



Deciphering biochemical basis of Qingkailing injection-induced anaphylaxis in a rat model by time-dependent metabolomic profiling based on metabolite polarity-oriented analysis

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ABSTRACT

Ethnopharmacological relevance: Qingkailing injection (QKLI) is prepared from eight traditional Chinese medicinal materials or their extracts, which is widely used in clinical practice to treat the upper respiratory inflammation, pneumonia, high fever and viral encephalitis, nonetheless, suffering from serious anaphylaxis.

Aim of study: This study aims to develop an integrative metabolomics approach for deciphering the biochemical basis of QKLI induced anaphylaxis (QKLI-IA).

Materials and methods: The accuracy of animal modeling, the coverage of detected metabolites and the timeliness of pathological reaction are three key factors for revealing the biochemical basis of disease with untargeted metabolomics. In this study, firstly, the allergic rats (responders) were first screened by passive cutaneous anaphylaxis experiment and then were utilized for modeling. To cover a wider range of metabolites, a large-scale untargeted metabolomics based on metabolites polarity-oriented analysis was performed using ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. Then, to evaluate the timeliness of QKLI-IA, a time-dependent metabolomic profiling including the early, mid and late anaphylaxis stages of QKLI-IA, was performed.

Results: Corresponding to early, mid and late anaphylaxis stages of QKLI-IA, 14, 9 and 4 potential biomarkers were identified, respectively. Metabolism pathway analysis revealed that QKLI-IA resulted in dynamic changes in serum amino acid, fatty acid, glycerolipid, and phospholipid metabolisms. Twenty-four metabolites were found with identical fluctuating trends across the three stages of QKLI-IA. The results indicate that the pathogenesis of QKLI-IA is closely related to arachidonic acid metabolism.

Conclusion: This research provides a methodology reference for revealing the biochemical basis of disease using metabolomic profiling and offers a new insight to understand the pathogenesis of QKLI-IA.

1. Introduction

Metabolomics was defined as a comprehensive analytical approach for the study of the complete set of endogenous metabolites (metabolome) present in a biological system (Arias et al., 2009). The major aim of metabolomics is to obtain an answer to, or insight into, a biological question. Therefore, metabolomics is suitable for revealing the biochemical changes of disease pathogenesis (Hu et al., 2012; Bujak et al., 2014). Anaphylaxis is an acute, systemic, metabolically abnormal reaction, and its developmental process is complicated (Brown et al., 2001; Singer, Zodda, 2015). Therefore, using metabolomics approach to explore biochemical changes is beneficial to help clarify the

biochemical basis of anaphylaxis. The accuracy of animal modeling, the coverage of untargeted metabolomics data and the timeliness of pathological reaction are 3 key factors in metabolomics research aimed at elucidating the biochemical basis of disease pathogenesis, and each factor has a major influence on the research results.

In pharmacological or nutritional studies, ‘intersubject variation’ is often observed among laboratory animals, in which the induced effect is so variable that the animals can be classified as either ‘responders’ or ‘nonresponders’ (Li et al., 2007). Generally, the success rate of animal modeling is not 100% due to the individual differences in rats. The conventional animal modeling method for anaphylaxis uses behavioral indicators, such as scratching nose, piloerection and convulsions, to

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judge if anaphylaxis has indeed occurred, instead of inspecting the antibodies produced in the sensitization stage, thus ignoring the effects of individual differences in rats. This omission can result in an inaccurate assessment of which animals are allergic and can make subsequent toxicology research unclear. Therefore, establishing a new method to distinguish allergic animals was definitely the foundation of this anaphylaxis pathogenesis study. In this paper, the preparation method of antiserum was first investigated then the responders were screened based on a passive cutaneous anaphylaxis experiment, thus guaranteeing the accuracy of subsequent allergy models and leading to reliable research results.

As yet, knowledge about the underlying biochemical basis of anaphylaxis is largely unclear. Therefore, large-scale untargeted metabolomic profiling is necessary. The only reported anaphylaxis metabolomics study in mice models was induced with peanuts (Chalcraft et al., 2014). The study aimed at detecting basic and polar metabolites using a series of two columns: ZIC HILIC and RP-Amide. Consequently, most of the potential biomarkers were phosphatidylcholines and lyso-phosphatidylcholines rather than other kinds of metabolites such as amino acids, fatty acids, glycerolipids and phospholipids, which are involved with the anaphylactic reaction. In our study, each serum sample was pretreated with two different polarity solvents according to the polarity of metabolites and was determined under different experimental conditions. This was called the metabolites polarity-oriented analysis method. By using this method, a variety of allergy-related metabolites were able to be detected.

It is important to select appropriate time points when utilizing metabolomic tools to study the underlying biological processes involved with acute metabolic diseases such as anaphylaxis. According to our preliminary experiment, we found that Qingkailing injection induced anaphylaxis (QKLI-IA) can last for 120 min. Therefore, on the basis of this result, we carried out a dynamic study to reveal the trajectory of the metabolomic profiles corresponding to the stages of anaphylaxis.

QKLI is a traditional Chinese medicine which is widely used in clinical practice to treat the upper respiratory inflammation, pneumonia, high fever and viral encephalitis (Huang, 2012; Chen et al., 2002; Li et al., 2005; Fan, Liu, 2011); nonetheless, some people can suffer serious anaphylaxis from this substance. Therefore, deciphering the biochemical basis of QKLI-IA is important to its clinical application and secondary development. This study may also provide a reference approach for the investigating of adverse reactions of traditional Chinese medicine.

2. Materials and methods

2.1. Establishment of QKLI-IA animal models

2.1.1. Animals

Specific pathogen free grade male Sprague-Dawley rats (200 ± 20 g) were purchased from Beijing Weitonglihua Laboratory Animal Technology Co., Ltd. (Beijing, China). All rats were kept under a constant temperature (23 ± 2 °C) and humidity (60 ± 5%) with a 12h/12h light/dark cycle in controlled rooms. The rats were fed standard laboratory chow with water ad libitum and were acclimated to the room for 1 week. All animal experiments were approved by the Animal Ethics Committee of Beijing University of Chinese Medicine (Beijing, China), and all procedures were performed according to the Helsinki Declaration.

2.1.2. Preparation method of antiserum

Eighteen rats were randomly divided into six groups which were saline control group, QKLI control group, Al(OH)₃ gel control group, Freund's complete adjuvant (FCA) control group, QKLI + Al(OH)₃ gel group, QKLI + FCA group. The rats in the saline control group were subcutaneously injected with 4.2 mL/kg 0.9% saline five times at two-

week intervals. The rats in the QKLI, Al(OH)₃ gel, FCA, QKLI + Al(OH)₃ gel and QKLI + FCA groups were injected with 4.2 mL/kg QKLI, 4.2 mL/kg Al(OH)₃ gel, 4.2 mL/kg FCA, 8.4 mL/kg QKLI + Al(OH)₃ gel and 8.4 mL/kg QKLI + FCA under the same conditions, respectively. Ten days after the first sensitization, whole blood was collected by orbital blood every three days until the 28th day. The whole blood was stand at room temperature for 30 min and then centrifuged (3000 rpm, 4 °C) for 15 min. The serum were collected and stored at −80 °C.

Another 18 rats were also randomly divided into six groups which were saline control group, QKLI control group, Al(OH)₃ gel control group, FCA control group, QKLI + Al(OH)₃ gel group, QKLI + FCA group. One day before the experiment, the back skin of rats were shaved, selected eight points on the shaved sides of rats, then 0.1 mL corresponding antiserum of each time point were injected intradermally to shaved sides of rats. Twenty-four hour after sensitization, the sera-transferred rats were challenged by intravenously injecting 8.4 mL/kg QKLI containing 1% Evan's Blue. 30 min after antigen challenge, rats were sacrificed. The skin at the reaction site was excised, if no blue spot excised the 3.0 × 1.5 cm² area of skin. The blue spot of the inner skin at the injection site was measured. Then the skin specimen was dissolved in 6 mL of acetone-saline (7:3) mixture solution. Twenty-four hour later, sample was centrifuged (2000 rpm, 4 °C) for 10 min. The absorbance of dye leakage extracted in the supernatant was measured at 610 nm with a tspectrophotometer. Blue spot with a diameter more than 5 mm was regarded as positive reaction.

2.1.3. Responders screening

Before the experiment, the back skin of rats was shaved, and 0.1 mL antiserum which contained QKLI antibody was injected intracutaneously. At 24 h after sensitization, sera-transferred rats were challenged by intravenously injecting 8.4 mL/kg QKLI containing 1% Evan's Blue. After thirty minutes the diameter of the blue spot on the skin was measured. Rats with a blue spot diameter of more than 3 mm were regarded as responders. Others were regarded as nonresponders.

2.1.4. Allergy model establishment and sample collection

In this study, twenty-eight qualified responders were selected and used to establish the allergy model and additional twenty-eight healthy rats were added as control group (CG). The rats of allergy group (AG) were sensitized to 1 mL antiserum, which contained QKLI antibody, by intraperitoneal injection. The CG rats were treated with an equal volume of 0.9% saline. Twenty-four hours later, all rats were challenged by intravenous injection with 8.4 mL/kg QKLI. At 0 min, 10 min, 30 min and 120 min after QKLI administration, seven rats were selected from each group randomly and anesthetized with 10% chloral hydrate, and the blood samples were obtained by abdominal aortic. Blood samples were stood at room temperature for 30 min and then centrifuged (3000 rpm, 4 °C) for 15 min, and then the serum was collected and stored at −80 °C.

2.2. Allergy model validations

Two indicatives of anaphylaxis, namely, histamine and β-hexosaminidase, were measured with enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions of the ELISA kits. Results were expressed as the mean ± standard deviation (SD). The CG and AG groups were compared by *t*-test. *P* < 0.05 was considered statistically significant.

2.3. Metabolites polarity-oriented analysis method

To obtain comprehensive biomarkers associated with anaphylaxis, the polar and weak polar fractions of rat serum were processed and analyzed. Full details on serum sample preparation, ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC Q-TOF/MS) analysis and validation are shown in

the supplementary materials.

2.4. UPLC Q-TOF/MS data processing and multivariate statistical analysis

Micromass MarkerLynx Application Manager Version 4.0 (Waters Corp., Milford, USA) was used for peak selection and peak alignment of the UPLC Q-TOF/MS raw data to obtain a three-dimensional data matrix which containing the retention time, m/z , and peak intensity of each sample, and then EZinfo 2.0 software (Waters Corporation, Milford, MA, USA) was used for principal components analysis (PCA) and orthogonal partial least-squared discriminant analysis (OPLS-DA) for the data matrix. SPSS 16.0 and Metaboanalysis 3.0 were used for statistical analyses. When test P values were less than 0.05, the metabolite differences were considered significant.

2.5. Metabolite identification and pathway analysis

Metabolite identification was performed for the most significant metabolite ions from the OPLS-DA models. The MS^E technique was used to assign the metabolite peaks. Some available biochemical databases, such as METLIN (<http://metlin.scripps.edu/>), KEGG (<http://www.genome.jp/kegg/>), LIPID MAPS (<http://www.lipidmaps.org/>), Chempider (<http://www.chemspider.com>) and HMDB (<http://www.hmdb.ca/>), were used to analyze and identify the potential biomarkers. Pathway analysis was performed using Metaboanalysis 3.0.

3. Results

3.1. Preparation method of antiserum

The results showed that the rats of QKLI + Al(OH)₃ gel group and QKLI + FCA group had blue spot produced at the injection site after 8.4 mL/kg QKLI containing 1% Evan's Blue injected (Table S1). However, the rats of saline control group, QKLI control group, Al(OH)₃ gel control group and FCA control group had no blue spot. The diameter of blue spot in QKLI + Al(OH)₃ gel group was more than 5 mm. Besides, the absorbance of dye leakage extracted in the supernatant of QKLI + Al(OH)₃ gel group were higher than all control groups (Fig. S1 and S2). These results indicated that rats which were sensitized by QKLI + Al(OH)₃ gel could produce IgE antibody, the levels of IgE were highest in the QKLI + Al(OH)₃ gel group at 25th day after the first sensitization. So the QKLI + Al(OH)₃ gel group were selected to prepare antiserum and blood samples were collected at 25th day after the first sensitization. Furthermore, in our study, we found the diameter of blue spot in rat inner skin was 2 mm larger than the outside.

3.2. Responders screened

Experimental results showed that only 28 rats (two-fifths of all rats in the study) were responders after QKLI administration, indicating that the success rate of animal modeling is not 100%. There were different responses of rats upon being challenged by QKLI. Therefore, to guarantee the accuracy of the allergy model, screening responders from QKLI-sensitized rats was the key step in the study of QKLI-IA.

3.3. Serum histamine and β -hexosaminidase analysis

Histamine and β -hexosaminidase are well known markers of mast cell degranulation and play important roles in eliciting anaphylactic response (Amin, 2012). The concentrations of histamine and β -hexosaminidase in serum were used as biochemical markers for evaluating anaphylaxis. Data are summarized in the supplementary materials (Table S2 - S3). Compared with the corresponding CG rats, levels of serum histamine were significantly increased in AG rats at the time points of 10 min and 30 min after QKLI challenge, but had no statistically significant difference at the 120 min time point (Fig. 1A). The

reason for this might be that the majority of the histamine was metabolized due to its very short half-life in serum. Significantly increased levels of β -hexosaminidase were observed in AG rats at 10 min after QKLI administration and remained at that level for the duration of the experiment (Fig. 1B). These results showed that all rats of AG were experienced anaphylaxis as expected, whereas rats of CG did not. Consequently, the significantly increased levels of histamine and β -hexosaminidase in rat serum of AG indicated that we successfully established the allergy animal model.

3.4. Serum metabolomic profiles

Serum samples were analyzed in both positive and negative ionization modes with the UPLC Q-TOF/MS. Typical base peak intensity (BPI) chromatograms of serum from CG and AG at the 10, 30 and 120 min time points after QKLI challenge exhibited good separation results under the optimized gradient elution procedure (Fig. S3–S5). As can be seen from these figures, most of the peaks, whether polar metabolites or weak polar metabolites, were evenly distributed on the chromatogram with little overlap, which reduced the suppression or strengthening effect between ions undoubtedly and provided a foundation for the accuracy of subsequent data analysis. Compared with the CG, the serum metabolic profiles of AG rats were changed, and some representative peaks are marked with red arrows. The difference in the metabolic profiles between the CG and AG indicated that QKLI-IA in rats caused changes to endogenous substances. To investigate changes in endogenous metabolite profiling caused by QKLI-IA, multiple pattern recognition methods have been used to phenotype the serum metabolome of rats.

3.5. Multivariate statistical analysis and candidate metabolite identification

Metabolic profiling of serum samples was acquired by UPLC Q-TOF/MS in both positive and negative ion modes. The metabolic trajectory of AG and CG rats at time points of 0 min, 10 min, 30 min and 120 min was showed by PCA scores plot, which revealed a clear separation at different time points as the anaphylactic reaction progressed (Fig. 2). For the polar part, the serum metabolic profiles of rats at 10 min post-challenge by QKLI began to separate from the pre-challenge; at 30 min, the metabolic profiles were separated obviously; at 120 min, the separation trend was not obvious. However, the metabolic profiles of CG were almost entirely overlapping with no identifiable trend able to be observed (Fig. 2A). For the weak polar part, serum metabolic profiles of rats had the same trend as that of the polar part (Fig. 2B). The tracks of metabolic profiles at the different time points clearly demonstrated that QKLI-IA caused different metabolic perturbations paralleling different pathobiological changes in the process of anaphylaxis. Furthermore, these data revealed distinct metabolomics profiles related to the stage of anaphylaxis development. To maximize the differences between CG and AG, and to determine the variables that contribute to the discrimination, OPLS-DA was further employed for metabolic data and determination of serum metabolites. The results showed that all AG and CG rats can be completely separated in the OPLS-DA scores plot, which indicated that serum metabolism was significantly changed in allergy rats (Supplementary Fig. S6–S8). To find the significantly altered metabolites at each time point that caused rats metabolic profiles changed, all selected metabolites were filtered by Student's t -test. The metabolites with a significant difference of $P < 0.05$ and a total fold change greater than ± 1.0 were kept and these data were plotted as a volcano plot for easy visualization (supplementary Fig. S9 - S11). The VIP (variable importance in the projection) value was then used to select the most representative biomarkers which were closely associated with QKLI-IA. Potential metabolites were selected based on $VIP > 2$. The information of identified metabolites is shown in the supplementary materials Table S4.

