treatment, the metabolomics profile found in asthma was significantly reversed, shifting much closer to that of the control group, indicating that SPA has therapeutic effects against asthma. Metabolomic pathway analysis by the ingenuity pathway analysis demonstrated that several pathways including fatty acid metabolism, lipid metabolism, and purine metabolism were significantly altered in asthma. This study offers new methodologies for the understanding of asthma and the mechanisms of SPA in treating asthma.

Received 11th January 2017,
Accepted 10th April 2017

DOI: 10.1039/c7mb00025a

rsc.li/molecular-biosystems

1 Introduction

Asthma is a chronic inflammatory lung disease affecting over 300 million people worldwide, and this number has been estimated to increase to 400 million by the year 2025.¹ Asthma leads to over 250 000 deaths annually worldwide. Therefore, there is a need to better understand asthma by identifying new biomarkers, therapeutic targets, and pharmacological agents to reduce the rapidly increasing morbidity and mortality related to the disease.

Surfactant protein A (SPA) is one of the predominant immune proteins in the airspaces. As a natural immune molecule belonging to the lectin family, SPA plays a role in the regulation of natural and adoptive immunotherapy of cells.² SPA also regulates the production of inflammatory cytokines and reactive species; moreover, it is thought to mediate a balance between upregulating inflammatory mediators to protect the host while preventing an exaggerated inflammatory response that could damage the delicate lung epithelium and impair gas exchange.^{3,4} In the pulmonary mucosa surface, pulmonary surfactant can reduce the surface tension of the liquid removal function, which is also involved in the maintenance of mucosal immune balance.

SPA can inhibit the maturation and antigen presentation function of dendritic cells.⁵ In addition, it can directly inhibit the proliferation of T cells by inducing the expression of regulatory cytokines of TGF- β .⁶ A large amount of evidence suggests that the decrease or inactivation of SPA may lead to the decrease of its protective effects.^{7,8} A number of clinical and experimental studies have also found that SPA is decreased^{9,10} or functionally inactivated in asthmatic patients and asthmatic animal models.¹¹ Therefore, SPA may be a potential therapeutic agent against asthma.

Metabolomics is the comprehensive analysis of small molecule metabolites in cells, tissues, organs, or organisms, being influenced by genetic modifications, pathological stimuli, or the environment.¹² Metabolomics has been proven to be an acceptable and reproducible platform, capable of capturing key molecular signatures and characteristics of diseases at different stages and progression.^{13,14} Metabolomics has also been used for asthma studies, which might offer novel and unique insights to understand asthma. To date, there have been several studies reporting the use of metabolomics in the discovery of diagnostic biomarkers in urine or exhaled breath condensate of patients with asthma.^{15–18} In these studies, subjects with asthma were discriminated from healthy populations, and some potential disease-relevant urinary biomarkers were identified. However, these studies are mainly useful for diagnostic purposes for identifying new biomarkers in the urine or breath. Although it is possible to obtain metabolomics data from many different sources (such as serum, urine, and exhaled breath condensate), serum is clinically particularly useful as it is a readily

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7mb00025a

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available and informative biofluid. In the search for insights into the mechanism of disease occurrence, it is important to detect as many molecules as possible. Compared to urine or exhaled breath condensate, there are more molecules in serum. Metabolite profiles of serum can be regarded as important indicators in the understanding of the mechanism of disease occurrence and can provide information for the identification of early and differential metabolic markers of a disease.

In this study, we employed an integrated approach of combining liquid chromatography (LC) mass spectrometry (MS) metabolomics methods to analyze the serum from experimental asthma. We further investigated the effects of SPA on the metabolic profiles of serum in the murine model of asthma. Our findings revealed substantial alterations in energy, lipid, and fatty acid metabolism in the serum obtained from an experimental model of asthma as well as potentially important metabolites for the characterization of asthma. We further demonstrated that SPA was able to reverse many of these changes in the serum metabolome in experimental asthma.

2 Materials and methods

2.1 Chemicals and reagents

LC-MS grade acetonitrile and methanol were purchased from Honeywell Burdick and Jackson (Muskegon, MI, USA). Mass spectroscopic grade formic acid was purchased from Fluka (Buchs, Switzerland). Ultra-pure water was obtained using a Milli-Q system from Millipore (Bedford, MA, USA). Reference standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Animals

Herein, thirty-six female BALB/c mice were purchased from the Nanjing branch of Beijing Weitonglihua Laboratory animal facility (permission number: SCXK-SU-2013-0005). All the animals were maintained under specific-pathogen-free conditions with a light/dark cycle of 12 h, had access to sterilized water and a standard laboratory diet *ad libitum*, and were used when aged 6–8 weeks and weighing 18 ± 2 g.

In the present study, the mice were randomly divided into a model, control group, and SPA groups ($n = 12$, per group). For the asthma group ($n = 12$), the immunization protocol was performed as follows: BALB/c mice were sensitized and challenged with ovalbumin (OVA) to develop allergic airway inflammation. On day 0 (day of first injection) and 14, mice were abdominally injected with $10 \mu\text{l}$ 10% chicken ovalbumin (OVA) (purity $\geq 98\%$, Sigma-Aldrich) in $250 \mu\text{l}$ 1% aluminum hydroxide $\text{Al}(\text{OH})_3$. On days 14, 25, 26, and 27, mice were anesthetized with an abdominal injection of 0.25% sodium pentobarbital (50 mg kg^{-1}) in normal saline, followed by an intranasal (i.n.) dose of $100 \mu\text{g}$ OVA in $30 \mu\text{l}$ phosphate-buffered saline (PBS). For the SPA groups, in addition to the immunization protocol described above, SPA ($10 \mu\text{g kg}^{-1}$, Cloud-Clone Corp., Houston, TX) dissolved in saline was intranasally injected on day 24 before a continuous OVA challenge from day 25 to 27. The corresponding control groups were intranasally injected with PBS only. All animals were euthanized on day 28. The study protocol was approved by the local institutional review board at the authors' affiliated institutions, and animal experiments were performed in compliance with the relevant laws and the institutional guidelines for the Animal Care and Use Committee of the Second Military Medical University.

