APJAI
*Asian Pacific Journal of*
Allergy and Immunology

ORIGINAL ARTICLE

# Metabolomics analysis of anaphylactoid reaction reveals its mechanism in a rat model

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## Abstract

**Background:** Anaphylactoid reactions, accounting for more than 77% of all immune-mediated immediate hypersensitivity reactions, have become a serious threat to public health, but their effect mechanism is not clear and diagnostic tests are limited. Comprehensive metabolite analysis may reveal the anaphylactoid effect mechanism systematically and provide reference for future diagnostic purposes.

**Methods:** Plasma from Brown Norway rats given intravenous injection of saline, compound 48/80 (2.5 mL/kg) or ovalbumin (20 mL/kg) in 20 s for the first time was used to study the effect mechanism of anaphylactoid reactions through metabolomics (UPLC-qTOF-MS/MS). Metabolomics integrated with proteomics data were used to analyze the anaphylactoid pathways by MetaboAnalyst followed by integrated pathway analysis.

**Results:** Thirty metabolites were identified through the METLIN database by MS/MS and 18 of them were confirmed by authentic standards. The results showed that adenosine, histamine, N-acetylhistamine, N(α)-γ-glutamylhistamine, malate and xanthine are important indices for anaphylactoid reactions. It could be concluded that the effect mechanism is mainly composed of histidine metabolism, arachidonic acid metabolism, energy metabolism, purine metabolism and other small molecules through 30 metabolites. Multiple linear regression analysis indicated that not only histamine but also N(α)-γ-glutamylhistamine and arachidonic acid could be used to evaluate anaphylactoid symptoms of animals. Furthermore, the citrate cycle, histidine metabolism and arachidonic acid metabolism could be the main pathways of anaphylactoid reactions as determined by MetaboAnalyst.

**Conclusion:** The results may provide a reference to improve diagnostic accuracy and predict and monitor treatment efficacy in anaphylactoid reactions in the clinical setting.

**Keywords:** Compound 48/80; Histamine; Anaphylactoid reaction; Metabolomics; Ovalbumin

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## Introduction

An anaphylactoid reaction (pseudoallergy or idiosyncratic reaction) is a nonimmune hypersensitivity reaction whose symptoms are similar to typical anaphylaxis reactions. The first "anaphylactoid" phenomenon was discovered in 1920 and was really affirmed in the 1990s.[1,2] Subsequently, it was determined that a series of substances including liposomes, analgesics, radiologic contrast agents, micelle solvents, vitamin K injection and *Ginkgo biloba* injection can induce anaphylactoid reactions.[3-5] Anaphylactoid reactions are generally recognized as occurring after first exposure to an antigen and are not mediated by pre-existing IgE antibodies.[6] As reported, they account for more than 77% of all immune-mediated immediate hypersensitivity reactions.[6] The anaphylactoid mechanism can be divided into a generation mechanism and an effect mechanism. The generation mechanism had been determined as direct stimulation by antigens[7] followed by activation of a coagulation sequence or the complement pathway,[8,9] but the effect mechanism is not completely clear; current research is

primarily focused on histamine and tryptase.[5,10,11] It is generally known that the effect mechanism is mostly caused by small molecules of blood; thus, metabolomics would be more conducive to explore the anaphylactoid mechanism.

Compound 48/80 (C4880) can induce a mast cell-dependent anaphylactoid reaction, commonly attributed to a direct, receptor-bypassing property to activate G to release histamine from mast cells.[12] Ovalbumin (OVA) is commonly used as the positive control for type I anaphylaxis reactions and can also induce an anaphylactoid reaction,[13] but its effect mechanism has not been studied. In addition, due to good susceptibility, Brown Norway (BN) rats were selected as an ideal animal for the evaluation of anaphylactoid reactions.[14] A previous study showed that C4880 is involved in the direct stimulation pathway, complement and kallikrein–kinin pathways through the coagulation pathway; OVA-induced anaphylactoid reaction could be a combination of the coagulation, classical complement and integrated pathways.[15] Thus, the anaphylactoid mechanisms of BN rats induced by C4880 or OVA were studied for the first time by comprehensive application of metabolomics. The objective of the work presented here was to address the following problems: 1) identification of metabolites of blood related to anaphylactoid reactions; 2) the effect mechanisms of anaphylactoid reactions; 3) pathway analysis of anaphylactoid reactions.

## Methods

### *Reagents*

HPLC-grade methanol, acetonitrile and formic acid were purchased from Merck (Germany). The following compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA): C4880 (purity > 98%), OVA, leucine-enkephalin, valine, tyrosine, adenosine, adenine, histamine, uric acid, glutathione, citric acid, hippuric acid, xanthine, arachidonic acid, serine, aspartic acid, alanine, α-ketoglutaric acid, leukotriene B4 (LTB4), palmitic acid and creatinine. Ultrapure water was prepared with a Milli-Q water purification system (Millipore, France).