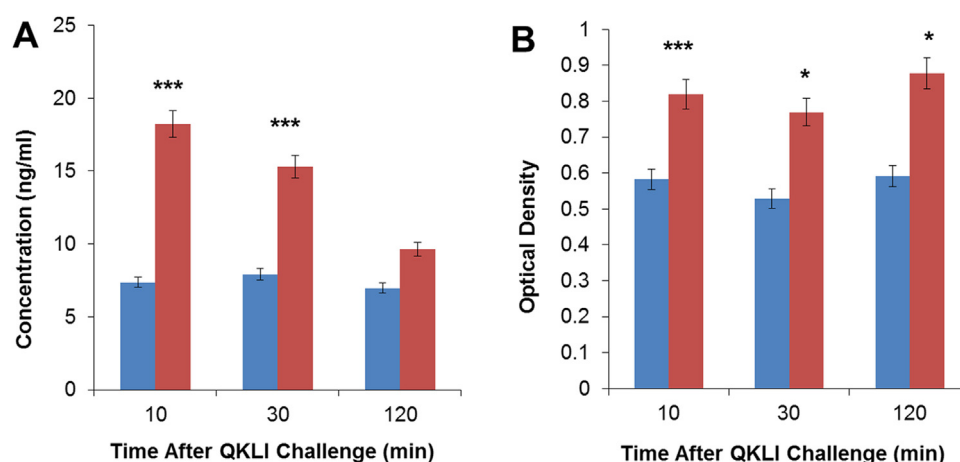


Fig. 1. Levels of serum histamine and β -hexosaminidase in CG and AG rats (blue = CG, red = AG; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (A) Histamine; (B) β -hexosaminidase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6. Metabolite abnormalities in early stage of QKLI-IA

Fifty-eight potential biomarkers which showed significant changes and were considered as strong contributors were distinguished from those of the controls at 10 min after QKLI administration, and included 33 metabolites in serum polar part (24 metabolites in the positive mode and 9 metabolites in the negative mode) and 25 metabolites in serum weak polar part (15 metabolites in the positive mode and 10 metabolites in the negative mode). PCA was performed and receiver operator characteristic (ROC) curves were generated to further explore the impact of these selected metabolites. Fig. 3A shows that the PCA of these selected metabolites could separate AG rats from CG rats completely. For comparing the predictive ability of these metabolites, area under the curve (AUC), which combines the sensitivity (true positive rate) and specificity (false positive rate), was determined. ROC analysis showed that 14 of the above 58 metabolites were identified as the top-ranked candidates, which having an AUC value more than 0.85 as well as having high sensitivity and specificity. This included creatine, 15-HETE, LysoPC (18:1), S1P, 5-HETE, arachidonic acid, palmitic acid, LysoPC (20:4), PC (16:0/22:6), DG (20:4/15:0/0:0), 12-HETE, PC (18:2/16:0), PC (18:0/22:6) and PC (18:0/20:3). The ROC curves for the evaluation of QKLI-IA with the individual metabolites and the combination of the fourteen metabolites are shown in Fig. 3B and C. The AUC value of the combined 14 biomarkers was 0.90, which demonstrated satisfactory performances in the early stage of QKLI-IA. Boxplots showing fluctuation trends of 14 metabolites are depicted in Fig. S12. These biomarkers responsible for the early stage of QKLI-IA play an important role in specific metabolic pathways. Therefore, finding the disturbed metabolic pathways could help us to better understand the pathogenesis of QKLI-IA. In our study, the biochemical pathways that responsible for the observed metabolic abnormalities were identified by ingenuity pathway analysis (IPA). The result revealed that 12 metabolites involved in arachidonic acid (AA) metabolism, glycerophospholipid metabolism, fatty acid metabolism, biosynthesis of unsaturated fatty acids, arginine and proline metabolism, glycine, serine and threonine metabolism were affected by QKLI-IA at 10 min after QKLI challenged (Fig. 3D). The results indicate that these 14 metabolites could be useful for investigating the pathogenesis of QKLI-IA.

3.7. Metabolite abnormalities in mid stage of QKLI-IA

Fifty-nine potential biomarkers were screened and identified at 30 min post-challenge, including 33 metabolites in polar part and 26 metabolites in weak polar part (Supplementary Table S4). Thirteen of these metabolites were markedly altered in the AG rats at time point

30 min only (Fig. 4A). The cluster diagram revealed that CG and AG rats could be completely separated by these thirteen selected metabolites. Except for L-phenylalanine, C16 sphinganine and DG (20:4/20:2/0:0), the VIP scores of the other ten metabolites were more than 1.0 (Fig. 4B). To assess these different metabolites in the mid stage of QKLI-IA, ROC curves were performed and results showed that, 9 of the above 13 metabolites were identified as the top-ranked candidates with an AUC value more than 0.80 (Fig. 4C), and included PC (22:6/18:0), tryptophan, sphinganine, PC (18:3/19:1), tyramine, LysoPC (18:4), phosphocholine, SM (d18:1/24:1), and DG (18:0/20:4). Biological pathway analysis revealed that the significant changes of the 9 metabolites were associated with the disturbed pathways of glycerophospholipid metabolism, sphingolipid metabolism, tryptophan metabolism, tyrosine metabolism, AA metabolism, alpha-linolenic acid metabolism and linoleic acid metabolism (Fig. 4D). As a result, these 9 metabolites were closely associated with the pathogenesis QKLI-IA at the 30 min time point.

3.8. Changes of metabolites in late stage of QKLI-IA

Forty-one potential biomarkers were screened and identified at 120 min post-challenge, including 23 metabolites in the polar part and 18 metabolites in the weak polar part (supplementary Table S4). Among these identified metabolites, 4 weak polar metabolites appeared only at this stage, showing significant perturbations. Heat maps revealed that these metabolites were altered markedly in AG rats at 120 min time point (Fig. S13A). Fig. S13B shows the PCA of selected metabolites that can separate AG from CG rats completely. To explore the suitability of the 4 metabolites, ROC curves were generated and the results demonstrate that these metabolites had high sensitivities and specificities with AUCs of 0.80 or greater (Fig. S13C). Biological pathway analysis revealed that these 4 metabolites are involved in glycerophospholipid metabolism, linoleic acid metabolism, alpha-linolenic acid metabolism and AA metabolism (Fig. S13D). Among the 4 disturbed pathways associated with the 4 markers, the highest impact was on the glycerophospholipid metabolism pathway. The detailed glycerophospholipid metabolism pathway is shown in Fig. S13E.

3.9. Abnormalities of metabolites from early to late stage of QKLI-IA

Among the selected metabolites, 24 were found in all three time points after QKLI challenge, and included AA, 12-HETE, 15-HETE and some phosphatidylcholines. Moreover, the same fluctuating trend was also found when comparing the corresponding CG: AA, 12-HETE, PC (20:3/18:0), and TG (12:0/20:2/22:3) were all markedly upregulated, and 15-HETE, LysoPC (20:4), LysoPC (18:2) were all significantly

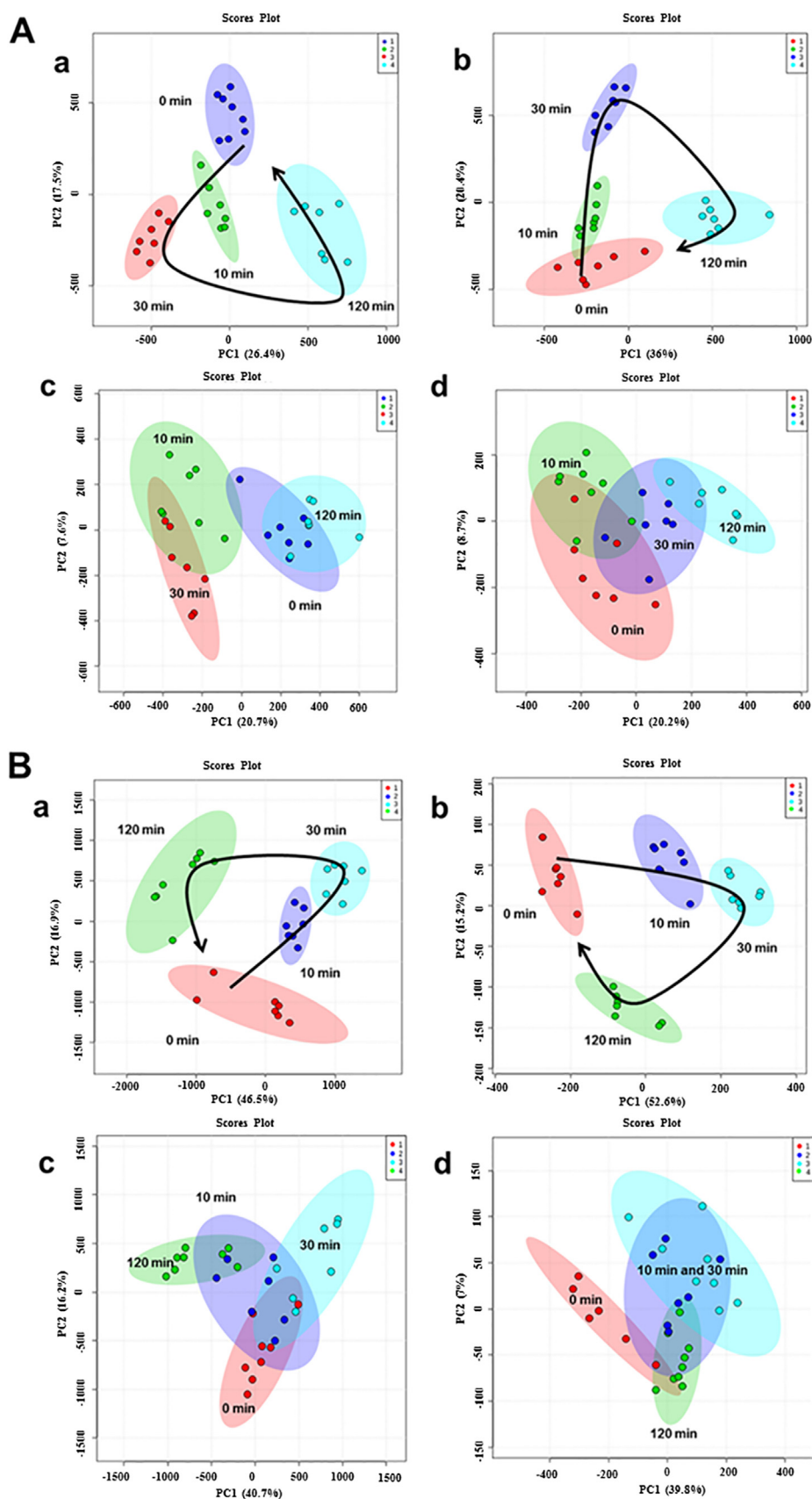


Fig. 2. PCA scores plot of polar metabolites (A) and weak polar metabolites (B). (a) AG in ES⁺; (b) AG in ES⁻; (c) CG in ES⁺; (d) CG in ES⁻.

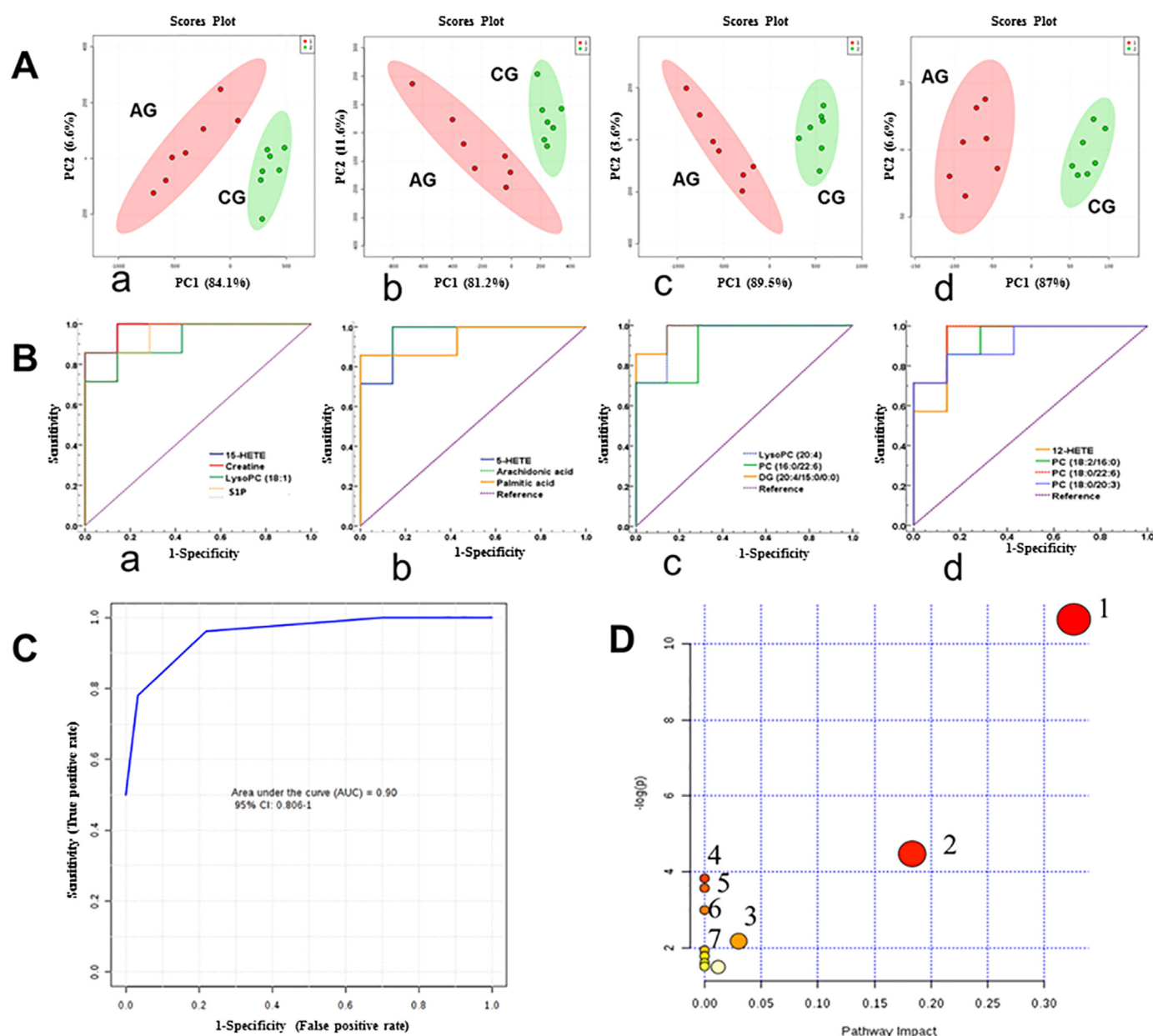


Fig. 3. PCA of 58 identified metabolites and diagnostic performance at the time point 10 min as well as associated metabolic pathways. (A) PCA of differential metabolites in serum polar part and weak polar part. The AG and CG rats could be separated completely based on these metabolites. (a) ES^+ mode of polar metabolites; (b) ES^- mode of polar metabolites; (c) ES^+ mode of weak polar metabolites; (d) ES^- mode of weak polar metabolites. (B) ROC curves for evaluating QKLI-IA with the individual metabolites and (C) the combination of the fourteen metabolites with high sensitivity and specificity. (D) Summary of pathway analysis. Each dot of the figure represents a related metabolic pathway. The color and size of each dot was related to the $-\log(p)$ value and pathway impact value, respectively. (1) AA metabolism; (2) glycerophospholipid metabolism; (3) sphingolipid metabolism; (4) biosynthesis of unsaturated fatty acids; (5) linoleic acid metabolism; (6) alpha-linolenic acid metabolism; (7) fatty acid metabolism.

downregulated at the 3 time points. Therefore, we speculated that these 24 biomarkers were directly related to QKLI-IA. To compare the contribution of the 24 altered metabolites at different stages of anaphylaxis, the unsupervised cluster analyses were performed which focusing on each individual metabolite species that exhibited statistically significant differences. Serum samples from AG rats were segregated into tight clusters at different time points (Fig. 5A). The heat map reflected the relative average concentrations of the 24 metabolites in the AG rats (Fig. 5B). To assess the fluctuations throughout the process of anaphylaxis, the 24 biomarkers which had the same changing trends in three AG were analyzed (Fig. 5C). The results of pathway analysis were similar to the single point analysis, and the 24 metabolites also related to AA metabolism, glycerophospholipid metabolism, and sphingolipid

metabolism, etc. Among the five disturbed pathways associated with the 24 markers, the impact on the AA metabolism pathway was the highest, indicating that QKLI-IA was mainly caused by the abnormalities in the AA metabolism pathway.