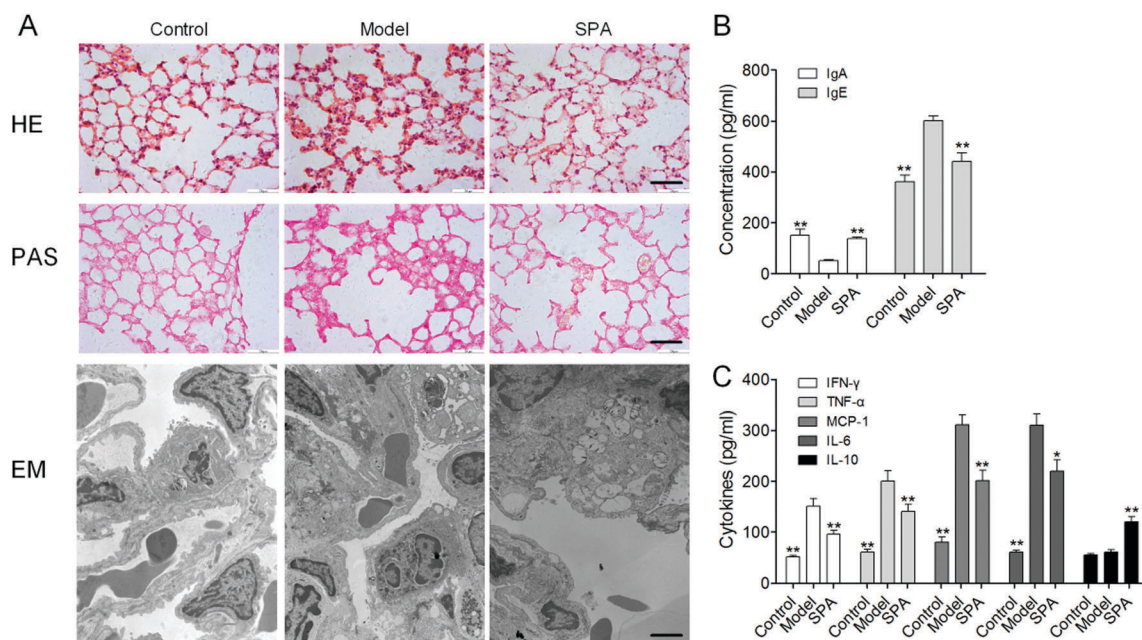


Fig. 1 SPA protects from OVA-induced allergic asthma in mice. Mice sensitized with OVA received SPA after the OVA challenge. (A) Tissue inflammation and global cell metaplasia analyzed on HE (magnification $\times 200$; scale bar = $50 \mu\text{m}$), PAS-stained (magnification $\times 200$; scale bar = $50 \mu\text{m}$) and lung sections by light microscopy electron microscope (magnification $\times 4000$; scale bar = $2 \mu\text{m}$). (B) Serum level of IgA and IgE were determined by ELISA. (C) Cytokine expression in serum. * $P < 0.05$ and ** $P < 0.01$ compared with OVA-induced allergic asthma mice ($n = 6$).

2.3 Sample collection

All animals were euthanized on the 28th day after a 10 h fast prior to euthanasia, and 0.5 ml blood samples were obtained. Serum was separated from entire blood *via* centrifugation at 3000 rpm at 4 °C for 10 min, and then, the serum was frozen at −80 °C for storage. Thawed once, 100 µl of the serum was added to 300 µl methanol (with the internal standard 2-chlorophenylalanine, 5 µg ml^{−1}), and then, the mixture was vigorously stirred for 3 min. After centrifugation at 13 000 rpm for 15 min at 4 °C, 150 µl of supernatant and the mixture was stored at −80 °C until analysis. Lung tissue samples were immediately removed, washed with physiological saline, and blotted dry. The right lobes of six lungs from each group were isolated for histopathological examination, and the others were frozen at 80 °C for subsequent processing.

2.4 Histopathology staining and ELISA

IgA, IgE, IFN-γ, TNF-α, MCP-1, IL-6, and IL-10 levels were assayed according to the manufacturer's instructions (eBioscience, San Diego, CA).

Lobules of lungs from 6 mice randomly selected from each group were fixed in 4% neutral buffered paraformaldehyde, dehydrated for 12 h, embedded in paraffin wax, cut into 3 µm-thick slices, and then examined *via* light microscopy (Olympus, Tokyo, Japan) for hematoxylin-eosin (HE) and periodic acid-Schiff stain (PAS); they were also examined using an

electron microscope (Hitachi Co. Ltd, Tokyo, Japan), operated at 75 kV.

2.5 LC-Q-TOF/MS

Serum metabolite profiling was performed using an Agilent 1290 infinity liquid chromatography system equipped with an Agilent 6538 Accurate-Mass QTOF/MS (Agilent Technologies, Santa Clara, CA, USA). Separation of all the samples was performed using an ACQUITY UPLC@HSS T3 C18 column (2.5 µm, 2.1 mm × 100 mm, Waters) with the column temperature set at 25 °C. The C18 column is not suitable for the separation of most polar small molecules because they will be almost simultaneously eluted from the column. It is ideal to use a HILIC-based column and C18 column for the separation of both non-polar compounds and polar compounds. The flow rate was 0.4 ml per min, and the mobile phase consisted of ultrapure water with 0.1% formic acid and acetonitrile. The following gradient program was used: 5% acetonitrile for 0–2 min; 5–95% acetonitrile for 2–13 min; 95% acetonitrile for 13–15 min; and re-equilibration step for 5 min. The sample injection volume was 3 µl.

Mass detection was performed in both the positive and negative ion modes with the following setting: drying gas (N₂) flow rate, 11 l min^{−1}; gas temperature, 350 °C; pressure of nebulizer gas, 45 psig; Vcap, 4000 V (−3000 V); fragmentor, 120 V; skimmer, 60 V; and scan range, *m/z* 80–1000. All the analyses were carried out using the instrument mass spray to ensure accuracy and reproducibility.