### *Animal experiments and sample collection*

Male BN rats (200 ± 20 g) were purchased from WeiTongLiHua Co. (Beijing, China; laboratory animal license, SCXK (army): 2012-0001). The animals were maintained under SPF laboratory conditions (Centre for Animal Experiment of Liaoning University of Traditional Chinese Medicine, Shenyang, China). The rats had ad libitum access to a standard laboratory diet and filtered tap water. All animal procedures were approved by the Liaoning Provincial Animal Welfare and Care Guideline in accordance with the National Institute of Health's guidelines regarding the principles of animal care (2004). Special care was taken to minimize animal discomfort during all procedures in accordance with a previous study.[15] The control group of BN rats (n = 8) received an intravenous (i.v.) injection of sterile normal saline in 20 s, the C4880 group of BN rats (n = 8) was treated with sterile C4880 (2.5 mL/kg, i.v. injection in 20 s) and the OVA group of BN rats (n = 8) was treated with sterile OVA (20 mL/kg, i.v. injection in 20 s); then, anaphylaxis symptoms were observed and evaluated by using a scoring system after the injection as stated previously.[14] The BN rats were sacrificed 30 min after injection; blood samples were collected into chilled tubes containing EDTA-K2, and centrifuged at 2,000 × g for 10 min at 4 °C to obtain plasma which was stored at −80 °C until analysis.

### *Sample preparation and UPLC-qTOF-MS analysis*

The plasma samples were thawed before analysis, and 500 μL acetonitrile was added into 200 μL plasma and vortex mixed for 30 s, followed by centrifugation at 11,600 × g for 3 min at 4 °C. Then, 500 μL free clear supernatant was collected, vacuum-dried and reconstituted at a ratio of 1:1 with 130 μL of acetonitrile–water; after centrifugation at 11,600 × g for 3 min, a 3 μL injection of sample was injected for UPLC-qTOF-MS analysis.

Plasma samples from the three groups were mixed together as the quality control (QC) samples. The QC sample was first analyzed once the instrument was calibrated to condition or equilibrate the analytical platform, and then analyzed after every eight samples to ensure the instrument was same as the pre-conditions during the analytical procedure.[16]

Chromatographic separation was performed on an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.7 μm, Waters Corp., Milford, MA, USA) and a BEH Amide column (2.1 × 100 mm, 1.7 μm, Waters Corp., Milford, MA, USA) using a Waters ACQUITY UPLC system equipped with a binary solvent delivery system. The column was maintained at 45 °C and eluted at a flowing rate of 0.3 mL/min, using a mobile phase of (A) 0.1% (by volume) formic acid in water and (B) methanol (gradient program of T3 column: 0–1 min, 0% B; 1–7 min, 0% B to 60% B; 7–10 min, 60% B to 100% B; 10–14 min, 100% B; 14–14.1 min, 100% B to 0% B; 14.1–16 min, 0% B), a mobile phase of (C) water and (D) acetonitrile (gradient program of amide column: 0–0.5 min, 5% D; 0.5–6 min, 5% D to 30% D; 6–8 min, 30% D to 50% D; 8–10 min, 50% D; 10–10.1 min, 50% D to 5% D; 10.1–12 min, 5% D) and curve: 6, separately. The column eluent was directed to the mass spectrometer without split.

Mass spectrometry was performed on a Waters Xevo G2 QToF quadrupole accelerated time-of-flight mass spectrometer (Waters Corp., Manchester, UK) with an electrospray ionization source (ESI) operating in positive (T3 column and amide column) and negative ion mode (T3 column) separately. The capillary voltage, sampling cone and extraction cone of the positive ion mode were set at 3.0 kV, 20.0 kV and 3.0 kV, and of the negative ion mode were set at 2.5 kV, 35.0 kV and 4.0 kV. Nitrogen was used as the drying gas, the desolvation gas rate was set to 800 L/h at a temperature of 450 °C, the cone gas rate was set at 20 L/h and the source temperature at 110 °C. The scan rate was set at 0.2 s. Leucine-enkephalin was used as the lock mass in all analysis (*m/z* 556.2771 for positive ion mode and *m/z* 554.2615 for negative ion mode). Data were collected in centroid mode from *m/z* 50 to *m/z* 1200.