4. Discussion

Identification of physiopathological mechanisms and metabolic disorders in the early and advanced stages is critical for the study of QKLI-IA pathogenesis. In the present study, the allergy index examination of serum showed that histamine and β -hexosaminidase were significantly increased in the AG rats indicating that, as expected, all rats of AG experienced anaphylaxis whereas rats of CG did not. The

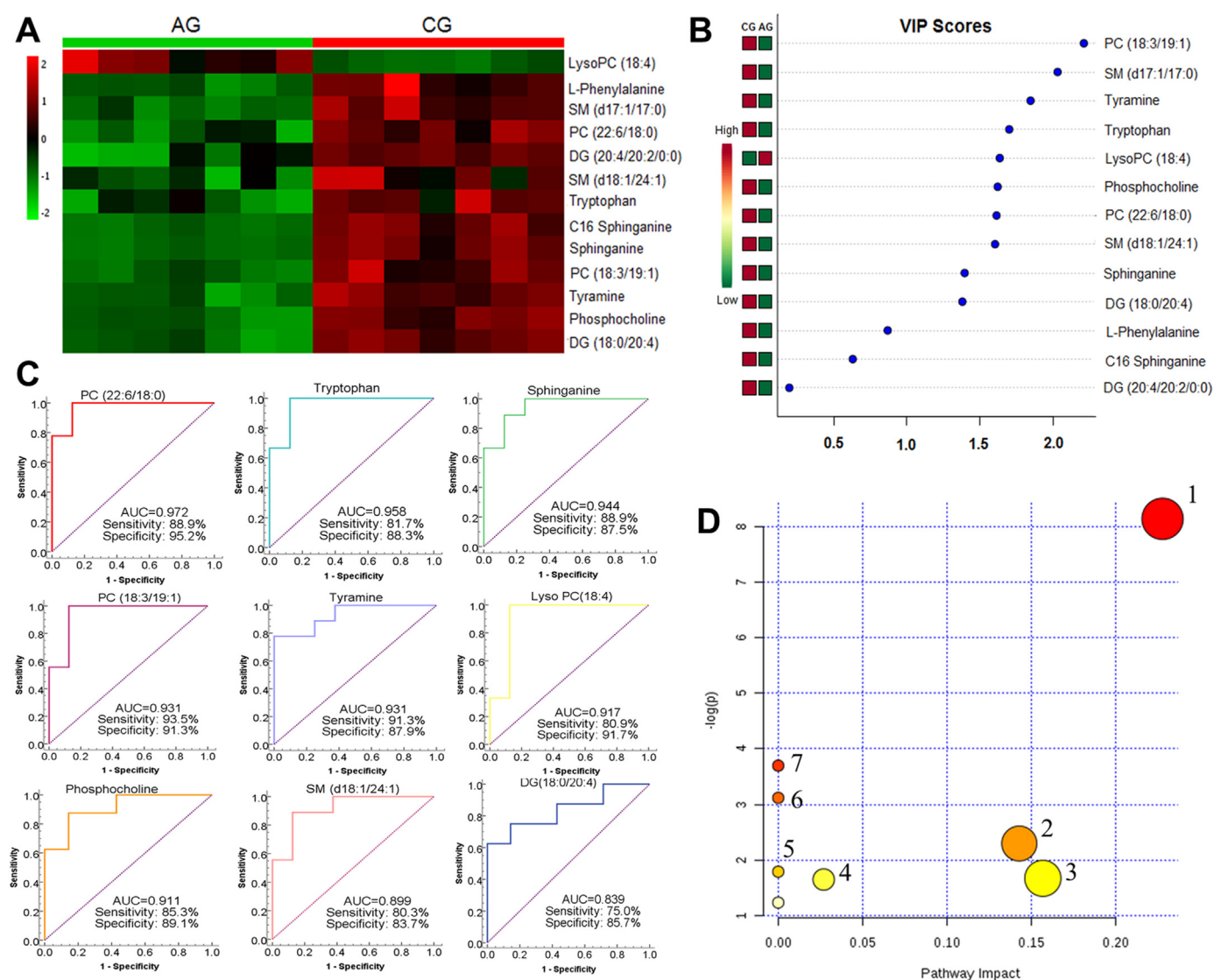


Fig. 4. Hierarchical cluster analysis and ROC curves of 9 identified metabolites as well as associated metabolic pathways at time point 30 min (A) Clustered heat map of 13 (only appeared at time points 30 min) out of 59 identified metabolites between AG and CG. Heat map displays the changing trend of these 13 metabolites. Each cell in the heat map represents the fold change between the two groups for a particular metabolite. It visualizes the level of each metabolite in each sample ranging from high (red) over average (black) to low (green). Rows represent each identified metabolite from AG and CG and columns represent individual experiments. (B) VIP scores with expression heat map from PLS-DA analysis. Green indicates reduction and red indicates increase. (C) The ROC curves of 9 out of 13 identified metabolites for evaluation of QKLI-IA with the individual biomarkers. The associated AUC, sensitivity and specificity values were indicated. (D) Summary of pathway analysis. (1) glycerophospholipid metabolism; (2) sphingolipid metabolism; (3) tryptophan metabolism; (4) tyrosine metabolism; (5) AA metabolism; (6) alpha-linolenic acid metabolism; (7) linoleic acid metabolism.

change in the allergy index level in allergy rats was accompanied by profound perturbations of serum amino acid, fatty acid, glycerolipid, and phospholipid metabolism in early, intermediate and advanced phases of QKLI-IA. Disturbances to glycerophospholipid and AA metabolism were observed in the serum at dynamic stages in the AG rats. Recent studies reported that the pathways of AA metabolism (Tao et al., 2010; Liu and Yokomizo, 2015), glycerophospholipid metabolism (Ried et al., 2013; Nauta et al., 2008; Wymann and Schneider, 2008), sphingomyelin metabolism (Aguilera-Romero et al., 2014; Oskertizian et al., 2007), fatty acid metabolism (Gdula-Argasińska et al., 2015) and tryptophan metabolism (Le and Broide, 2006; Frumento et al., 2002) were related to anaphylaxis. According to the metabolic pathway analysis from our study, we have summarized the pathways which are likely to be associated with QKLI-IA, as well as the trends of potential biomarkers (Fig. 6).

AA, is a polyunsaturated fatty acid. It has as an important role in the

pathogenesis of many allergy based diseases, such as asthma and skin rashes (Mateu et al., 2015; Yui et al., 2015). Anaphylaxis can result in an altered metabolism of AA (Kadowaki et al., 2008). In our study, an increased level of AA was observed at three time points post-challenge. AA is a precursor of many inflammatory mediators. Some AA metabolites, such as prostaglandins (PGs), leukotrienes (LTs) and hydroxyleicosatrienoic acid (HETE) are associated with the development of inflammation. We found that some AA metabolites were significantly altered in rats of AG compared with CG, among which the levels of 12-HETE and 5-HETE increased. 12-HETE is metabolized by AA through the 12-LOX pathway. When the 12-LOX enzyme is over expressed, unsaturated fatty acids such as AA and linoleic acid are converted into bioactive 12-HETE, and inflammatory occurs (Porro et al., 2014). 5-HETE is a pro-inflammatory mediator which can promote leukotriene synthesis (Meng et al., 2015). Decreased levels of 15-HETE were found at the 3 time points of the AG, likely due to its anti-inflammatory

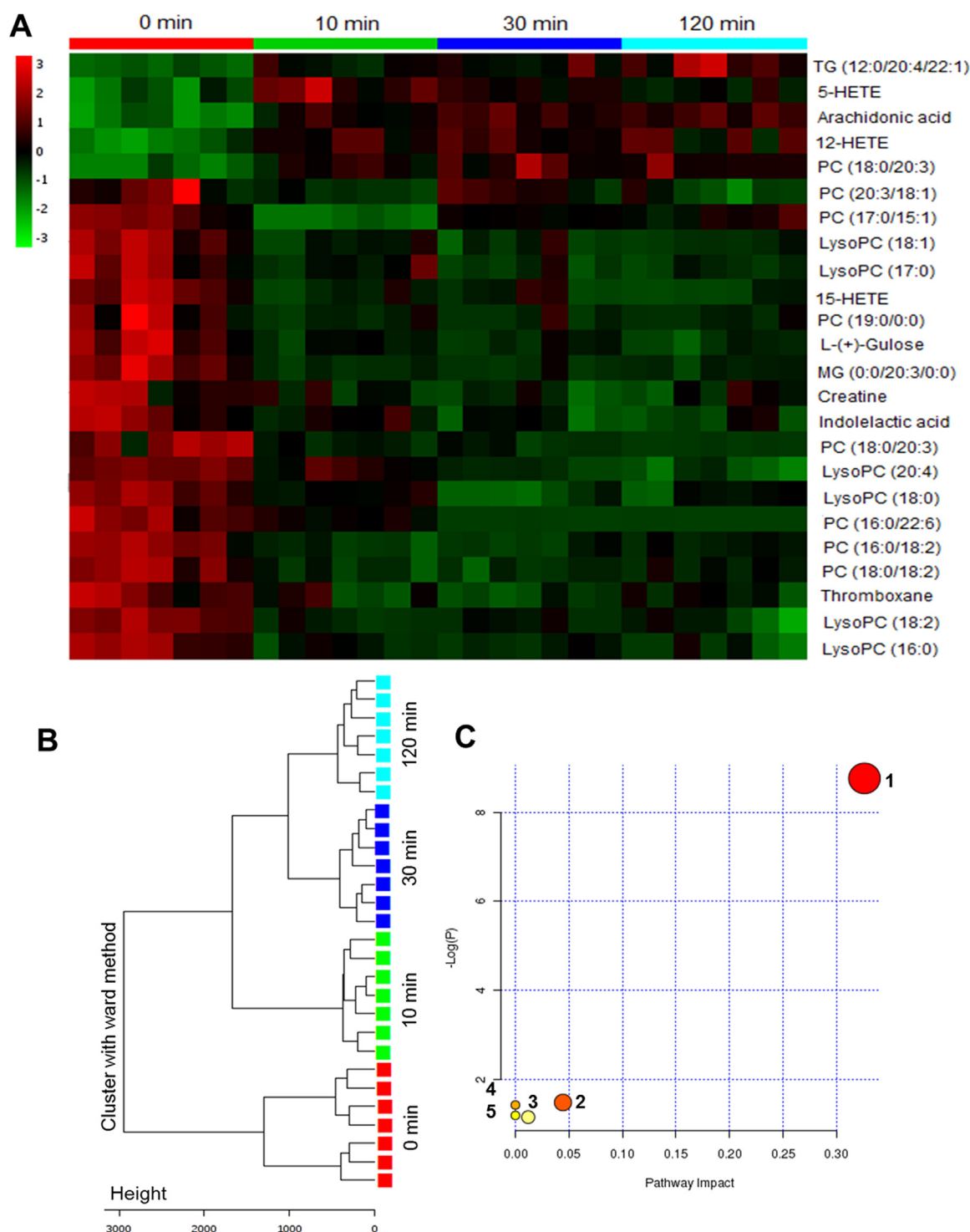


Fig. 5. Hierarchical cluster analysis and metabolic pathway analysis of 24 metabolites. (A) Dendrograms of hierarchical clustering of different AG at 0 min, 10 min, 30 min and 120 min (B) Hierarchical clustering heat map of the 24 differential metabolites, with the degree of change in the different groups marked with colors including up-regulation (red) and down-regulation (green). (C) Summary of pathway analysis. (1) AA metabolism; (2) glycerophospholipid metabolism; (3) arginine and proline metabolism; (4) biosynthesis of unsaturated fatty acids; (5) glycine, serine and threonine metabolism.

capabilities (Kowal et al., 2008). In addition, AA can be metabolized to LTs by the LOX pathway and metabolized to PGs by the COX pathway. LTs and PGs are important inflammatory mediators in allergic diseases (Ried et al., 2013); LTC₄ can cause vasoconstriction, bronchospasm and further contributes to edema, while PGE₂ can cause arteries to dilate, which further contributes to swelling. AA metabolites had low abundance and short half-life, usually only tens of seconds to minutes, which limited the detection of AA metabolites (Frumento et al., 2002). In our

study, LTs and PGs could not be detected, likely for these two reasons.

Glycerophospholipids are lipid compounds consisting of glycerol, fatty acid and phosphoric acid. They are the main components of cell membranes. Depending on the different substituents of glycerol-3-phosphate, glycerophospholipids can be classified into phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylserine (PS). Recent studies reported that PC plays an important role in the pathogenesis of inflammation (Liu and

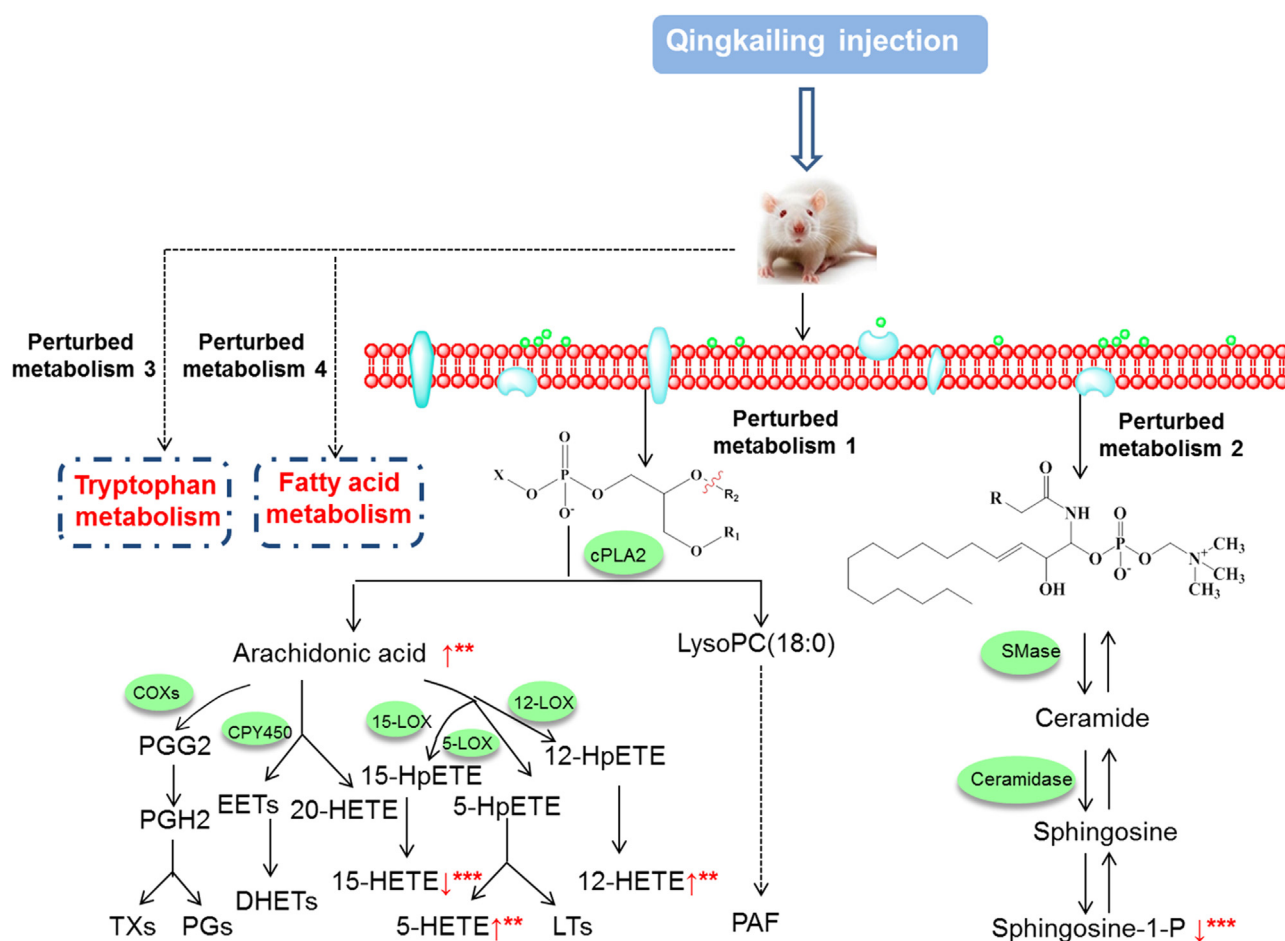


Fig. 6. The perturbed metabolic pathways associated with QKLI-IA. ↑: Compared with the CG, the metabolite was up-regulated in the AG. ↓: Compared with the CG the metabolite was down-regulated in the AG. ** $P < 0.01$, *** $P < 0.001$ compared with CG.