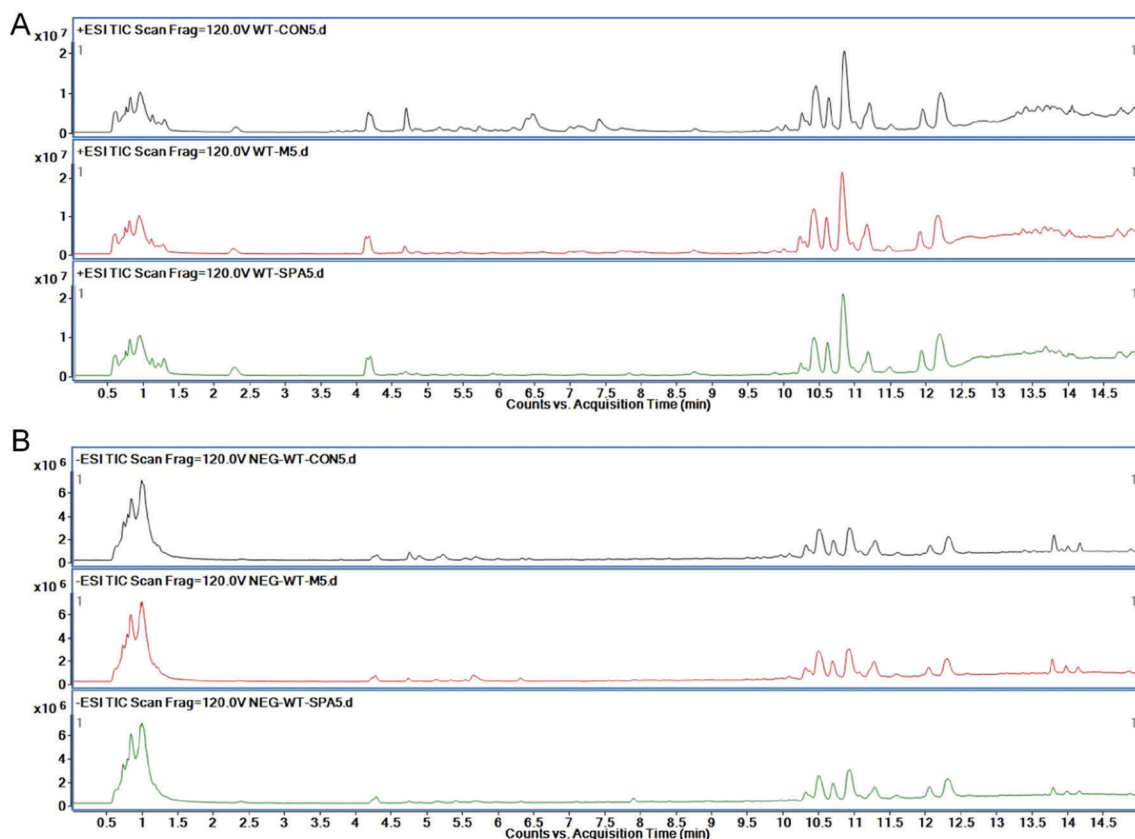


Fig. 2 The total ion chromatogram of control group and model group, SPA group (A) ESI+; (B) ESI−.

Leucine enkephalin was used as the instrument reference mass (m/z 121.0509, 922.0098 in ES+ and 119.0363, 966.0007 in ES−) at the concentration of 100 ng ml^{−1} for all the analyses. The MS/MS analysis was carried out in the targeted MS/MS mode with the collision energy ranging from 10 eV to 40 eV.

2.6 Sequence analysis

Serum samples were run in random order, and the pooled quality control (QC) sample was analyzed for every 6 serum samples. If differences were detected between QC control results (either previous results or within the analysis), the analysis was stopped, the ionization source and the column were cleaned, and the mass spectrometer was recalibrated. Additional samples were not analyzed until the QC analyses were satisfactory.

2.7 Data processing and identification of the metabolites

For metabolomics analysis, spectrometric data were converted to mzData formats *via* Agilent Masshunter Qualitative Analysis B.04.00 software (Agilent Technologies, USA) and input to XCMS for peak finding, peak alignment, and peak normalization across all the samples. The excel table was exported into the SIMCA-P software (version 13.0; Umetrics AB, Umea, Sweden) for multivariate data analysis. Data sets were mean-centered and Par-scaled to adjust the importance of high and low-abundance metabolites to an equal level before carrying out

the statistical analysis. Unsupervised multivariate analysis principle component analysis (PCA) was first performed on LC-MS data as an unbiased statistical method to observe intrinsic trends. To achieve the maximum separation among the groups, supervised multivariate analysis partial least-squares discriminant analysis (PLS-DA) was applied. The goodness of the fit was quantified by R^2Y , while the predictive ability was indicated by Q^2 . Comparison of serum metabolic profiles from the asthma model, control, and SPA groups revealed substantial differences in the PLS-DA score plot (Fig. 3). Potential differential metabolites were selected according to the Variable Importance in the Projection (VIP) values, and the variables with $VIP > 1$ were considered to be influential for the separation of the samples. In addition, the student test was performed to determine if the differential metabolites obtained from PLS-DA modeling were statistically significant ($P < 0.05$) among the groups at the univariate analysis level. All the results were presented as mean $\pm$ SEM, multiple comparisons were carried out by one-way ANOVA analysis of variance followed by Dunnett's test, and $P < 0.05$ was considered statistically significant. The structures of the potential biomarkers were elucidated using the high-resolution MS and MS/MS spectra and by searching various databases including KEGG (<http://www.genome.jp/kegg>) and HMDB (<http://www.hmdb.ca>). The MSMS data has been provided as ESI⁺ Pathway analysis and