### *Data processing*

The raw MS spectra were first analyzed using MarkerLynx Applications Manager version 4.1 (Waters Corp., Manchester, UK), which allowed deconvolution, alignment and data reduction to give a list of mass and retention time pairs with

corresponding peak area for all the detected peaks from each file in the data set. The main parameters in MarkerLynx were set as follows: retention time range, 0–14 min; mass range, 50–1200 Da; XIC window, 0.05 Da; automatically calculate peak width and peak-peak baseline noise; use the raw data during the deconvolution procedure; marker intensity threshold (count), 1000; mass tolerance, 0.05 Da; retention time windows, 0.1 min; noise elimination level, 6; deisotopic peaks.

The processed data list was then exported and processed by partial least squares discriminant analysis (PLS-DA) in the software package SIMCA-P (v12.0, Umetrics, Umea, Sweden).

### *Statistical analysis*

All values were expressed as mean ± SD. The significance of differences among the means of the C4880, OVA and control groups was compared through one-way ANOVA test followed by Dunnett's test using the Statistical Package for Social Sciences program (SPSS 20.0, Chicago, IL, USA). Correlation analysis and multiple linear regression analysis were also used to assay the symptom scores of individual BN rats with the metabolites through bivariate and multinomial logistic regression tests using SPSS 20.0. The significance threshold was set at $p < 0.05$ for this test.

## Results

### *Animal behavior study*

The symptoms of BN rats in control groups were regarded as normal; the symptoms of BN rats in the C4880 group showed dyspnea, unsteady gait, myasthenia of limbs, convulsions, spasm and death; the symptoms of BN rats in the OVA group exhibited trembling, nose scratching and shortness of breath. The scores for each group are shown in **Table 1**, and the results indicated that the rats in the OVA and C4880 groups exhibited a serious anaphylactoid reaction.

### *Principal component analysis (PCA) and PLS-DA processing of UPLC-qTOF-MS data*

Within metabolomics, PCA and PLS-DA approaches are frequently used to distinguish metabolic differences between classes. In this work, both PCA and PLS-DA were tried and exhibited satisfactory classification (as shown in **Figures 1 and 2**). The supervised pattern recognition (PLS-DA, which is a classical linear projection method for mapping data to a lower dimension with grouping) was focused more on the actual class discriminating variation in the data compared to the unsupervised approach (PCA, which is a classical linear projection method for mapping data to a lower dimension without grouping). As a consequence, the PLS-DA method was employed to determine the specific variation among the control, C4880 and OVA groups. When the supervised pattern recognition was employed, the integrity of the mathematical model was evaluated first before being used for further interpretation. Commonly, R2Y provides an estimate of how well a model fits the Y data, whereas Q2 is an estimate of how well a model predicts the Y. Both Q2 and R2Y close to 1 indicate an excellent model, whereas their poor ratios are likely to be the onset of model over-fitting.[17] The Q2 and R2 values were calculated from the results in the SIMCA-P package, which were good to fit and predict, respectively. The system stability was monitored by the QC samples which clustered together