Yokomizo, 2015). The fatty acid on C₁/C₂ of PC could be hydrolyzed by cytosolic phospholipase A₁/A₂ (cPLA₁/A₂) when the cell membrane was stimulated by physicochemical and pathological factors. The fatty acids, such as FFA C20:2, FFA C20:3, FFA C20:4 and FFA C22:6, are precursors of bioactive lipid mediators (Perez-Chacon et al., 2010; Harizi et al., 2008). These fatty acids can be converted into downstream metabolites of AA (Ried et al., 2013). Lysophosphatidylcholine (LysoPC), a kind of lipid compound, is hydrolyzed from PC. It has been shown that LysoPC is associated with inflammation (Perez-Chacon et al., 2010; Harizi et al., 2008). It can regulate many important physiological and pathological pathways by high affinity binding to G protein-coupled receptors which are expressed on the cell membranes of monocytes and macrophages. In addition, LysoPC can be indirectly converted into platelet-activating factor (PAF), which can induce chemotaxis of inflammatory cells causing inflammation (Gill et al., 2015). In our study, PAF could not be detected likely due to very low abundance.

Tryptophan is the only amino acid associated with autoimmune diseases. The disturbed tryptophan metabolism pathway is related to the over expression of the rate-limiting enzyme IDO. Previous research has found that tryptophan levels are down regulated when the immune system is activated. In our study, decreased levels of tryptophan were found in rats at 30 min post-challenge.

Sphingosine 1-phosphate (S1P), a sphingolipid metabolite, is a highly bioactive lipid mediator which is closely related to inflammatory reactions. In our study, a decreased level of S1P was found in rats of the AG, which is consistent with reported literature.

In summary, the present study revealed abnormal metabolites in the

intermediate and advanced stages of QKLI-IA, providing information for the exploration of anaphylaxis pathogenesis. The significantly altered levels of metabolites at three time points during anaphylaxis indicated that disturbed metabolism pathways of AA metabolism, glycerophospholipid metabolism, fatty acid metabolism, sphingomyelin metabolism and tryptophan metabolism were involved in QKLI-IA to some extent, and among which, the disturbed AA metabolism pathway was closely associated with the pathogenesis of QKLI-IA.

5. Conclusion

In conclusion, the present study developed an integrative metabolomics approach for deciphering the biochemical basis of QKLI-IA, which fully considered three key factors, namely the accuracy of animal modeling, the coverage of detected metabolites and the timeliness of pathological reaction. The pathway analysis results revealed that the pathogenesis of QKLI-IA was closely associated with the disturbance of AA metabolism pathway. The findings of our study offer a new insight in the understanding of the pathogenesis of QKLI-IA and provide a reference approach for the study of adverse reactions to traditional Chinese medicine.

Acknowledgements

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Conflict of interest

The authors declare no competing financial interests.

Author contributions

Xiaoyan Gao and Lingling Qin wrote the manuscript; Lingling Qin, Zhixin Zhang, Huizhen Zhao, Wenjuan Zhou, Ziyi Xie, Honghong Yu and Zhenghai Huang performed the experiments; Lingling Qin and Zhixin Zhang analyzed the raw data; Xiaoyan Gao undertook the study design. All authors read and approved the final version of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2018.07.013.

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Supporting Materials

Deciphering biochemical basis of Qingkailing injection-induced anaphylaxis in a rat model by time-dependent metabolomic profiling based on metabolite polarity-oriented analysis

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16 **1. Metabolites polarity-oriented analysis method**

17 **Serum sample preparation.** To obtain comprehensive biomarkers which associated with anaphylaxis, the polar
18 part and weak polar part of rat serum were processed and analyzed, respectively.

19 For the polar part of serum, precipitation of protein was performed by the addition of the methanol solution. The
20 150 μ L serum was mixed with 450 μ L methanol solution then vortexed 30 s. After 10 min, samples were
21 centrifuged at 12000 rpm at 4°C for 10 min. The 400 μ L supernatant was evaporated to dryness under a gentle
22 stream of nitrogen and reconstituted with 75 μ L water and then vortexed 30 s. After 10 min, samples were
23 centrifuged at 12000 rpm at 4°C for 10 min. The prepared supernatant samples were removed to the injection vial
24 for ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC
25 Q-TOF/MS) analysis.

26 For the weak polar part of serum, precipitation of protein was performed by the addition of the
27 chloroform/methanol (2:1, v: v) solution. The 300 μ L serum was mixed with 900 μ L chloroform/methanol (2:1, v:
28 v) solution and then vortexed 2 min. After 10 min, samples were centrifuged at 12000 rpm at 4°C for 10 min. The
29 200 μ L chloroform layer was evaporated to dryness under a gentle stream of nitrogen and reconstituted with 100
30 μ L isopropanol/acetonitrile (1:1, v: v) and then vortexed 30 s. After 10 min, samples were centrifuged at 12000
31 rpm at 4°C for 10 min. The prepared supernatant samples were removed to the injection vial for
32 UPLC-Q-TOF/MS analysis.

33 **UPLC Q-TOF/MS analysis.** For the polar metabolites analysis, separation of metabolites was performed on a
34 Waters Acquity™ Ultra Performance LC system (Waters Corp., Milford, MA) that was equipped with an HSS T3
35 column (2.1 mm $\times$ 100 mm, 1.8 μ m, Waters, UK). The injection volume was 2.5 μ L and the mobile phase flow rate
36 was 0.30 mL $\cdot$ min⁻¹. The mobile phase consisted of phase A (0.1% formic acid in water) and phase B (0.1% formic
37 acid in acetonitrile). The gradient was as follows: 0-1.0 min, 100% A; 1.0-5.0 min, 100-40% A; 5.0-8.0 min, 40-0%

38 A; 8.0-12.0 min, 0% A; 12.0-14.0 min, 0-100% A. The auto-sampler and analytical column were maintained at
39 temperatures of 4°C and 45°C, respectively. Mass spectrometry was performed on a Xevo™ G2 Q/TOF (Waters
40 MS Technologies, Manchester, UK). An electrospray ionisation source (ESI) interface was used and was set in
41 both positive ion and negative ion mode. Data were collected from m/z 50 to m/z 1200. For positive ion mode, the
42 capillary and cone voltage were set at 3 kV and 28 V. The desolvation gas was set at 800 L/h at a temperature of
43 400°C. The cone gas was set at 35 L/h, and the source temperature was set at 100°C. For negative ion mode, the
44 capillary and cone voltage were set at 2.2 kV and 30 V. The desolvation gas was set at 800 L/h at a temperature of
45 400°C. The cone gas was set at 35 L/h and the source temperature was set at 100°C.

46 For the weak polar metabolites analysis, separation of metabolites was performed on a Waters Acquity™ Ultra
47 Performance LC system (Waters Corp., Milford, MA) that was equipped with a CSH C18 column (2.1 mm×100
48 mm, 1.8 µm, Waters, UK). The injection volume was 2.5 µL and the mobile phase flow rate was 0.30 mL·min⁻¹.
49 The mobile phase consisted of phase A (60: 40 acetonitrile: water, with 10 mM ammonium acetate) and phase B
50 (10: 90 acetonitrile: isopropanol, with 10 mM ammonium acetate). The gradient was as follows: 0-2.0 min, 60-57%
51 A; 2.0-2.1 min, 57-50% A; 2.1-12.0 min, 50-46% A; 12.0-12.1 min, 46-30% A; 12.1-18.0 min, 30-1% A,
52 18.0-18.1 min, 1-60% A. The auto-sampler and analytical column were maintained at temperatures of 4°C and
53 45°C, respectively. Mass spectrometry was performed on a Xevo™ G2 Q-TOF (Waters MS Technologies,
54 Manchester, UK). An ESI interface was used and was set in both positive ion and negative ion mode. Data were
55 collected from m/z 50 to m/z 1200. For positive ion mode, the capillary and cone voltage were set at 3.2 kV and 30
56 V. The desolvation gas was set at 800 L/h at a temperature of 400°C, the cone gas was set at 30 L/h and the source
57 temperature was set at 120°C. For negative ion mode, the capillary and cone voltage were set at 2.3 kV and 30 V.
58 The desolvation gas was set at 800 L/h at a temperature of 400°C. The cone gas was set at 40 L/h and the source
59 temperature was set at 120°C.

60 2. Validation of the UPLC Q-TOF/MS Conditions

61 Aliquots (100 μ L) of serum samples were mixed to generate pooled quality control (QC) samples. The
62 repeatability was evaluated by the relative standard deviations (RSDs) of retention time, m/z and peak area of six
63 different QC samples, which were produced with the same preparation method. The system stability test was
64 measured by the RSDs of retention time, m/z and peak area of QC samples, which were placed every 6 samples
65 during the entire sample analysis procedure.

66 For polar metabolites, extracted ion chromatographic peaks of ten ions with the retention time and m/z pairs of
67 1.48-204.1234, 3.84-188.0724, 7.93-357.2799, 9.29-381.2986, 12.38-835.6665 in positive ion mode and
68 1.00-341.1085, 3.86-203.0820, 4.73-322.9495, 7.94-783.5795, 9.30-281.2493 in negative ion mode were selected
69 for method validation. For weak polar metabolites, extracted ion chromatographic peaks of ten ions with the
70 retention time and m/z pairs of 1.58-496.3401, 7.49-782.5682, 10.39-786.5990, 13.55-813.6835, 16.28-369.3524
71 in positive ion mode and 1.55-480.3091, 4.19-283.2636, 7.29-826.5597, 10.09-830.5918, 14.79-694.6361 in
72 negative ion mode were selected for method validation.

73 For the polar part analysis, the relative standard deviations (RSDs) of the retention time for repeatability and
74 system stability were found to be 0-0.51% and 0-0.53% in positive ion mode and 0-0.75% and 0-0.57% in
75 negative ion mode, respectively. The RSDs of the m/z for the repeatability and system stability were within the
76 ranges of 0% and 0% in positive ion mode and 0% and 0% in negative ion mode, respectively. The RSDs of the
77 peak area for the repeatability and system stability were within the ranges of 4.60-12.07% and 3.47-12.83% in
78 positive ion mode and 4.39-13.99% and 10.16-12.46% in negative ion mode, respectively. For the weak polar part
79 analysis, the RSDs of the retention time for repeatability and system stability were found to be 0-0.07% and
80 0.12-1.02% in positive ion mode and 0-0.29% and 0.04-0.36% in negative ion mode, respectively. The RSDs of
81 the m/z for the repeatability and system stability were within the ranges of 0% and 0% in positive ion mode and 0%

82 and 0% in negative ion mode, respectively. The RSDs of the peak area for the repeatability and system stability
83 were within the ranges of 4.85-12.35% and 10.7-14.94% in positive ion mode and 6.56-14.84% and 8.53-14.64%
84 in negative ion mode, respectively. These results indicated that the developed method showed good repeatability
85 and stability for the serum metabolomics analysis.

Supplementary Figures

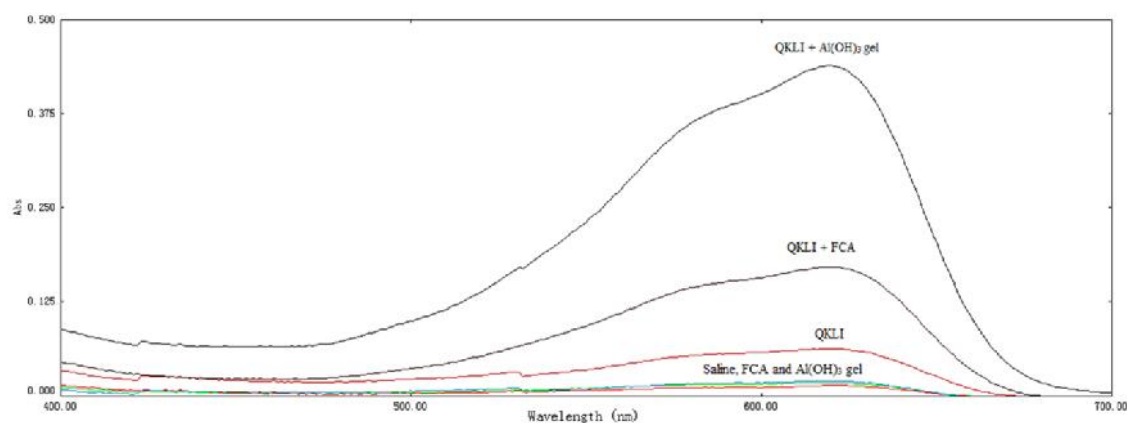


Fig. S1 The blue spot UV absorption spectrum of six groups of rats.

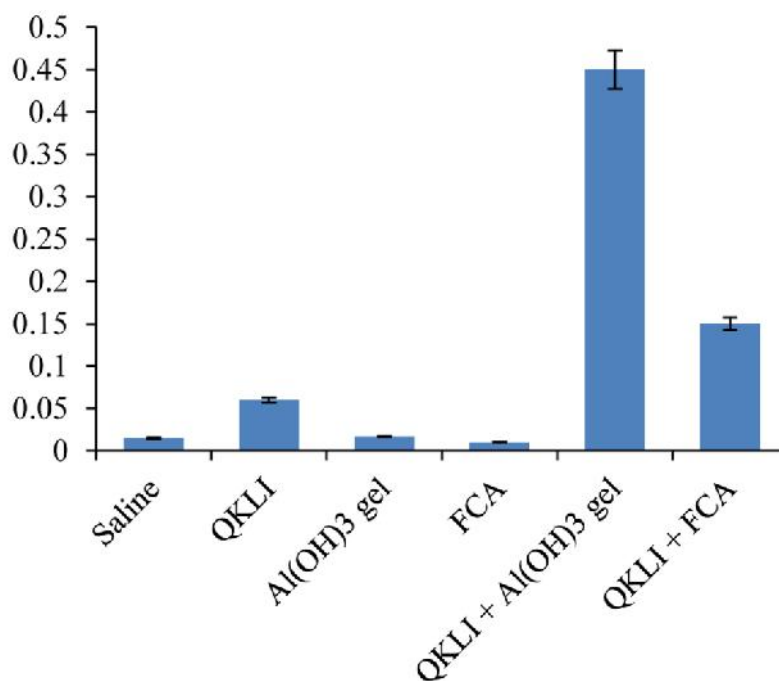


Fig. S2 Absorbance of dye leakage extracted in the blue spot supernatant of six groups of rats.