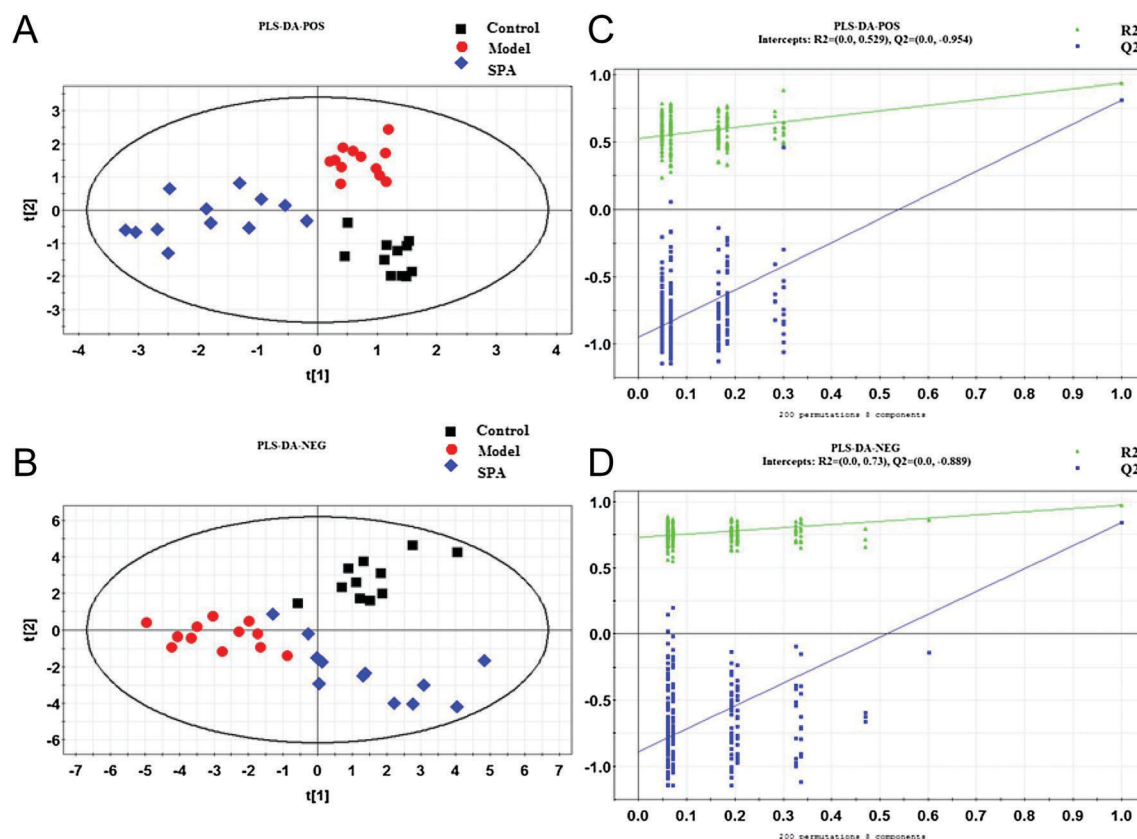


Fig. 3 PLS-DA score plot (■) Control group, (●) Model group, (◆) SPA group. (A) POS ($R^2X = 0.366$, $R^2Y = 0.828$, and $Q^2 = 0.785$); (B) NEG ($R^2X = 0.837$, $R^2Y = 0.974$, and $Q^2 = 0.829$); (C) POS, a 999-times permutation test for the corresponding model. The Y-axis intercepts were: R^2 (0, 0.529), Q^2 (0, −0.954). (D) NEG, a 999-times permutation test for the corresponding model. The Y-axis intercepts were: R^2 (0, 0.73), Q^2 (0, −0.889).

Table 1 The potential biomarkers of OVA-induced asthma detected by UPLC-Q-TOF/MS and their variation tendency