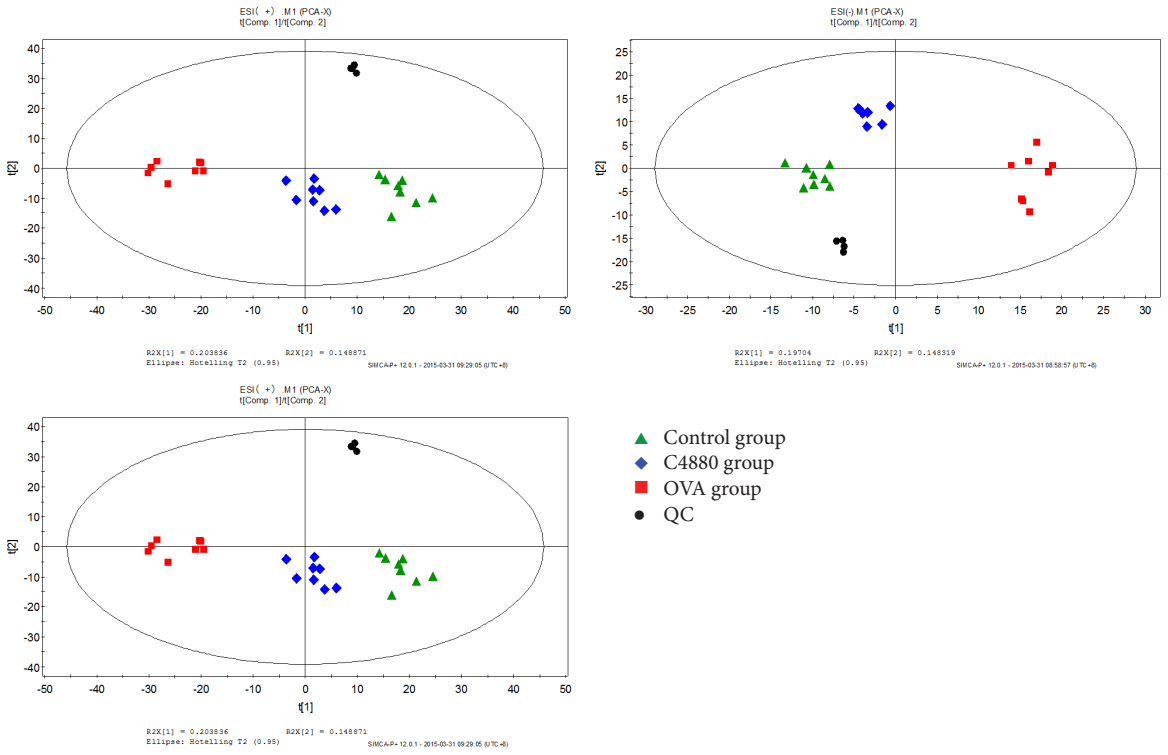


**Figure 1. PCA scores plots of rat plasma data (a) T3 positive mode: Comparison of normal controls, C4880 group and OVA group (b) T3 negative mode: Comparison of normal controls, C4880 group and OVA group (c) amide positive mode: Comparison of normal controls, C4880 group and OVA group**

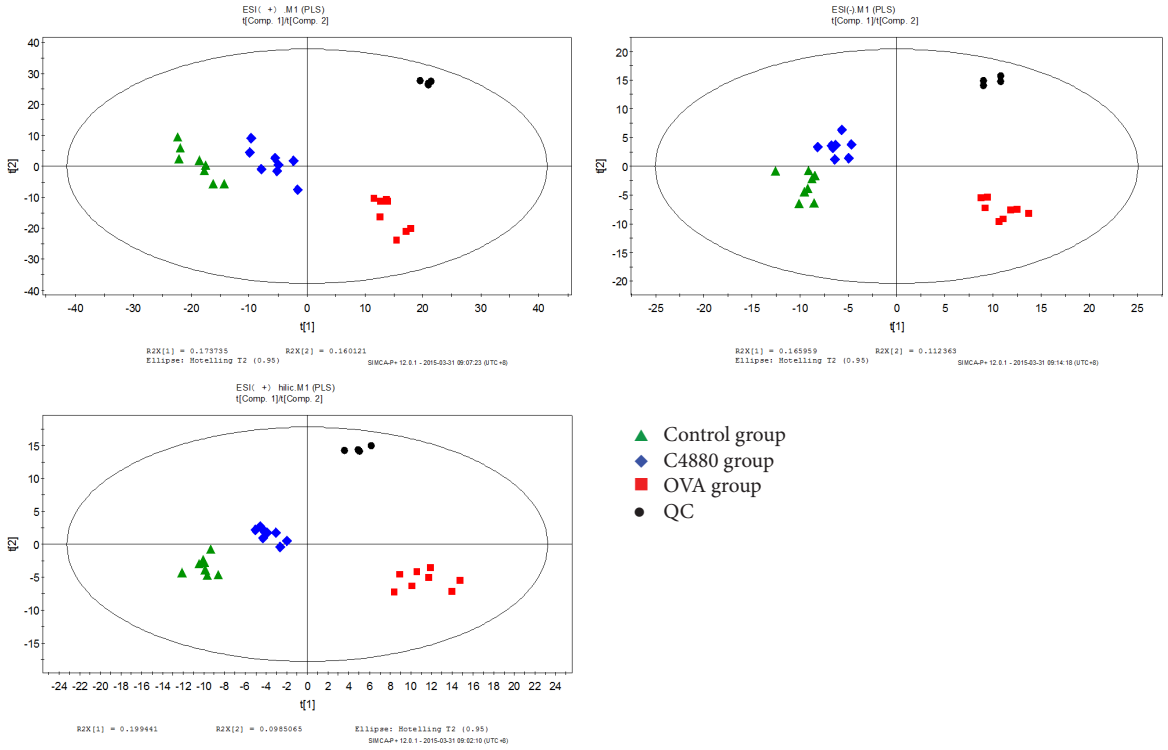


**Figure 2. PLS-DA scores plots of rat plasma data. (a) T3 positive mode: Comparison of normal controls, C4880 group and OVA group (Q2Y(cum)=0.931, R2X(cum) = 0.404, R2Y(cum)= 0.99). (b) T3 negative mode: Comparison of normal controls, C4880 group and OVA group (Q2Y(cum)=0.948, R2X(cum) = 0.415, R2Y(cum)= 0.991). (c) Amide positive mode: Comparison of normal controls, C4880 group and OVA group (Q2Y(cum)=0.885, R2X(cum) = 0.368, R2Y(cum)= 0.989)**