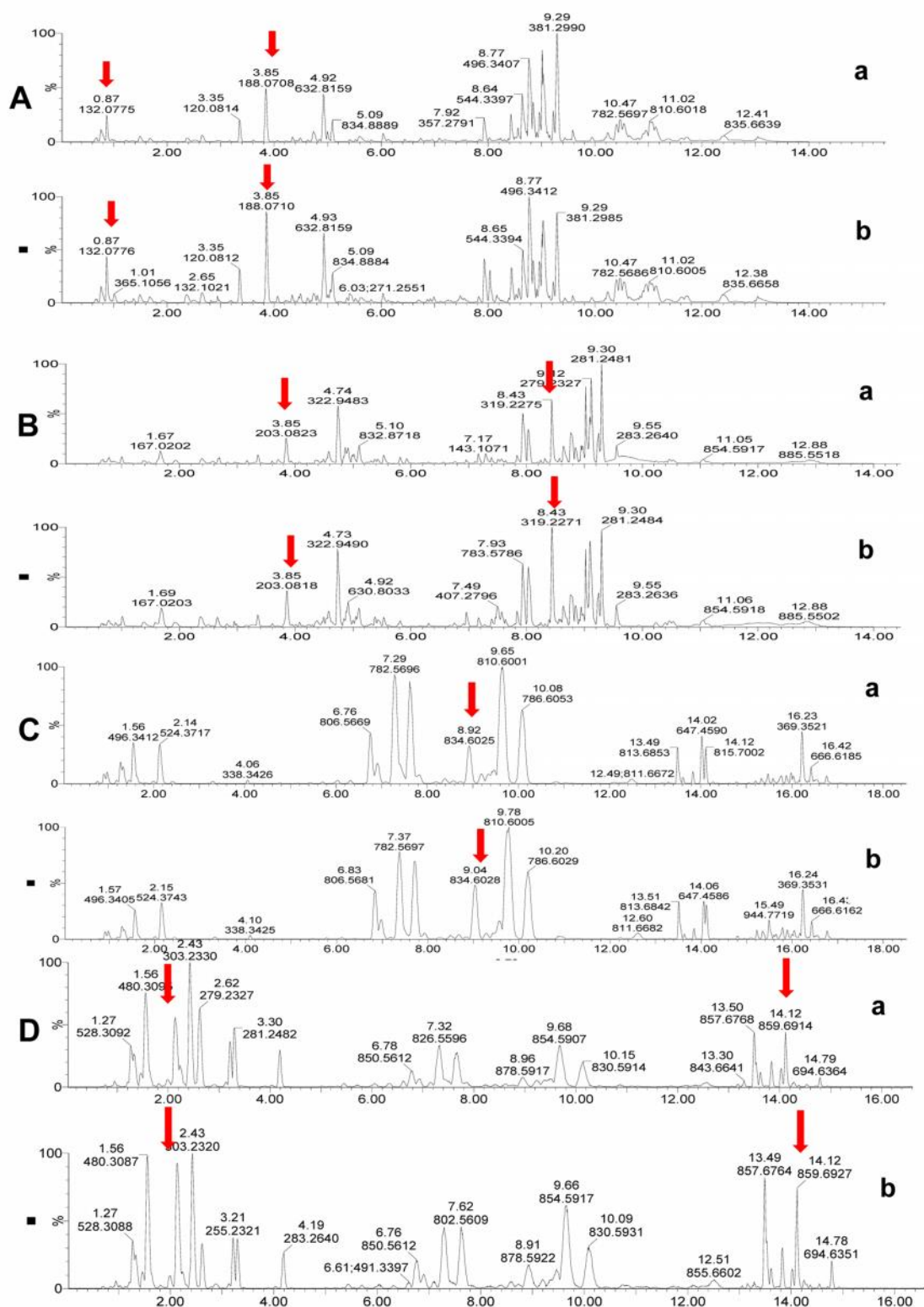


Fig. S3 Typical base peak intensity (BPI) chromatograms obtained from rats serum in control groups (a) and allergy groups (b) at 10 min post-challenge by QKLI. (A) For polar metabolites ES⁺; (B) For polar metabolites ES⁻; (C) For weak polar metabolites ES⁺; (D) For weak polar metabolites ES⁻.

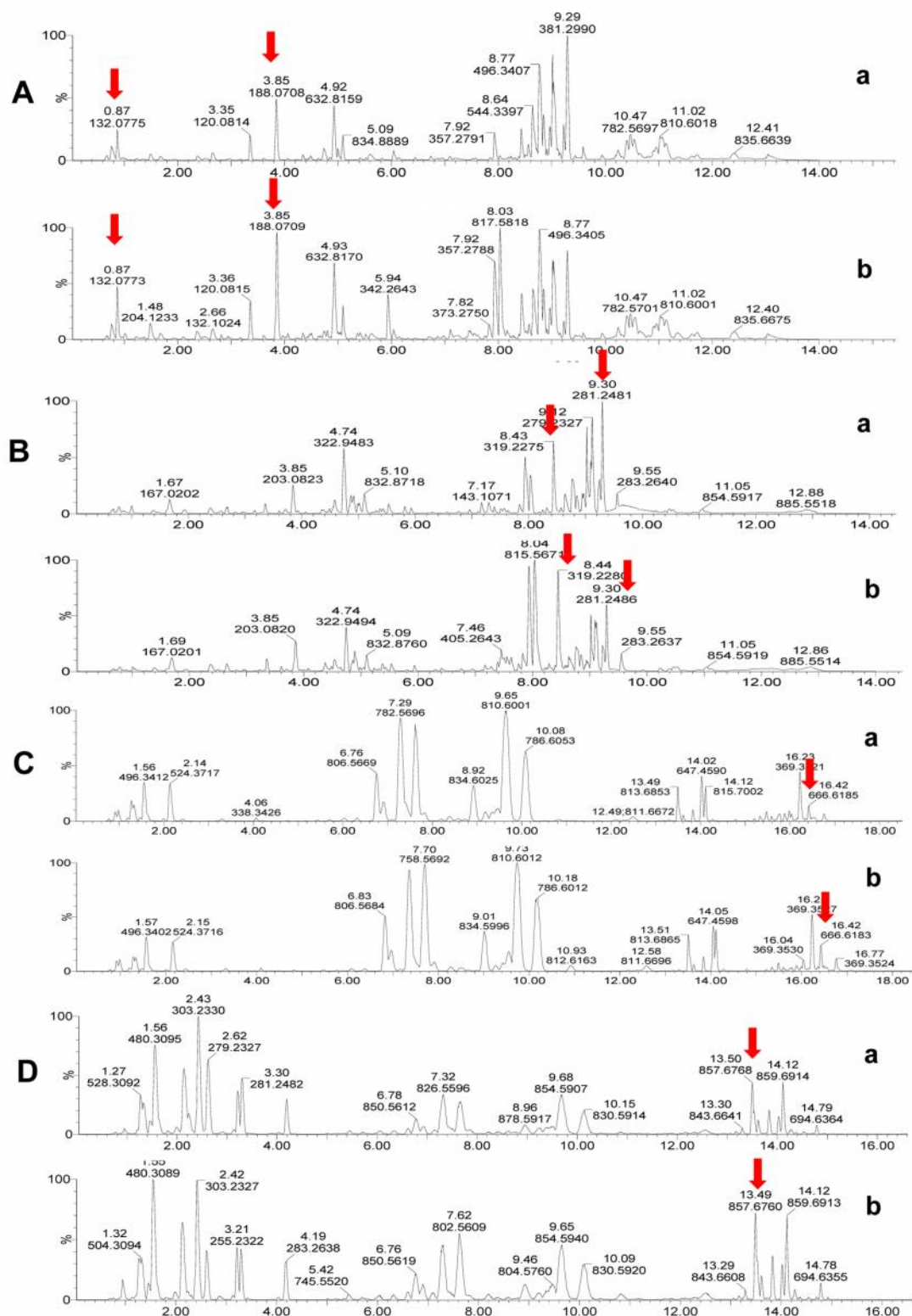


Fig S4 Typical base peak intensity (BPI) chromatograms obtained from rats serum in control groups (a) and allergy groups (b) at 30 min post-challenge by QKLI. (A) For polar metabolites ES⁺; (B) For polar metabolites ES⁻; (C) For weak polar metabolites ES⁺; (D) For weak polar metabolites ES⁻.

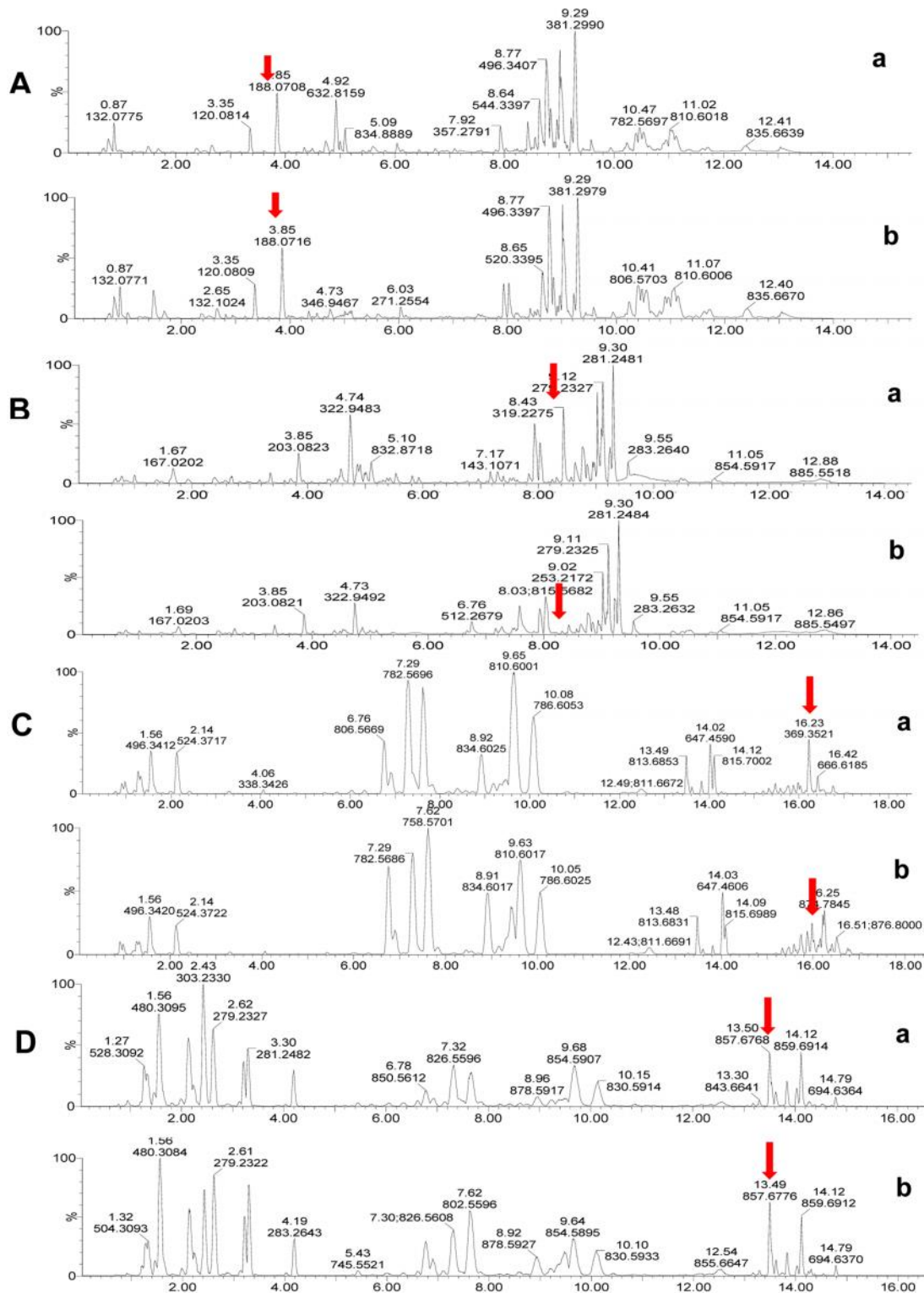
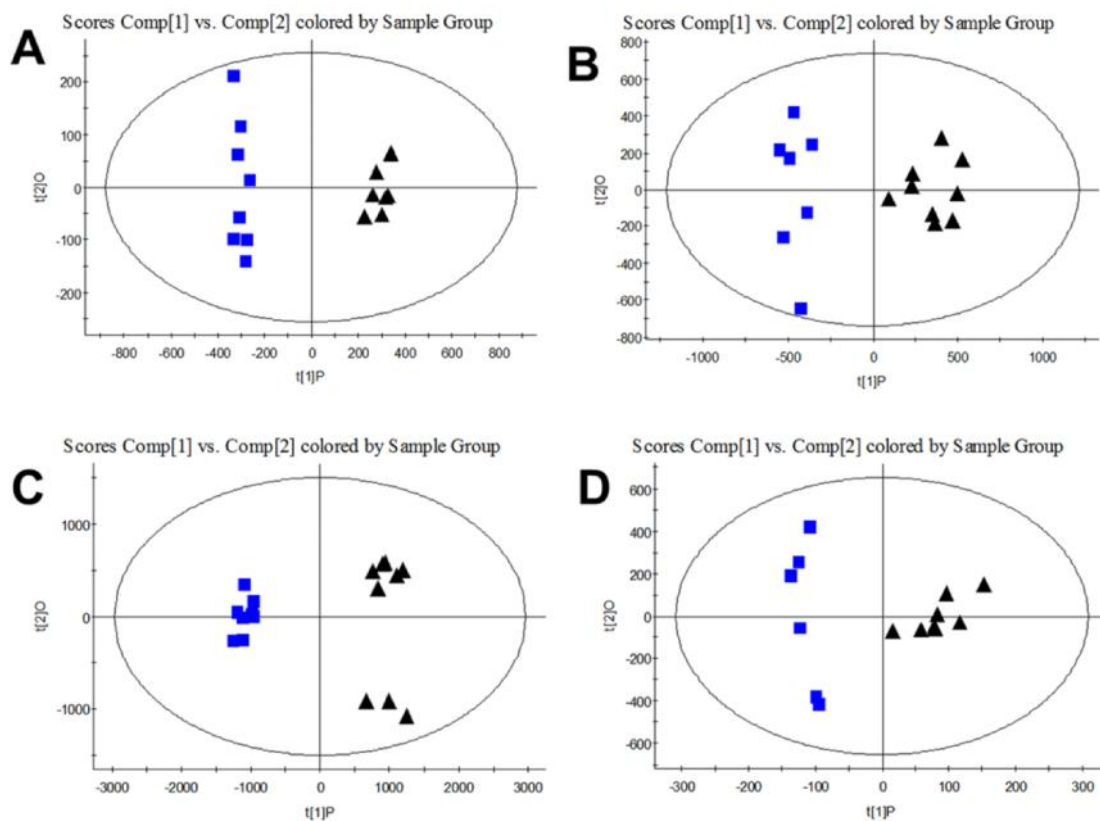