No.	<i>m/z</i>	RT (min)	VIP	<i>P</i> (MC)	FC (C/M)	<i>P</i> (MS)	FC (S/M)	Name	Formula	Adduct	Trend		Related pathway
											(M/C)	(S/M)	
1	118.086	0.88	2.5	2.71×10^{-5}	0.75	5.91×10^{-3}	1.19	Valine	C ₃ H ₁₁ NO ₂	[M + H] ⁺	↑ ↓	Valine, leucine and isoleucine degradation	
2	296.067	0.73	1.61	4.17×10^{-5}	1.43	1.77×10^{-1}	0.87	5-Aminoimidazole ribonucleotide	C ₈ H ₁₄ N ₃ O ₇ P	[M + H] ⁺	↓ ↑	Purine metabolism	
3	348.071	1.13	1.28	8.61×10^{-4}	0.63	1.17×10^{-2}	1.39	Adenosine monophosphate	C ₁₀ H ₁₄ N ₅ O ₇ P	[M + H] ⁺	↑ ↓	Purine metabolism	
4	165.055	1.26	1.12	8.81×10^{-3}	0.74	4.72×10^{-2}	1.25	Phenylpyruvic acid	C ₉ H ₈ O ₃	[M + H] ⁺	↑ ↓	Phenylalanine metabolism	
5	544.34	10.49	9.49	3.90×10^{-6}	0.74	2.92×10^{-8}	1.52	LysoPC(20:4)	C ₂₈ H ₅₀ NO ₇ P	[M + H] ⁺	↑ ↓	Lipid metabolism	
6	570.356	10.91	2.77	2.68×10^{-7}	0.28	1.19×10^{-4}	1.92	LysoPC(22:5)	C ₃₀ H ₅₂ NO ₇ P	[M + H] ⁺	↑ ↓	Lipid metabolism	
7	480.309	10.93	1.16	1.07×10^{-6}	1.18	1.87×10^{-5}	0.88	LysoPC(15:0)	C ₂₂ H ₄₈ NO ₇ P	[M - H] ⁻	↑ ↓	Lipid metabolism	
8	548.372	11.54	2.26	1.40×10^{-2}	0.75	2.83×10^{-4}	1.59	LysoPC(20:2)	C ₂₈ H ₅₄ NO ₇ P	[M + H] ⁺	↑ ↓	Lipid metabolism	
9	546.354	12.21	1.53	9.65×10^{-3}	1.06	8.57×10^{-2}	0.96	LysoPC(20:3)	C ₂₈ H ₅₄ NO ₇ P	[M + H] ⁺	↑ ↓	Lipid metabolism	
10	572.369	12.47	1.41	1.78×10^{-4}	0.68	3.15×10^{-5}	1.54	LysoPC(22:4)	C ₃₀ H ₅₄ NO ₇ P	[M + H] ⁺	↑ ↓	Lipid metabolism	
11	500.277	9.88	1.61	5.11×10^{-9}	3.57	7.05×10^{-1}	0.85	LysoPE(0:0/20:5)	C ₂₅ H ₄₂ NO ₇ P	[M + H] ⁺	↑ ↓	Lipid metabolism	
12	504.309	10.5	1.07	3.10×10^{-6}	1.30	1.92×10^{-1}	0.92	LysoPE(0:0/20:2)	C ₂₅ H ₄₂ NO ₇ P	[M + H] ⁺	↑ ↓	Lipid metabolism	
13	536.371	11.79	1.47	1.43×10^{-4}	0.61	1.35×10^{-4}	1.61	LysoPE(0:0/22:1)	C ₂₇ H ₅₄ NO ₇ P	[M + H] ⁺	↑ ↓	Lipid metabolism	
14	568.362	12.17	4.33	3.25×10^{-4}	1.32	1.30×10^{-6}	0.69	PC(18:0/0:0)	C ₂₆ H ₅₄ NO ₇ P	[M + FA - H] ⁻	↑ ↓	Lipid metabolism	
15	524.372	12.2	10.85	1.07×10^{-4}	1.14	9.42×10^{-8}	0.83	PAF C-16	C ₂₆ H ₅₄ NO ₇ P	[M + H] ⁺	↑ ↓	Lipid metabolism	
16	508.341	12.3	1.09	5.14×10^{-9}	1.32	5.14×10^{-9}	0.76	LysoPE(20:0/0:0)	C ₂₅ H ₅₂ NO ₇ P	[M + H] ⁺	↑ ↓	Lipid metabolism	
17	550.388	12.47	2.26	2.54×10^{-3}	0.76	7.22×10^{-4}	1.37	PC(2:0/O-18:1)	C ₂₈ H ₅₆ NO ₇ P	[M + H] ⁺	↑ ↓	Lipid metabolism	
18	204.123	0.83	5.79	6.52×10^{-6}	1.61	6.21×10^{-1}	0.91	Acetylcarnitine	C ₉ H ₁₇ NO ₄	[M + H] ⁺	↑ ↓	Insulin resistance	
19	132.076	0.97	1.26	2.47×10^{-2}	1.15	7.20×10^{-1}	0.96	Creatine	C ₄ H ₉ N ₃ O ₂	[M + H] ⁺	↑ ↓	Glycine, serine and threonine metabolism	
20	218.139	1.45	1.31	1.61×10^{-3}	0.65	9.01×10^{-2}	1.25	Propionyl-L-carnitine	C ₁₀ H ₁₉ NO ₄	[M + H] ⁺	↑ ↓	Fatty acyls	
21	372.311	9.34	1.32	7.46×10^{-3}	1.56	3.71×10^{-1}	0.81	Myristoylcarnitine	C ₂₁ H ₄₁ NO ₄	[M + H] ⁺	↑ ↓	Fatty acyls	
22	398.327	9.59	1.62	2.51×10^{-4}	1.92	7.55×10^{-1}	0.87	<i>trans</i> -Hexadec-2-enoyl carnitine	C ₂₃ H ₄₃ NO ₄	[M + H] ⁺	↑ ↓	Fatty acyls	
23	426.358	10.37	2.6	1.25×10^{-2}	1.64	2.46×10^{-1}	0.75	Vaccenyl carnitine	C ₂₅ H ₄₇ NO ₄	[M + H] ⁺	↓ ↑	Fatty acyls	
24	400.343	10.16	2.81	2.42×10^{-4}	1.54	1.02×10^{-1}	0.81	Palmitoyl-L-carnitine	C ₂₃ H ₄₅ NO ₄	[M + H] ⁺	↑ ↓	Fatty acid metabolism	
25	277.218	13.5	1.8	1.28×10^{-5}	2.13	9.07×10^{-1}	0.93	Octadecatrienoic acid	C ₁₈ H ₃₀ O ₂	[M - H] ⁻	↑ ↓	Fatty acid metabolism	
26	279.233	14.15	2.96	4.16×10^{-3}	1.79	4.11×10^{-1}	0.78	Octadecadienoic acid	C ₁₈ H ₃₂ O ₂	[M - H] ⁻	↑ ↓	Fatty acid metabolism	
27	281.249	14.99	1.34	2.63×10^{-7}	0.13	1.27×10^{-5}	3.23	Octadecenoic acid	C ₁₈ H ₃₄ O ₂	[M - H] ⁻	↑ ↓	Fatty acid metabolism	
28	317.212	11.33	1.2	9.47×10^{-4}	7.14	6.03×10^{-1}	0.41	12-oxo-5Z,8Z,10E,14Z- eicosatetraenoic acid	C ₂₀ H ₃₀ O ₃	[M - H] ⁻	↑ ↓	Fatty acid metabolism	
29	301.218	13.38	3.18	5.11×10^{-9}	5.00	6.16×10^{-1}	0.76	Eicosapentaenoic acid	C ₂₀ H ₃₀ O ₂	[M - H] ⁻	↓ ↑	Biosynthesis of unsaturated fatty acids	
30	329.249	14.13	1.59	1.52×10^{-7}	4.55	7.58×10^{-1}	0.75	Docosapentaenoic acid	C ₂₂ H ₃₄ O ₂	[M - H] ⁻	↓ ↑	Biosynthesis of unsaturated fatty acids	
31	258.11	0.78	1.01	4.86×10^{-3}	1.30	8.63×10^{-2}	0.85	5-Methylcytidine	C ₁₀ H ₁₅ N ₃ O ₅	[M + H] ⁺	↓ ↑	Biosynthesis of amino acids	
32	116.071	0.79	1.24	2.39×10^{-4}	0.66	7.74×10^{-1}	1.05	Proline	C ₅ H ₉ NO ₂	[M + H] ⁺	↑ ↓	Arginine and proline metabolism	

M/C: model group compared to control group; S/M: SPA group compared to model group; ↑: up-regulated, ↓: down-regulated.

networks were presented by IPA software (IPA, Ingenuity® Systems, <http://www.ingenuity.com>).