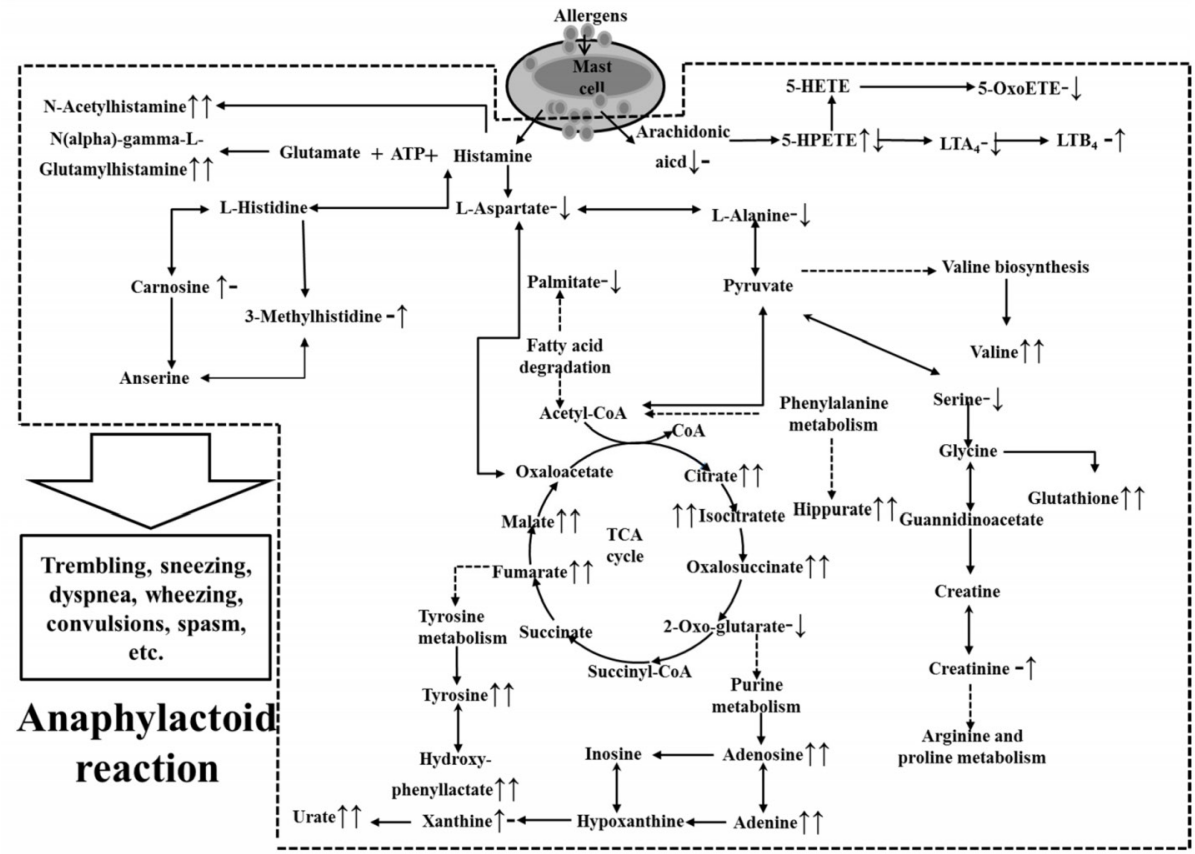


**Figure 3. The effect mechanism study of anaphylactoid reaction according to KEGG nomenclature using metabolomics. The levels of potential biomarkers were labeled with "↑" up-regulated , "↓" down-regulated and "-" no significant difference comparing with control group, the left was C4880 group and the right was OVA group**