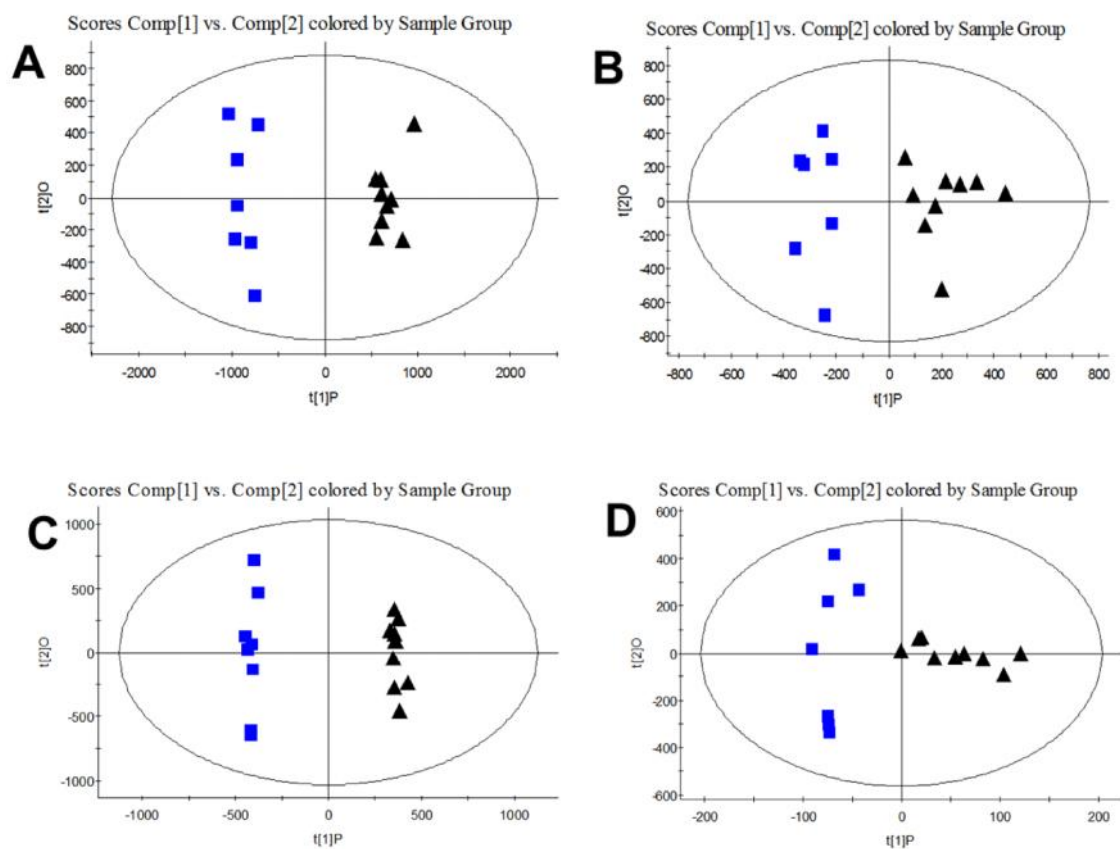
Fig. S5 Typical base peak intensity (BPI) chromatograms obtained from rats serum in control groups (a) and allergy groups (b) at 120 min post-challenge by QKLI. (A) For polar metabolites ES^+ ; (B) For polar metabolites ES^- ; (C) For weak polar metabolites ES^+ ; (D) For weak polar metabolites ES^- .



104

105 **Fig. S6** OPLS-DA score plot comparing allergy group (▲) to control group (■) at 10 min post-challenge. (A) For
 106 polar metabolites ES^+ ; (B) For polar metabolites ES^- ; (C) For weak polar metabolites ES^+ ; (D) For weak polar
 107 metabolites ES^- .

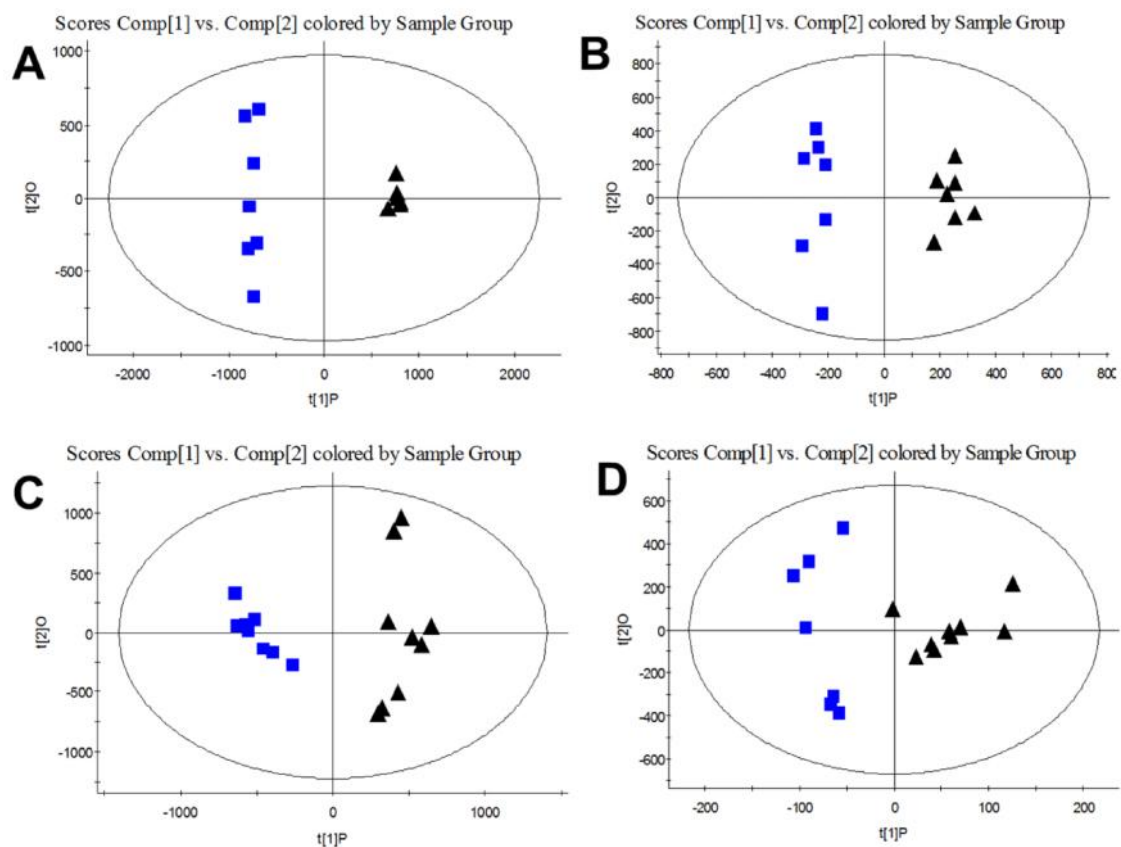
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109

110 **Fig. S7** OPLS-DA score plot comparing allergy group (▲) to control group (■) at 30 min post-challenge. (A) For
 111 polar metabolites ES^+ ; (B) For polar metabolites ES^- ; (C) For weak polar metabolites ES^+ ; (D) For weak polar
 112 metabolites ES^- .

113

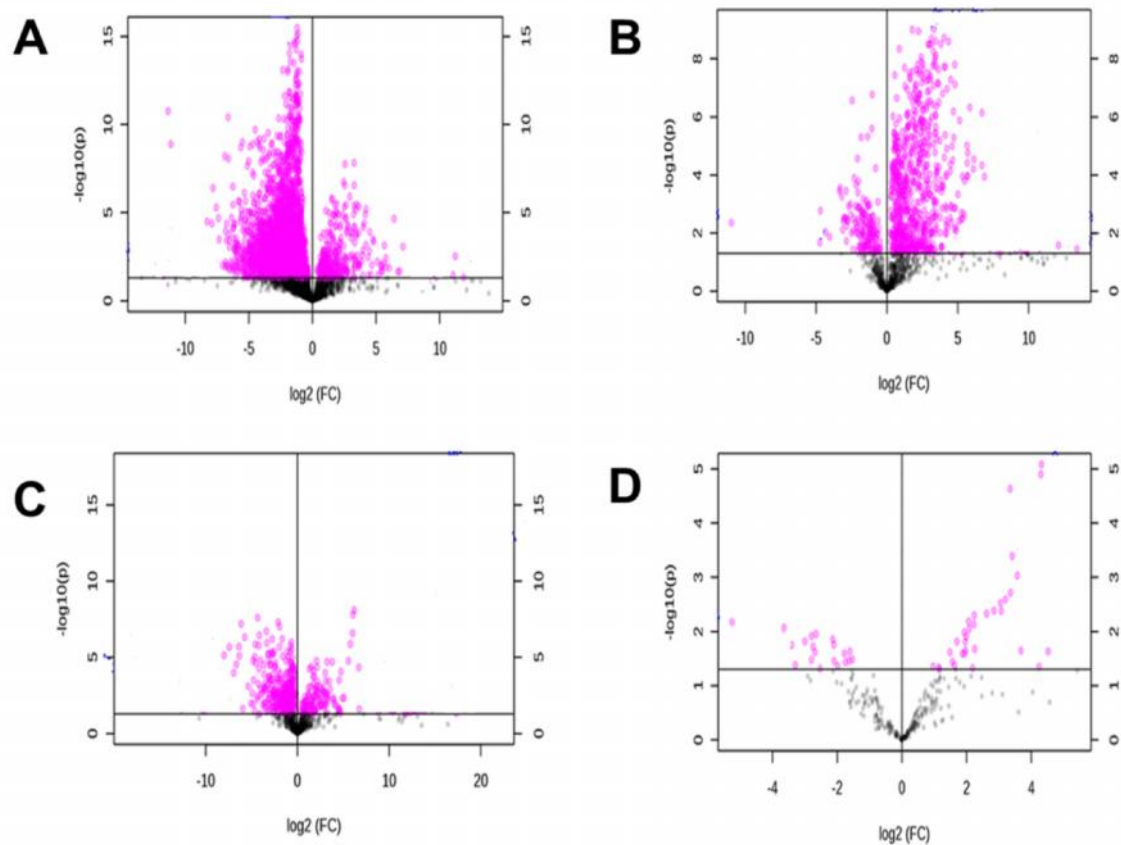


114

115 **Fig. S8** OPLS-DA score plot comparing allergy group (▲) to control group (■) at 120 min post-challenge. (A)

116 For polar metabolites ES^+ ; (B) For polar metabolites ES^- ; (C) For weak polar metabolites ES^+ ; (D) For weak polar

117 metabolites ES^- .



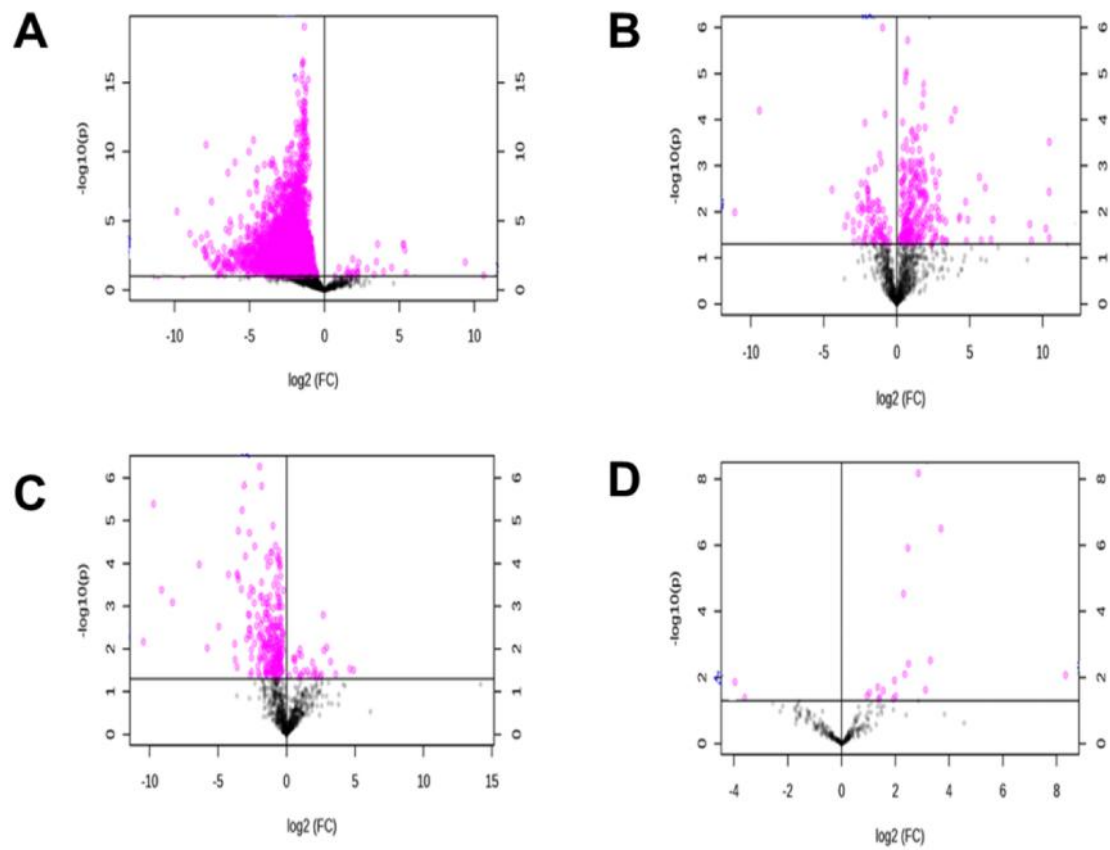
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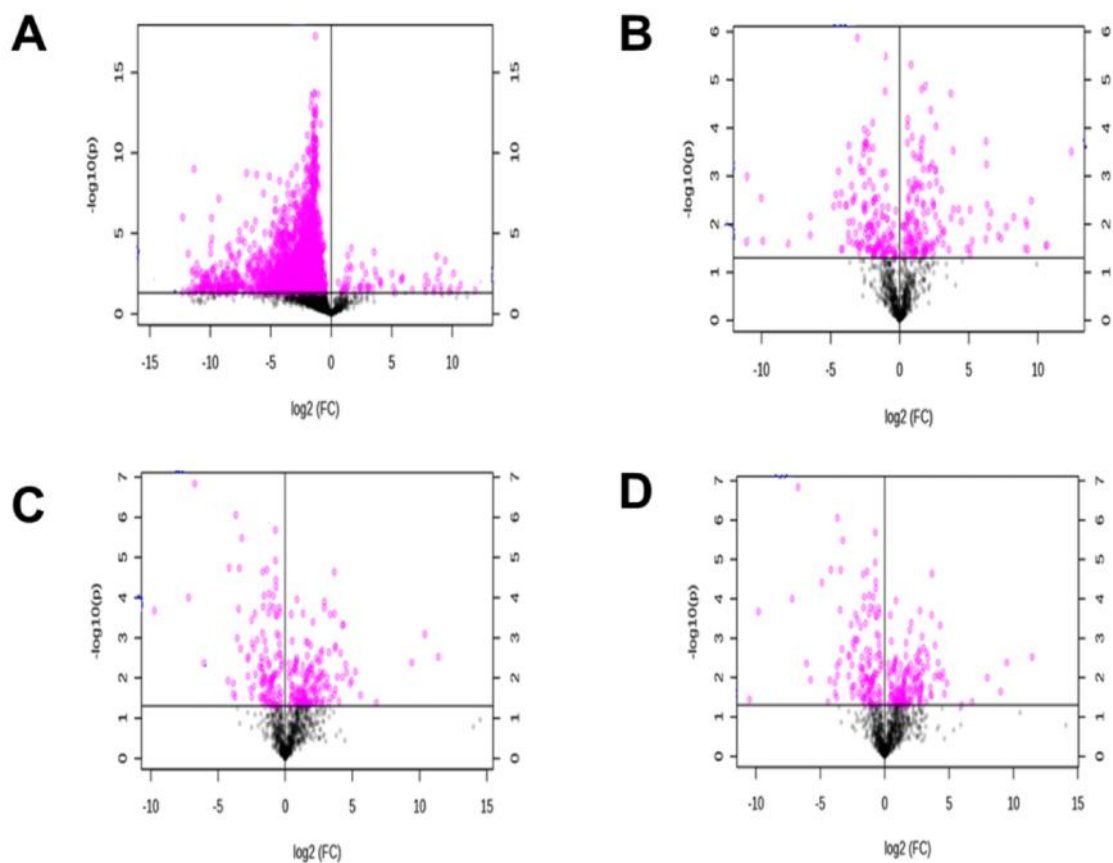
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Fig. S9 Volcano plots of metabolites at 10 min post-challenge comparing allergy group to control group. (A) For polar metabolites ES^+ ; (B) For polar metabolites ES^- ; (C) For weak polar metabolites ES^+ ; (D) For weak polar metabolites ES^- .