3 Results

3.1 Histopathological changes in lungs of animals among different groups

The OVA-challenged mice showed marked infiltration of inflammatory cells into perivascular and connective tissues, lumen narrowing, and mucosa thickening, compared with the normal mice (Fig. 1A). These results demonstrate the success of the procedure of OVA-challenging in discriminating the model group from the normal group. The SPA group showed less infiltration of inflammatory cells than the model group, and SPA significantly decreased the abnormal alveolar structure, structural disorder, and cell debris and fibroblasts in the lung sections, as determined by electron microscopy observation (Fig. 1A). In addition, SPA treatment significantly reduced the IgA level and increased the IgE level (Fig. 1B). Moreover, the expression of cytokines IFN- γ , TNF- α , MCP-1, and IL-6 were significantly decreased after SPA treatment compared with that in the

asthma group, and anti-inflammatory factor IL-10 was significantly increased (Fig. 1C).

3.2 Original chromatogram based on LC-Q-TOF MS

LC-Q-TOF MS was applied to obtain the metabolic profiles of the serum of the asthma group, SPA group, and control group in the positive and negative ESI mode. The total ion current data obtained for each group for each ionization mode is shown in Fig. 2. The well-overlapping spectrum of all the samples (Fig. 2) demonstrated the excellent stability and reproducibility of chromatographic separation and mass measurement during the entire sequence.

3.3 Modeling the data

Pattern recognition analysis was used to model the data and reveal the differences between the asthma, SPA group, and control group. PCA, as an unsupervised multivariate analysis, could actually reflect the clustering circumstance of the samples. To track the specific changes between the three groups, these variables were performed with the supervised multivariate analysis PLS-DA. The scores of the PLS-DA plot (Fig. 3) showed

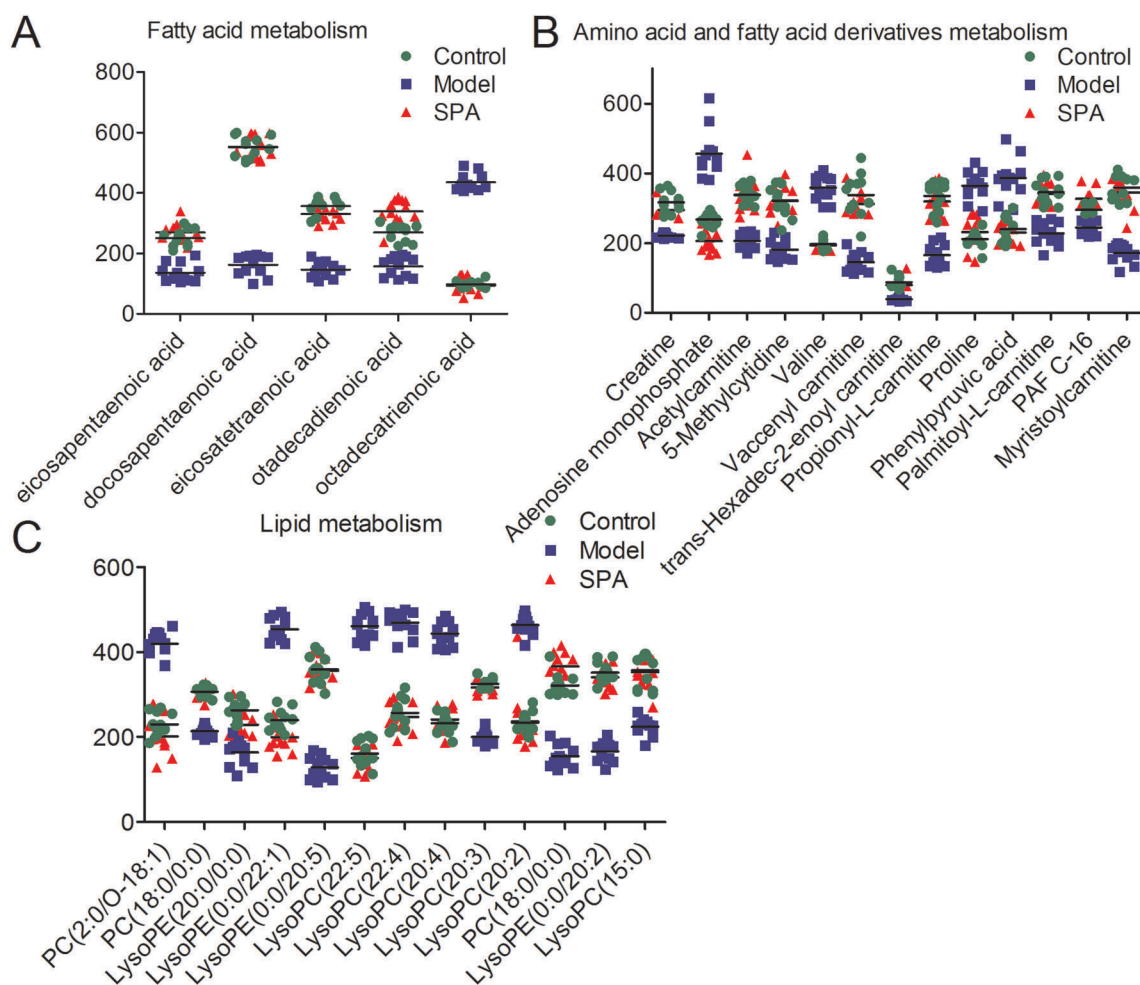


Fig. 4 Expression levels of the significantly changed metabolites. (A) Fatty acids metabolism; (B) amino acids and fatty acid derivatives metabolism; (C) lipids metabolism.

clearer separation between the healthy controls, the asthma model group, and the SPA group. Our model described 97.4% of the variation in the response Y ($R^2Y = 0.974$) and predicted 82.9% of the variation in the response Y ($Q^2 = 0.829$) for the two-component model.