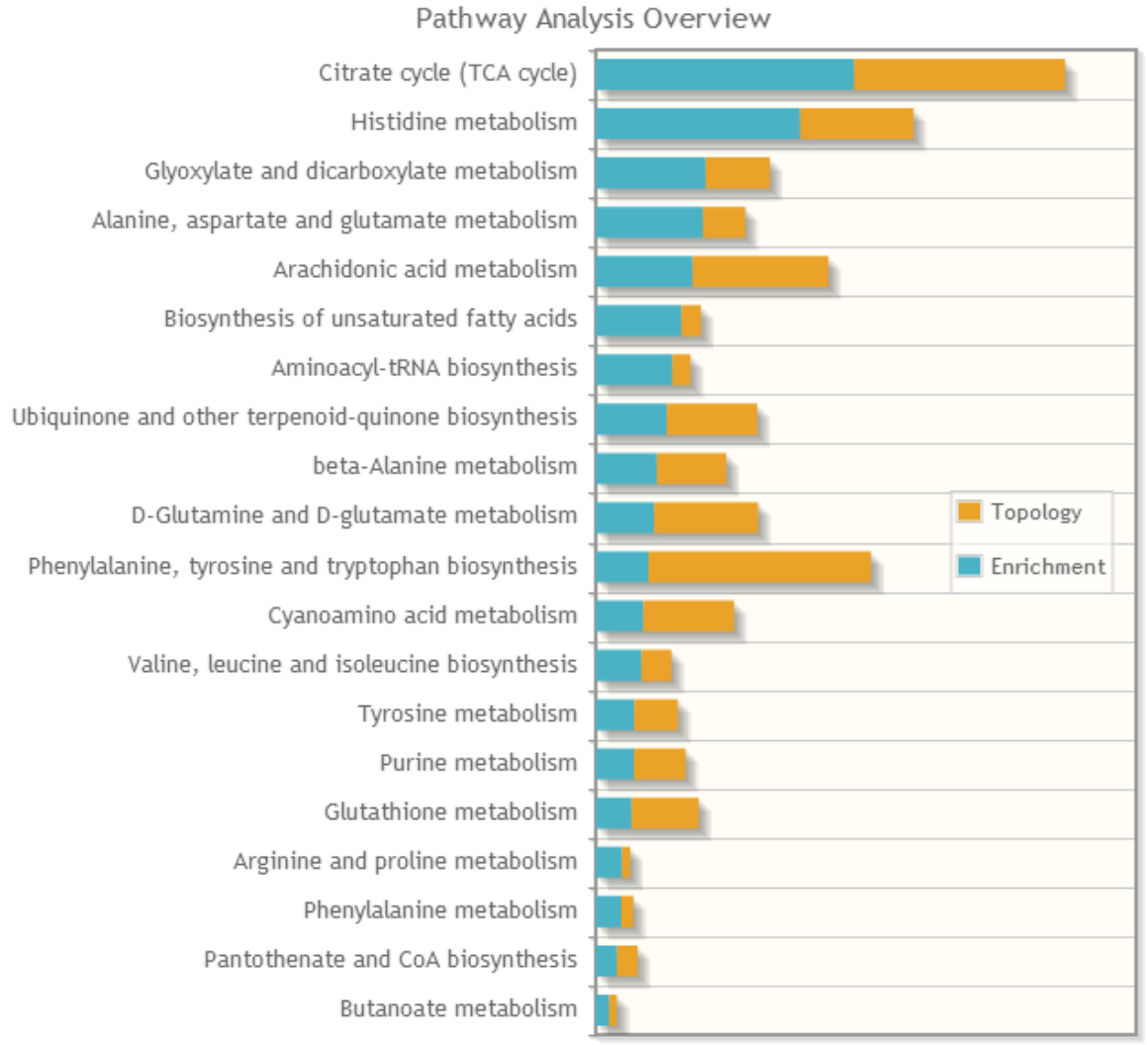


**Figure 4. The topology and enrichment of protein and metabolites data based on the pathway overrepresentation analysis**

tightly in the PCA score plot,[18] and the result indicated that the system stability was accommodative for this metabolomics study (**Figure 1**).

***Identification of potential biomarkers***

Variables (biomarkers) that significantly contributed to the clustering and discrimination were identified according to a threshold of variable importance in the projection (VIP) values (VIP > 1), which could be generated after PLS-DA processing using MarkerLynx software. In order to select potential biomarkers worthy of preferential study in the next step, these differential metabolites were validated using one-way ANOVA test. The critical p-value was set to 0.05 for significantly differential variables in this study. Following the criteria above, 30 significantly differential biomarkers were selected for further study.

Identification of these biomarkers was then carried out as follows, and the results are listed in **Table 2**.

Firstly, the calculated mass, mass deviation (mDa and ppm), DBE (total number of rings and double bonds in a molecule), i-FIT value (the likelihood that the isotopic pattern of the elemental composition matches a cluster of peaks in the spectrum) and elemental compositions associated with the measured mass of the candidate metabolites were generated and studied using MarkerLynx. The freely accessible METLIN (http://metlin.scripps.edu), KEGG (http://www.genome.jp) and HMDB (http://www.hmdb.ca) databases were also used to identify the structure of the metabolites.