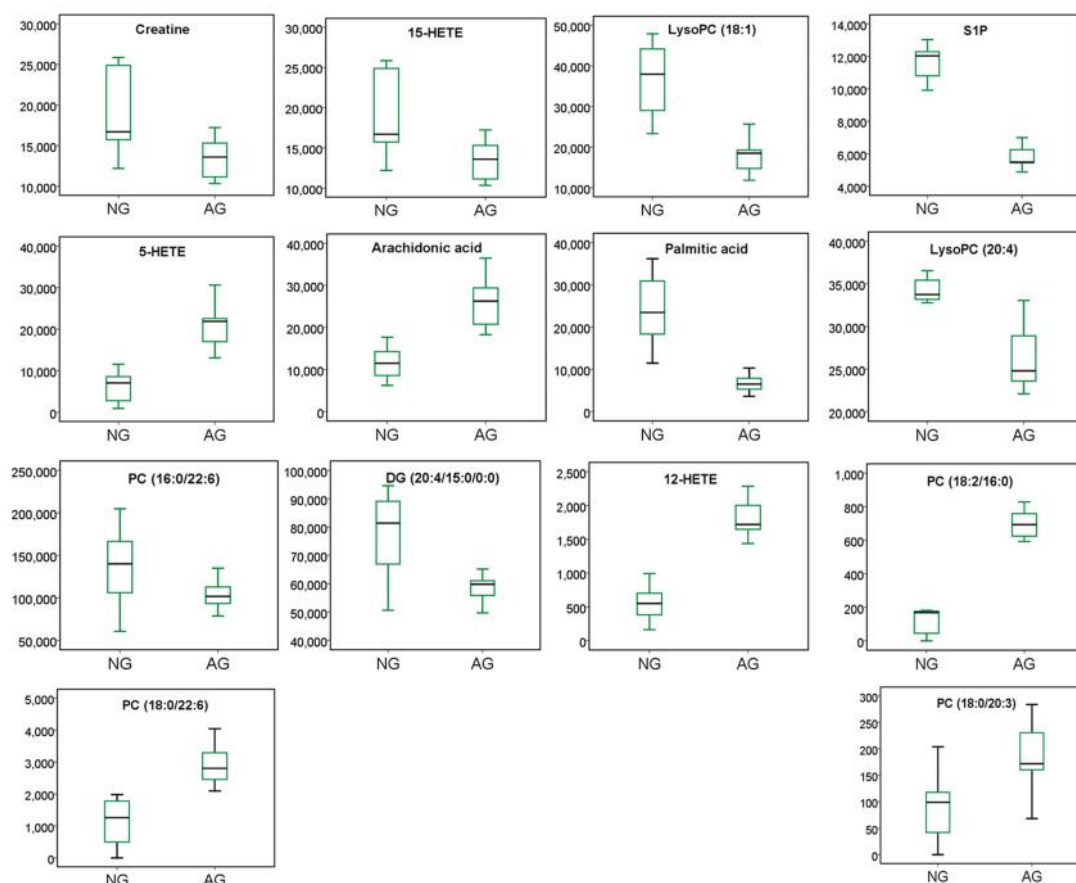


122
 123 **Fig. S10** Volcano plots of metabolites at 30 min post-challenge comparing allergy group to control group. (A) For
 124 polar metabolites ES^+ ; (B) For polar metabolites ES^- ; (C) For weak polar metabolites ES^+ ; (D) For weak polar
 125 metabolites ES^- .



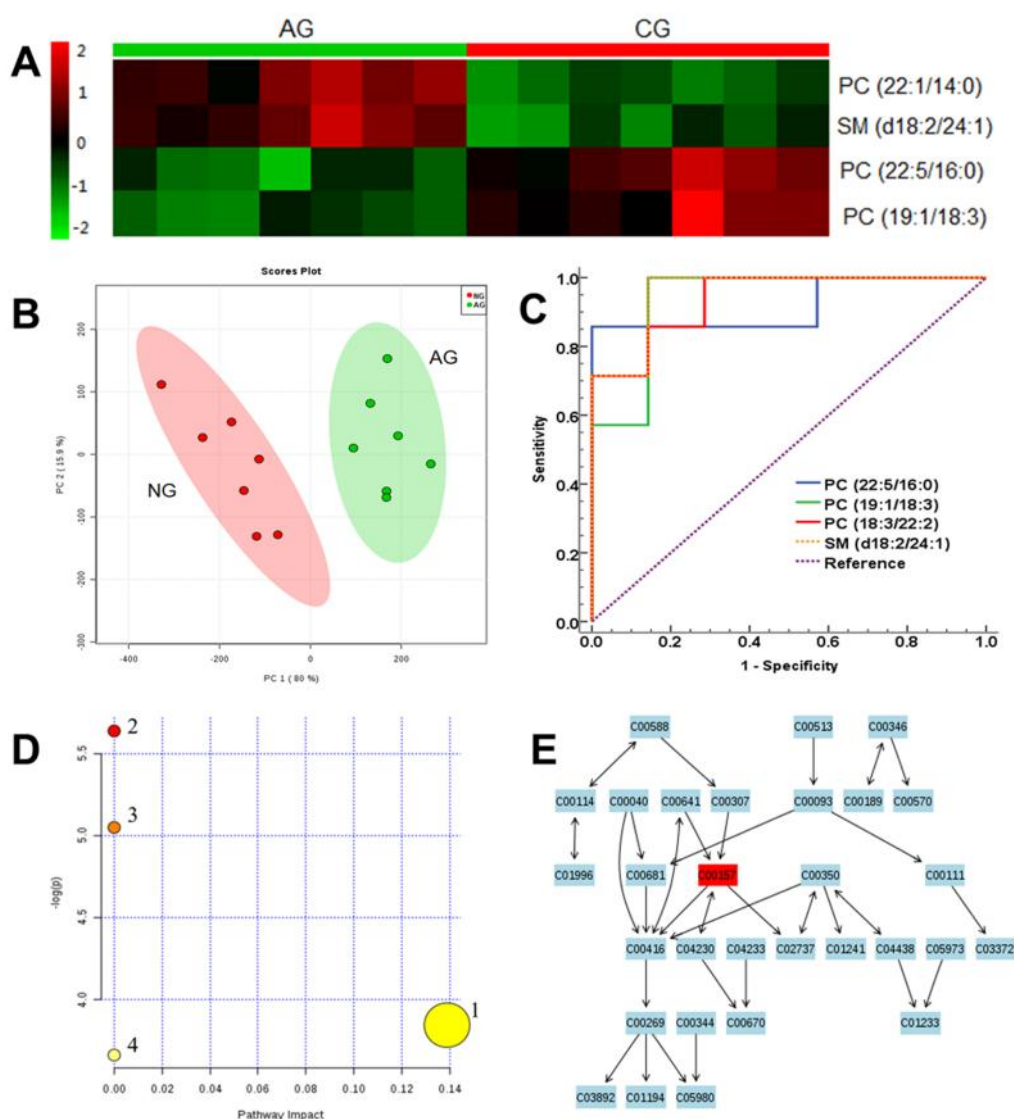
126

127 **Fig. S11** Volcano plots of metabolites at 120 min post-challenge comparing allergy group to control group. (A)
 128 For polar metabolites ES⁺; (B) For polar metabolites ES⁻; (C) For weak polar metabolites ES⁺; (D) For weak polar
 129 metabolites ES⁻.



130

131 **Fig. S12** Boxplots of 14 top-ranked metabolites in serum of control and allergy groups. The metabolites were
 132 creatine, 15-HETE, LysoPC (18:1), S1P, 5-HETE, arachidonic acid, palmitic acid, LysoPC (20:4), PC (16:0/22:6),
 133 DG (20:4/15:0/0:0), 12-HETE, PC (18:2/16:0), PC (18:0/22:6) and PC (18:0/20:3), respectively.



134

135 **Fig. S13** Hierarchical cluster analysis, ROC curves and PCA plot of 4 identified metabolites as well as associated
 136 metabolic pathway in late stage of anaphylaxis. **(A)** Clustered heat map of 4 metabolites appeared at 120 min. **(B)**
 137 PCA analysis showed separation of control group (red) and allergy group (green). **(C)** ROC curves of 4
 138 metabolites. **(D)** Summary of pathway analysis. (1) glycerolphospholipid metabolism; (2) linoleic acid
 139 metabolism; (3) alpha-Linolenic acid metabolism; (4) arachidonic acid metabolism. **(E)** The detailed pathway of
 140 glycerolphospholipid metabolism based on KEGG database.

141

Supplementary Tables

142

Table S1. The size of blue spot in rat skin after immune serum injected

Group	No.	time point (days)	The diameter of blue spot (mm)
Saline	1	--	--
	2	--	--
	3	--	--
QKLI	1	--	--
	2	--	--
	3	--	--
Al(OH) ₃ gel	1	--	--
	2	--	--
	3	--	--
FCA	1	--	--
	2	--	--
	3	--	--
QKLI + Al(OH) ₃ gel	1	at 19th and 25th day	3.0 mm, 5.0 mm
	2	at 19th and 25th day	2.0 mm, 6.0 mm
	3	at 25th day	5.3 mm
QKLI + FCA	1	at 16th and 25th day	1.0 mm, 4.0 mm
	2	at 19th and 25th day	1.0 mm, 4.0 mm
	3	at 25th day	2.0 mm

143

144

Table S2. Serum levels of histamine of rats in allergy groups and control groups

Time (min)	Control (ng/ml)	Allergy (ng/ml)	Fold change	p-value
10	7.3766 ± 2.978	18.2359 ± 8.0212	2.47	P < 0.001
30	7.9159 ± 0.7714	15.3083 ± 3.3043	1.93	P < 0.001
120	6.9749 ± 0.9544	9.6243 ± 31.0309	1.38	P > 0.05

145

146

Table S3. Serum levels of β-hexosaminidase of rats in allergy groups and control groups

Time (min)	Control (OD)	Allergy (OD)	Fold change	p-value
10	0.5823 ± 0.0032	0.8190 ± 0.0063	1.41	P < 0.001
30	0.5288 ± 0.0018	0.7696 ± 0.0260	1.46	P < 0.01
120	0.5913 ± 0.0022	0.8779 ± 0.0580	1.48	P < 0.01

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Table S4. Metabolites selected as biomarkers and their changing trends.

Time-point	No.	tR	m/z	Formula	Name	Trend	FC
10 min	#1	0.7378	203.0530	C ₆ H ₁₂ O ₆	L-(+)-Gulose	↓***	0.53
	#2	0.8659	132.0773	C ₄ H ₉ N ₃ O ₂	Creatine	↓*	0.69
	#3	3.8376	188.0710	C ₁₁ H ₁₁ NO ₃	Indolelactic acid	↓**	0.71
	4	7.9217	785.5913	C ₄₅ H ₈₉ N ₂ O ₆ P	SM(d18:1/22:1)	↑***	2.44
	5	8.0212	817.5806	C ₄₈ H ₈₅ O ₁₀ P	PG(22:1/20:4)	↑****	11.00
					Arachidonic acid		
	6	8.0267	355.2635	C ₂₂ H ₃₆ O ₂	ethyl ester	↑****	2.07
	#7	8.4274	343.2246	C ₂₀ H ₃₂ O ₃	15-HETE	↓****	0.38
					Sphingosine		
	8	8.4583	380.2565	C ₁₈ H ₃₈ NO ₅ P	1-phosphate	↓****	0.49
	9	8.6290	520.3403	C ₂₆ H ₅₀ NO ₇ P	LysoPC(18:2)	↓****	0.51
	10	8.7642	496.3404	C ₂₄ H ₅₀ NO ₇ P	PC(16:0/0:0)	↓****	0.55
	#11	8.8364	522.3556	C ₂₆ H ₅₂ NO ₇ P	LysoPC(18:1)	↓****	0.49
	#12	8.9030	510.3553	C ₂₅ H ₅₂ NO ₇ P	LysoPC(17:0)	↓**	0.49
	#13	9.0071	546.3532	C ₂₆ H ₅₄ NO ₇ P	LysoPC(18:0)	↓**	0.43
	14	9.0933	550.3873	C ₂₈ H ₅₆ NO ₇ P	PC(20:1/0:0)	↓****	0.34
	#15	9.1115	560.3690	C ₂₇ H ₅₆ NO ₇ P	PC(19:0/0:0)	↓**	0.24
	#16	9.2809	359.3159	C ₂₃ H ₄₀ O ₄	MG(0:0/20:3/0:0)	↓****	0.33
	#17	9.5907	360.3241	C ₂₀ H ₄₀ O	Thromboxane	↓****	0.49
	18	10.3964	806.5689	C ₄₆ H ₈₀ NO ₈ P	PC(22:6/16:0)	↓**	0.57

#19	10.4556	804.5505	C ₄₄ H ₈₀ NO ₈ P	PC(16:0/20:4)	↓****	0.46
#20	10.5446	758.5681	C ₄₂ H ₈₀ NO ₈ P	PC(16:0/18:2)	↓****	0.59
21	10.6352	784.5835	C ₄₄ H ₈₂ NO ₈ P	PC(18:2/18:1)	↓****	0.42
22	10.8939	760.5848	C ₄₂ H ₈₂ NO ₈ P	PC(16:0/18:1)	↓****	0.55
#23	11.0143	832.5815	C ₄₆ H ₈₄ NO ₈ P	PC(20:3/18:1)	↓**	0.60
#24	11.1454	808.5813	C ₄₄ H ₈₄ NO ₈ P	PC(18:0/18:2)	↓****	0.57
25	7.9397	391.2848	C ₂₂ H ₃₆ O ₂	Adrenic acid	↑****	1.65
#26	8.4343	319.2274	C ₂₀ H ₃₂ O ₃	5-HETE	↑**	1.33
				Ursodeoxycholic		
27	8.4594	391.2848	C ₂₄ H ₄₀ O ₄	acid	↑****	6.09
28	9.0146	253.2167	C ₁₆ H ₃₀ O ₂	Palmitoleic acid	↓**	0.34
#29	9.0950	303.2325	C ₂₀ H ₃₂ O ₂	Arachidonic acid	↑*	1.36
30	9.1124	279.2327	C ₁₈ H ₃₂ O ₂	Linoleic acid	↓*	0.26
31	9.2353	255.2325	C ₁₆ H ₃₂ O ₂	Palmitic acid	↓*	0.36
32	9.2939	281.2484	C ₁₈ H ₃₄ O ₂	Oleic Acid	↓*	0.32
33	10.9317	612.3294	C ₃₀ H ₅₀ NO ₇ P	LysoPC(22:6)	↓**	1.21
#1	1.2706	544.3399	C ₂₈ H ₅₀ NO ₇ P	LysoPC(20:4)	↓****	0.74
#2	1.3249	520.3400	C ₂₆ H ₅₀ NO ₇ P	LysoPC(18:2)	↓****	0.70
#3	1.5555	496.3401	C ₂₄ H ₅₀ NO ₇ P	LysoPC(16:0)	↓****	0.71
#4	6.7532	806.5684	C ₄₆ H ₈₀ NO ₈ P	PC(16:0/22:6)	↓****	0.75
5	6.8825	832.5846	C ₄₈ H ₈₂ NO ₈ P	PC(20:5/20:2)	↓****	0.06
6	7.6460	758.5695	C ₄₂ H ₈₀ NO ₈ P	PC(18:2/16:0)	↓**	0.33