3.4 Identification of potential biomarkers

To identify potential biomarkers, PLS-DA was finally chosen as an effective approach of filtering the unrelated variations. In this study, a variable with its VIP value larger than 1 was selected as the potential marker. Then, the T -test was employed in the following step to select significantly different variables ($P < 0.05$). In this way, 32 metabolites were found to significantly change in the serum of the asthma group, compared to those in the serum of the control group (Table 1). From the preliminary analysis, these differential metabolites were mainly attributable to small organic acids, amino acids, fatty acids, lipids, and other substances (Fig. 4).

As a result, 32 metabolites in serum samples were recognized as potential biomarkers related to OVA-induced asthma. They were eicosapentaenoic acid, octadecatrienoic acid, docosapentaenoic acid, octadecadienoic acid, LysoPC (22:6), LysoPC (20:4), LysoPE (0:0/20:2), LysoPC (22:5), LysoPC (15:0), LysoPC (20:2), LysoPE (0:0/22:1), and PC (18:0/0:0) (Fig. 5). Ingenuity pathway analysis and networks were presented by IPA software. The network was built based on the differentiated metabolites between the asthma and control groups (Fig. 6). The five top canonical pathways

include glycerophospholipid metabolism, fatty acid metabolism, purine metabolism, arginine and proline metabolism, and valine, leucine, and isoleucine degradation (Table 1).

4 Discussion

Metabolomics, especially MS-based metabolomics, is an emerging analytical tool in human disease research, due to its high sensitivity and capability to simultaneously measure many metabolic biomarkers in a single-biological sample.¹⁹ Recent studies have used metabolomics to analyze the urine or breath condensate of patients with asthma and demonstrated that this approach could offer opportunities for disease-related biomarker discovery.^{20–23}

In this study, serum derived from OVA-induced asthma group was analyzed using an LC-MS-based metabolomics approach. We hypothesized that asthmatic conditions would lead to alteration of metabolites in the asthma group, which can be reflected in the serum. SPA was administered to the model group to investigate the effects of reversion of the disorder in the serum metabolism induced by asthma. Our results indicated that the serum metabolic signatures of the OVA-induced asthma and control group were different. Moreover, there were major alterations in the model of asthma that were substantially reversed by SPA, suggesting that they are disease-relevant metabolic changes. As is well known, infection is an important cause of asthma as microorganisms not only consume SPA, but also cause the apoptosis of alveolar type II epithelial cells that secrete SPA,

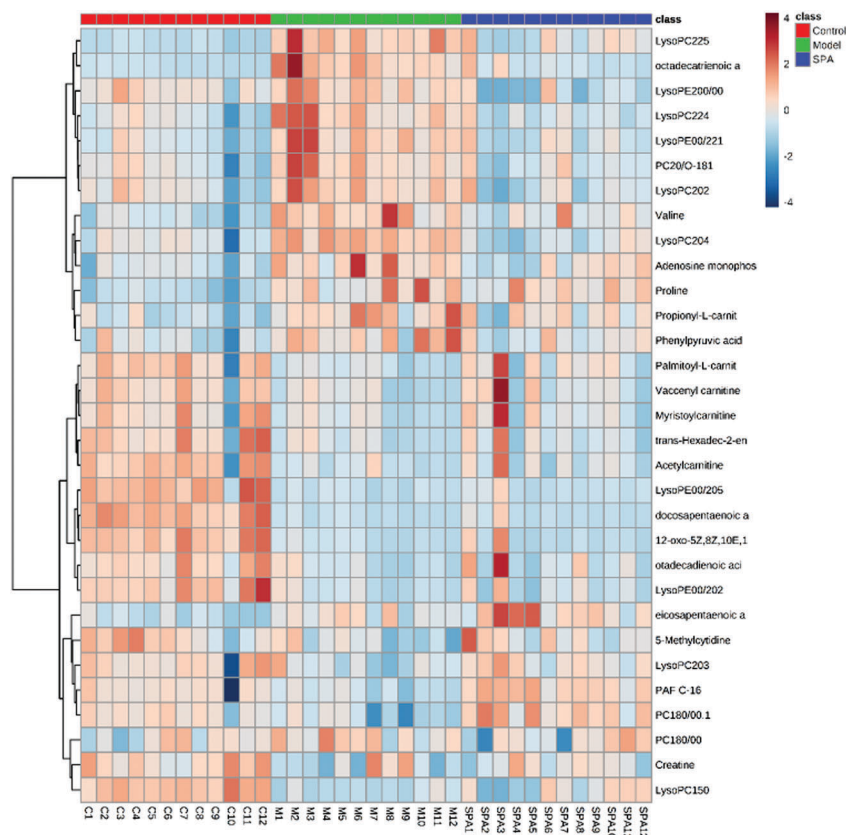
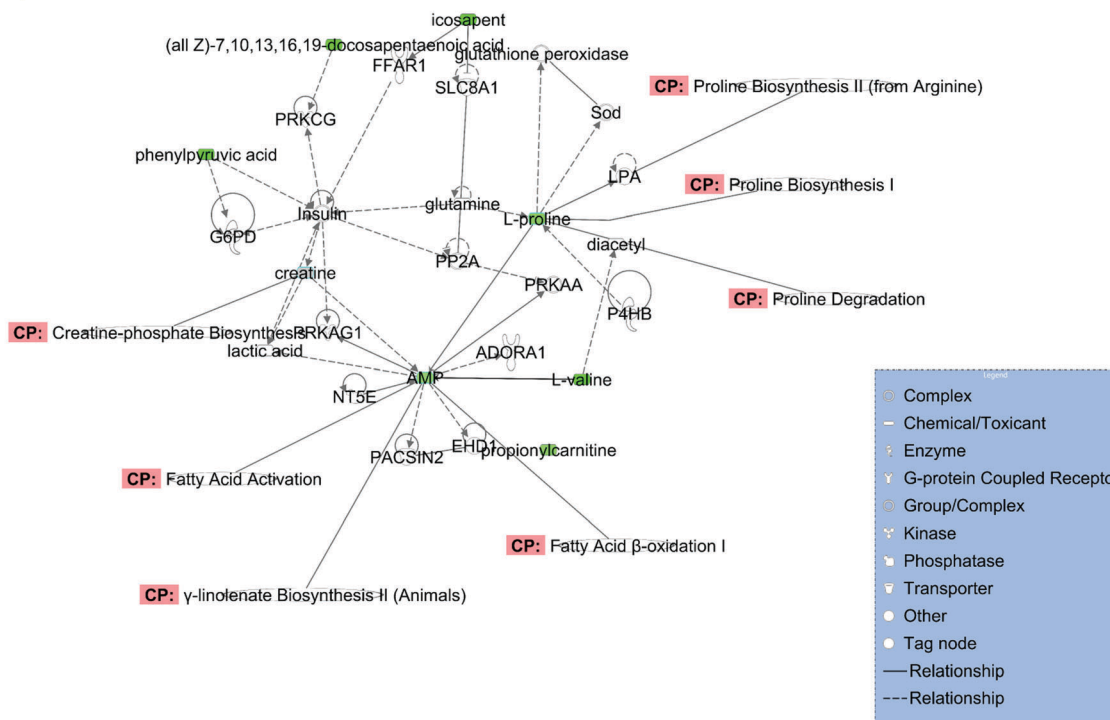