As a result, 30 metabolites were identified on the basis of accurate elemental compositions and context of retention time with the available databases. Due to the absence of fragmentation information for some metabolites, as compensation, 18 of them were confirmed with available

**Table 1. The symptom scores of BN rats**

| group | Dosage (mg/kg) | Judgment of the typical symptom score /count | | | | | Symptom scores |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | |
| Control | - | 10 | 0 | 0 | 0 | 0 | 0 |
| C4880 | 10 | 0 | 0 | 0 | 7 | 1 | 3.12±0.35 |
| OVA | 400 | 0 | 6 | 2 | 0 | 0 | 1.25±0.46 |

**Table 2. Potential biomarkers related to non-allergic hypersensitivity detected by UPLC-Q/TOF MS[a]**

| No. | $t_R$ (min) | *m/z* | Ion mode | Elemental composition | Identification results | KEGG | Control group | C4880 group | OVA group | C4880 group* | OVA group* | VIP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 5.40 | 118.0866 | $[M+H]^+$ | $C_5H_{11}NO_2$ | valine [b] | C00183 | 1.36±0.15 | 1.63±0.18 | 1.92±0.3 | + (↑) | + (↑) | 1.21 |
| 2 | 5.53 | 182.0817 | $[M+H]^+$ | $C_9H_{11}NO_3$ | tyrosine [b] | C00082 | 18.1±1.81 | 21.55±1.97 | 25.14±3.79 | + (↑) | + (↑) | 1.23 |
| 3 | 3.58 | 268.1045 | $[M+H]^+$ | $C_{10}H_{13}N_5O_4$ | adenosine [b] | C00212 | 0.21±0.04 | 0.77±0.14 | 0.45±0.09 | + (↑) | + (↑) | 2.30 |
| 4 | 3.52 | 136.0599 | $[M+H]^+$ | $C_5H_5N_5$ | adenine [b] | C00147 | 0.23±0.04 | 0.31±0.06 | 0.32±0.06 | + (↑) | + (↑) | 1.25 |
| 5 | 7.34 | 112.0872 | $[M+H]^+$ | $C_5H_9N_3$ | histamine [b] | C00388 | 0.17±0.03 | 8.36±1.59 | 1.7±0.33 | + (↑) | + (↑) | 2.38 |
| 6 | 1.11 | 169.036 | $[M+H]^+$ | $C_5H_4N_4O_3$ | uric acid [b] | C00366 | 2.02±0.4 | 2.76±0.34 | 2.77±0.55 | + (↑) | + (↑) | 1.23 |
| 7 | 1.01 | 308.0926 | $[M+H]^+$ | $C_{10}H_{17}N_3O_6S$ | glutathione [b] | C00051 | 0.25±0.05 | 0.47±0.09 | 0.6±0.11 | + (↑) | + (↑) | 1.35 |
| 8 | 1.11 | 191.0193 | $[M-H]^-$ | $C_6H_8O_7$ | citric acid [b] | C00158 | 9.42±0.78 | 11.55±1.69 | 19±3.24 | + (↑) | + (↑) | 1.37 |
| 9 | 5.19 | 178.0505 | $[M-H]^-$ | $C_9H_9NO_3$ | hippuric acid [b] | C01586 | 15.57±3.08 | 26.44±5.28 | 52.33±9.89 | + (↑) | + (↑) | 1.42 |
| 10 | 4.13 | 154.0977 | $[M+H]^+$ | $C_7H_{11}N_3O$ | N-Acetylhistamine | HMDB (13253) | 0.43±0.06 | 1.47±0.29 | 1.7±0.34 | + (↑) | + (↑) | 1.82 |
| 11 | 7.59 | 241.13 | $[M+H]^+$ | $C_{10}H_{16}N_4O_3$ | N(alpha)-gamma-L-Glutamylhistamine | C04138 | 0.58±0.1 | 1.44±0.27 | 1.01±0.2 | + (↑) | + (↑) | 2.18 |
| 12 | 4.65 | 181.0501 | $[M-H]^-$ | $C_9H_{10}O_4$ | Hydroxyphenyllactic acid | C03672 | 1.77±0.3 | 2.49±0.46 | 7.09±1.01 | + (↑) | + (↑) | 1.47 |
| 13 | 1.81 | 191.0194 | $[M-H]^-$ | $C_6H_8O_7$ | Isocitrate | C00311 | 0.77±0.14 | 1.53±0.30 | 2.76±0.46 | + (↑) | + (↑) | 1.46 |
| 14 | 1.81 | 189.0005 | $[M-H]^-$ | $C_6H_6O_7$ | Oxalosuccinate | C05379 | 0.11±0.02 | 0.21±0.04 | 0.31±0.06 | + (↑) | + (↑) | 1.47 |
| 15 | 1.09 | 115.0039 | $[M-H]^-$ | $C_4H_4O_4$ | Fumarate | C00122 | 1.13±0.19 | 1.83±0.31 | 1.68±0.31 | + (↑) | + (↑) | 1.52 |
| 16 | 1.08 | 133.0136 | $[M-H]^-$ | $C_4H_6O_5$ | Malate | C00711 | 2.42±0.47 | 4.01±0.74 | 3.53±0.64 | + (↑) | + (↑) | 1.53 |
| 17 | 7.72 | 227.1144 | $[M+H]^+$ | $C_9H_{14}N_4O_3$ | Carnosine | C00386 | 0.66±0.12 | 0.9±0.16 | 0.67±0.16 | + (↑) | — | 1.50 |
| 18 | 4.42 | 153.041 | $[M+H]^+$ | $C_5H_4N_4O_2$ | xanthine [b] | C00385 | 0.23±0.05 | 0.51±0.09 | 0.2±0.04 | + (↑) | — | 2.13 |
| 19 | 9.84 | 335.2217 | $[M-H]^-$ | $C_{20}H_{32}O_4$ | 5-HPETE | C05356 | 16.58±2.47 | 21.85±3.32 | 7.75±1.48 | + (↑) | + (↓) | 1.43 |
| 20 | 11.18 | 303.2326 | $[M-H]^-$ | $C_{20}H_{32}O_2$ | arachidonic acid [b] | C00219 | 601.5±34.76 | 566.35±30.36 | 618.88±50.14 | + (↓) | — | 1.07 |
| 21 | 6.77 | 106.0502 | $[M+H]^+$ | $C_3H_7NO_3$ | serine [b] | C00065 | 10.09±0.9 | 9.13±1.57 | 8.64±0.89 | — | + (↓) | 1.04 |
| 22 | 6.81 | 134.0452 | $[M+H]^+$ | $C_4H_7NO_4$ | aspartic acid [b] | C00402 | 0.57±0.13 | 0.51±0.08 | 0.4±0.1 | — | + (↓) | 1.02 |
| 23 | 6.04 | 90.0554 | $[M+H]^+$ | $C_3H_7NO_2$ | alanine [b] | C01401 | 5.79±0.75 | 5.86±0.29 | 4.27±0.52 | — | + (↓) | 1.22 |
| 24 | 1.11 | 145.0142 | $[M-H]^-$ | $C_5H_6O_5$ | α- ketoglutaric acid [b] | C00026 | 0.59±0.11 | 0.57±0.1 | 0.41±0.05 | — | + (↓) | 1.06 |