	7	7.8392	784.5845	C ₄₄ H ₈₂ NO ₈ P	PC(18:1/18:2)	↓**	0.20
	8	8.616	796.5829	C ₄₅ H ₈₂ NO ₈ P	PC(19:1/18:3)	↓**	0.08
	9	9.2352	734.5693	C ₄₀ H ₈₀ NO ₈ P	PC(16:0/16:0)	↓**	0.28
	#10	10.0140	764.5731	C ₄₀ H ₇₈ NO ₈ P	PC(17:0/15:1)	↓**	0.48
	11	10.0179	750.5574	C ₃₉ H ₇₆ NO ₈ P	PC(16:0/15:1)	↓***	0.19
	12	12.4323	788.6162	C ₄₄ H ₈₆ NO ₈ P	PC(22:1/14:0)	↓*	0.31
	13	14.0426	647.4585	C ₃₈ H ₆₆ O ₅	DG(20:4/15:0/0:0)	↓*	0.67
	14	15.4938	944.7698	C ₅₇ H ₁₀₀ O ₆	TG(12:0/20:4/22:1)	↑***	2.59
	#15	16.1819	898.7845	C ₅₇ H ₁₀₀ O ₆	TG(12:0/20:2/22:3)	↑**	4.63
	#16	1.2983	319.2274	C ₂₀ H ₃₂ O ₃	12-HETE	↑**	1.33
	17	1.9376	462.2992	C ₂₃ H ₄₈ O ₇ P	LysoPE(0:0/18:0)	↓*	0.19
	18	2.0833	277.2169	C ₁₈ H ₃₀ O ₂	C18:3n-7,10,13	↓**	0.10
	19	2.6227	279.2323	C ₁₈ H ₃₂ O ₂	Linoelaidic acid	↓**	0.27
	20	6.7634	790.5393	C ₄₆ H ₈₀ NO ₈ P	PC(16:0/22:6)	↑*	2.97
	21	7.6369	742.5383	C ₄₂ H ₈₀ NO ₈ P	PC(18:2/16:0)	↑*	2.29
	22	8.5941	812.5814	C ₄₄ H ₈₂ NO ₇ P	PC(O-16:0/20:4)	↑*	3.42
	23	8.7456	838.5963	C ₄₆ H ₈₄ NO ₇ P	PC(O-18:1/20:4)	↑*	2.99
	24	8.9445	878.5920	C ₄₈ H ₈₄ NO ₈ P	PC(18:0/22:6)	↑*	2.03
	#25	10.8272	856.6081	C ₄₆ H ₈₆ NO ₈ P	PC(18:0/20:3)	↑*	4.13
30 min	#1	0.7429	203.0531	C ₆ H ₁₂ O ₆	L-(+)-Gulose	↓***	0.42
	#2	0.8662	132.0772	C ₄ H ₉ N ₃ O ₂	Creatine	↓**	0.52
	3	3.3539	166.0867	C ₉ H ₁₁ NO ₂	L-Phenylalanine	↓**	0.59

4	3.3555	120.0812	C ₈ H ₁₁ NO	Tyramine	↓**	0.60
#5	3.8495	188.0711	C ₁₁ H ₁₁ NO ₃	Indolelactic acid	↓**	0.55
6	3.8500	205.0977	C ₁₁ H ₁₂ N ₂ O ₂	L-Tryptophan	↓**	0.52
#7	8.4278	343.2247	C ₂₀ H ₃₂ O ₃	15-HETE	↓****	0.33
8	8.6022	566.3218	C ₂₈ H ₅₀ NO ₇ P	PC(20:4/0:0)	↓****	0.37
9	8.6308	520.3399	C ₂₆ H ₅₀ NO ₇ P	LysoPC(18:2)	↓****	0.45
10	8.764	496.3399	C ₂₄ H ₅₀ NO ₇ P	PC(16:0/0:0)	↓****	0.44
#11	8.8382	522.3556	C ₂₆ H ₅₂ NO ₇ P	LysoPC(18:1)	↓****	0.41
#12	8.9025	510.3553	C ₂₅ H ₅₂ NO ₇ P	LysoPC(17:0)	↓****	0.30
#13	9.011	546.3532	C ₂₆ H ₅₄ NO ₇ P	LysoPC(18:0)	↓**	0.35
#14	9.1132	560.3686	C ₂₇ H ₅₆ NO ₇ P	PC(19:0/0:0)	↓**	0.23
#15	9.2826	359.3161	C ₂₃ H ₄₀ O ₄	MG(0:0/20:3/0:0)	↓****	0.31
#16	9.5906	360.3244	C ₂₀ H ₄₀ O	Thromboxane	↓**	0.38
17	10.2324	703.5748	C ₃₉ H ₇₉ N ₂ O ₆ P	SM(d17:1/17:0)	↓****	0.47
18	10.3963	806.5689	C ₄₆ H ₈₀ NO ₈ P	PC(22:6/16:0)	↓**	0.50
#19	10.4608	804.5504	C ₄₄ H ₈₀ NO ₈ P	PC(16:0/20:4)	↓****	0.41
#20	10.5429	758.5684	C ₄₂ H ₈₀ NO ₈ P	PC(16:0/18:2)	↓****	0.54
21	10.6032	830.5663	C ₄₆ H ₈₂ NO ₈ P	PC(20:3/18:2)	↓****	0.38
22	10.6397	784.584	C ₄₄ H ₈₂ NO ₈ P	PC(18:2/18:1)	↓****	0.37
23	10.7997	734.569	C ₄₀ H ₈₀ NO ₈ P	PC(16:0/16:0)	↓****	0.35
24	10.8983	760.5845	C ₄₂ H ₈₂ NO ₈ P	PC(16:0/18:1)	↓**	0.50
25	10.9542	856.5821	C ₄₈ H ₈₄ NO ₈ P	PC(22:6/18:0)	↓****	0.44

#26	11.0486	832.6005	C ₄₆ H ₈₄ NO ₈ P	PC(20:3/18:1)	↓****	0.49
#27	11.1466	786.5815	C ₄₄ H ₈₄ NO ₈ P	PC(18:0/18:2)	↓****	0.52
28	12.4004	835.6658	C ₄₇ H ₉₃ N ₂ O ₆ P	SM(d18:1/24:1)	↓**	0.44
29	3.8476	203.0819	C ₁₁ H ₁₂ N ₂ O ₂	Tryptophan	↓**	0.49
30	7.5884	514.2834	C ₂₆ H ₄₆ NO ₇ P	LysoPC(18:4)	↑*	7.53
#31	8.4328	319.2273	C ₂₀ H ₃₂ O ₃	5-HETE	↑**	1.44
32	9.0141	253.2167	C ₁₆ H ₃₀ O ₂	Palmitoleic acid	↓*	0.46
#33	9.0956	303.2325	C ₂₀ H ₃₂ O ₂	Arachidonic acid	↑**	1.49
1	0.9723	274.2747	C ₁₆ H ₃₅ NO ²	C16 Sphinganine	↓****	0.74
2	1.1839	302.306	C ₁₈ H ₃₉ NO ₂	Sphinganine	↓****	0.69
#3	1.2706	544.3397	C ₂₈ H ₅₀ NO ₇ P	LysoPC(20:4)	↓****	0.66
#4	1.3222	520.3402	C ₂₆ H ₅₀ NO ₇ P	LysoPC(18:2)	↓**	0.75
5	1.4758	184.0736	C ₅ H ₁₄ NO ₄ P	Phosphocholine	↓****	0.59
#6	1.5560	496.6725	C ₂₄ H ₅₀ NO ₇ P	LysoPC(16:0)	↓**	0.70
7	1.6163	522.3557	C ₂₆ H ₅₂ NO ₇ P	LysoPC(18:1)	↓****	0.67
8	2.1329	524.3716	C ₂₆ H ₅₄ NO ₇ P	LysoPC(18:0)	↓**	0.74
9	2.4070	538.3870	C ₂₇ H ₅₆ NO ₇ P	PC(19:0/0:0)	↓****	0.37
10	3.2928	381.2981	C ₂₃ H ₄₀ O ₄	MG(0:0/20:3/0:0)	↓****	0.68
11	4.0649	338.3424	C ₂₀ H ₄₀ O	Thromboxane	↓*	0.64
#12	6.7478	806.5864	C ₄₆ H ₈₀ NO ₈ P	PC(16:0/22:6)	↓*	0.61
13	7.1074	627.5346	C ₄₁ H ₇₂ O ₅	DG(18:0/20:4)	↓**	0.46
14	7.3077	782.5688	C ₄₄ H ₈₀ NO ₈ P	PC(16:0/20:4)	↓**	0.79

	15	7.7727	834.6004	C ₄₈ H ₈₄ NO ₈ P	PC(22:6/18:0)	↓***	0.45
	16	8.4042	796.5843	C ₄₅ H ₈₂ NO ₈ P	PC(18:3/19:1)	↓***	0.41
	17	8.4703	784.5842	C ₄₄ H ₈₂ NO ₈ P	PC(18:1/18:2)	↓*	0.17
	#18	10.0276	764.5731	C ₄₀ H ₇₈ NO ₈ P	PC(17:0/15:1)	↓***	0.63
	19	10.0348	750.5575	C ₃₉ H ₇₆ NO ₈ P	PC(16:0/15:1)	↓*	0.71
	20	14.0275	647.4587	C ₃₈ H ₆₆ O ₅	DG(20:4/15:0/0:0)	↓***	0.71
	#21	16.1508	898.7850	C ₅₇ H ₁₀₀ O ₆	TG(12:0/20:2/22:3)	↑*	4.08
	#22	1.2944	319.2275	C ₂₀ H ₃₂ O ₃	12-HETE	↑***	1.48
	23	2.4033	667.5327	C ₄₃ H ₇₂ O ₅	DG(20:4/20:2/0:0)	↑*	1.74
	24	7.6328	742.5384	C ₄₂ H ₈₀ NO ₈ P	PC(18:2/16:0)	↑***	2.46
	25	6.7572	790.5394	C ₄₆ H ₈₀ NO ₈ P	PC(16:0/22:6)	↑*	2.88
	#26	10.8186	856.6080	C ₄₆ H ₈₆ NO ₈ P	PC(18:0/20:3)	↑*	5.51
120 min	#1	0.7361	203.0530	C ₆ H ₁₂ O ₆	L-(+)-Gulose	↓****	0.49
	#2	0.8637	132.0773	C ₄ H ₉ N ₃ O ₂	Creatine	↓*	0.65
	#3	3.8472	188.0709	C ₁₁ H ₁₁ NO ₃	Indolelactic acid	↓**	0.59
	#4	8.4268	343.2246	C ₂₀ H ₃₂ O ₃	15-HETE	↓****	0.13
	5	8.6026	566.3220	C ₂₈ H ₅₀ NO ₇ P	PC(20:4/0:0)	↓****	0.39
	6	8.6308	520.3399	C ₂₆ H ₅₀ NO ₇ P	LysoPC(18:2)	↓****	0.50
	7	8.7620	496.3404	C ₂₄ H ₅₀ NO ₇ P	LysoPC(16:0)	↓****	0.50
	#8	8.8375	522.3557	C ₂₆ H ₅₂ NO ₇ P	LysoPC(18:1)	↓****	0.42
	#9	8.9017	510.3552	C ₂₅ H ₅₂ NO ₇ P	LysoPC(17:0)	↓****	0.31
	#10	9.0116	546.3711	C ₂₆ H ₅₄ NO ₇ P	LysoPC(18:0)	↓**	0.32

11	9.0920	550.3878	C ₂₈ H ₅₆ NO ₇ P	PC(20:1/0:0)	↓****	0.22
#12	9.1126	560.3689	C ₂₇ H ₅₆ NO ₇ P	PC(19:0/0:0)	↓**	0.24
#13	9.2819	359.6048	C ₂₃ H ₄₀ O ₄	MG(0:0/20:3/0:0)	↓****	0.35
#14	9.5908	360.3242	C ₂₀ H ₄₀ O	Thromboxane	↓**	0.42
15	10.3965	806.5687	C ₄₆ H ₈₀ NO ₈ P	PC(22:6/16:0)	↓*	0.70
#16	10.4568	804.5506	C ₄₄ H ₈₀ NO ₈ P	PC(16:0/20:4)	↓****	0.43
#17	10.5464	758.5684	C ₄₂ H ₈₀ NO ₈ P	PC(16:0/18:2)	↓****	0.67
18	10.6014	830.5661	C ₄₆ H ₈₂ NO ₈ P	PC(20:3/18:2)	↓****	0.37
19	10.6381	784.5843	C ₄₄ H ₈₂ NO ₈ P	PC(18:2/18:1)	↓****	0.44
#20	11.0218	832.5816	C ₄₆ H ₈₄ NO ₈ P	PC(20:3/18:1)	↓**	0.52
#21	11.1468	786.5990	C ₄₄ H ₈₄ NO ₈ P	PC(18:0/18:2)	↓****	0.52
	15.4942	944.7690	C ₅₇ H ₁₀₀ NO ₆	TG(12:0/20:4/22:1)	↑**	2.01
#22	8.4348	319.2272	C ₂₀ H ₃₂ O ₃	5-HETE	↑**	1.13
#23	9.0987	303.2355	C ₂₀ H ₃₂ O ₂	Arachidonic Acid	↑*	1.24
#1	1.2689	544.3400	C ₂₈ H ₅₀ NO ₇ P	LysoPC(20:4)	↓****	0.60
#2	1.3201	520.3405	C ₂₆ H ₅₀ NO ₇ P	LysoPC(18:2)	↓****	0.73
#3	1.5476	496.3403	C ₂₄ H ₅₀ NO ₇ P	LysoPC(16:0)	↓****	0.73
#4	6.7513	806.5683	C ₄₆ H ₈₀ NO ₈ P	PC(16:0/22:6)	↓****	0.59
5	7.3833	808.5845	C ₄₆ H ₈₂ NO ₈ P	PC(22:5/16:0)	↓**	0.58
6	8.3852	796.5843	C ₄₅ H ₈₂ NO ₈ P	PC(19:1/18:3)	↓*	0.47
7	9.4019	760.5845	C ₄₂ H ₈₂ NO ₈ P	PC(16:0/18:1)	↑**	1.81
#8	10.0147	764.5732	C ₄₀ H ₇₈ NO ₈ P	PC(17:0/15:1)	↓*	0.75

9	10.7301	836.6155	C ₄₈ H ₈₆ NO ₈ P	PC(18:3/22:2)	↑*	4.29
10	12.3872	788.6158	C ₄₄ H ₈₆ NO ₈ P	PC(22:1/14:0)	↑**	1.67
11	12.4258	811.6677	C ₄₇ H ₉₁ N ₂ O ₆ P	SM(d18:2/24:1)	↑**	1.77
#12	16.1444	898.7853	C ₅₇ H ₁₀₀ O ₆	TG(12:0/20:2/22:3)	↑**	6.35
#13	1.3002	319.2274	C ₂₀ H ₃₂ O ₃	12-HETE	↑**	1.35
14	1.9537	301.2163	C ₁₈ H ₃₂ O ₂	C18:2n-11,14	↓*	0.22
15	1.9376	462.2992	C ₂₃ H ₄₈ NO ₇ P	LysoPE(0:0/18:0)	↓*	0.19
16	7.6463	742.5378	C ₄₂ H ₈₀ NO ₈ P	PC(18:2/16:0)	↑*	2.36
17	8.9452	878.5920	C ₄₈ H ₈₄ NO ₈ P	PC(18:0/22:6)	↑*	1.92
#18	10.8311	856.6086	C ₄₆ H ₈₆ NO ₈ P	PC(18:0/20:3)	↑*	5.52

149 ↑: Compared with the control group using the peak areas, the metabolite was up-regulated in the allergy group.

150 ↓: Compared with the control group using the peak areas, the metabolite was down-regulated in the allergy group.

151 **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

152 #: The same biomarkers at the three time points of anaphylaxis.