Fig. 5 Heat maps of the significantly changed metabolites in different groups.

Path Designer Network 1



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Fig. 6 Biological network, canonical pathways and functions related to the identified metabolites. In the network, molecules are represented as nodes, and the biological relationship between two nodes is represented as a line. Red symbols represent up-regulated metabolites; green symbols represent down-regulated metabolites. Solid lines between molecules show a direct physical relationship between molecules, while dotted lines show indirect functional relationships.

which damages the SPA-mediated immune regulation.²⁴ On the contrary, glucocorticoids can inhibit the inflammatory response of asthma by promoting the secretion of SPA and other surface active proteins.²⁵ Based on the study of SPA deficient animals, the effects of SPA on allergic inflammation and asthma in the lungs were further clarified.⁵ For example, in the ovalbumin (OVA)-induced asthma model, the airway inflammation was more severe in SPA knockout mice compared with that in wild-type mice, and IL-4, IgE, and IL-5 levels were increased in broncho alveolar lavage fluid (BALF) while IL-4 and IgE levels were decreased after exogenous SPA treatment.⁴ In the present study, SPA treatment significantly reduced the IgA level and increased the IgE level compared with those in the asthma group and regulated the related metabolic pathways and cytokines.

In this study, a major alteration observed in animals with asthma was glycerophospholipid metabolism. Some researchers reported that the LysoPCs (such as LysoPC (16:0) and LysoPC (15:0)) levels in asthma were significantly reduced using LC-MS-based metabolomics investigation.²⁶ In our study, LysoPCs metabolites were significantly reduced in the serum of the OVA-induced asthma mice compared to those in the control group. The disorder of LysoPCs would lead to energy metabolism alterations and is related to interruptions of the cell membrane and the occurrence of inflammation with OVA-induced allergic asthma. According to the histopathological results, marked infiltration of inflammation was found in the model group

(Fig. 1) and confirmed by our findings. Moreover, a decreased amount of the metabolites of creatine in the model group suggested that energy metabolism was promoted *via* the urea cycle. Creatine is involved in muscular protein turnover and energy supply to muscles such as the airway smooth muscles.²⁷

Purine metabolism is important in energy metabolism and the metabolites of purine, including hypoxanthine, adenosine monophosphate (AMP), uric acid, xanthine, and inosine, are correlated with inflammation of chronic obstructive pulmonary disease.^{28,29} Uric acid is the end product of purine metabolism. Kool and his colleagues noted that increased uric acid levels occur in BALF with the airways of allergen-challenged asthmatic patients and mice.³⁰ Of particular interest is the fact that a recent study reported that uric acid levels in serum increase at the onset of asthma exacerbations in patients and subside during the course of varying degrees of the exacerbation (mild, moderate, and severe).³¹ In this study, the increased level of adenosine monophosphate in the model group was observed and it in agreement with the inflammatory reaction (Fig. 4 and 5).

In general, fatty acids are important constituents of cell membranes and the largest energy reserve in the body. Numerous studies have confirmed that long-chain fatty acids can activate inflammatory and innate immune responses of peripheral cells.^{32–34} A recent study reported that dodecanoic acid exhibits significant anti-inflammatory activities by the inhibition of NF-κB activation and the phosphorylation of the mitogen-activated

protein (MAP) kinases.³⁵ A previous study reported that asthmatic patients consume higher fatty acids (SFAs) and myristic acid in their daily energy intake.³⁶ In the current study, the decreased docosapentaenoic acid, eicosapentaenoic acid, octadecatrienoic acid, and otadecadienoic acid found in the model group (as shown in Fig. 4 and 5) could be linked to the energy requirement and may specifically participate in the body's response to inflammation.

5 Conclusions

A UPLC/MS-based serum metabolomics method was established and used to study the overall metabolic changes in the serum of OVA-induced asthmatic mice. The metabolic pathways perturbed in the OVA-induced animal model have been presented. Herein, thirty-two differential metabolites involving 9 metabolic pathways were identified as potential biomarkers associated with the asthma. The network of these metabolites related to the pathogenesis of asthma was established by IPA for the first time. The IPA analysis suggested that the major changes were the inflammation responses in the pathophysiologic process of experimental asthma. Our findings provide a promising method for examining the metabolite profiling related to asthma and the interventional effects of SPA, which has the potential to help understand the pathogenesis of asthma and the mechanisms of SPA in asthma therapy.

Conflict of interest

There are no competing interests.

Acknowledgements

The work was supported by the Natural Science Foundation of China (81370135).

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