[a] "↑" means a higher level of biomarkers, whereas "↓" represents a lower level of biomarkers. All data were representing intensities values of biomarkers. "+" means a statistically significant difference ($p < 0.05$), whereas "-" represents no statistically significant difference. [b] Confirmed with authentic standards. * Compared to normal control. Valine, tyrosine, adenosine, adenine, glutathione, xanthine, serine, aspartic acid, alanine, Creatinine and 3-Methylhistidine were detected and identified from BEH Amide column; the others were detected and identified from HSS T3 column.

**Table 2. Potential biomarkers related to non-allergic hypersensitivity detected by UPLC-Q/TOF MS[a] (Continues)**

| No. | $t_R$ (min) | *m/z* | Ion mode | Elemental composition | Identification results | KEGG | Control group | C4880 group | OVA group | C4880 group* | OVA group* | VIP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 9.92 | 335.2219 | $[M-H]^-$ | $C_{20}H_{32}O_4$ | leukotriene B4 [b] | C02165 | 0.01±0 | 0.01±0 | 1.8±0.3 | — | + (↑) | 1.51 |
| 26 | 11.34 | 255.2337 | $[M-H]^-$ | $C_{16}H_{32}O_2$ | palmitic acid [b] | C00249 | 902.07±19.17 | 885.47±49.01 | 665.45±103.98 | — | + (↓) | 1.32 |
| 27 | 0.87 | 114.0663 | $[M+H]^+$ | $C_4H_7N_3O$ | Creatinine [b] | C00791 | 11.66±1.37 | 11.45±0.71 | 15.18±1.1 | — | + (↑) | 1.44 |
| 28 | 7.60 | 170.0929 | $[M+H]^+$ | $C_7H_{11}N_3O_2$ | 3-Methylhistidine | C01152 | 39.62±5.33 | 41.88±4.06 | 57.96±5.4 | — | + (↑) | 1.26 |
| 29 | 10.23 | 317.2113 | $[M-H]^-$ | $C_{20}H_{30}O_3$ | 5-OxoETE | C14732 | 38.41±6.91 | 40.31±6.86 | 16.68±1.58 | — | + (↓) | 1.08 |
| 30 | 10.38 | 317.2113 | $[M-H]^-$ | $C_{20}H_{30}O_3$ | LTA4 | C00909 | 12.6±2.4 | 14.72±2.03 | 5.95±1.16 | — | + (↓) | 1.23 |

[a] "↑" means a higher level of biomarkers, whereas "↓" represents a lower level of biomarkers. All data were representing intensities values of biomarkers. "+" means a statistically significant difference ($p < 0.05$), whereas "-" represents no statistically significant difference. [b] Confirmed with authentic standards. * Compared to normal control. Valine, tyrosine, adenosine, adenine, glutathione, xanthine, serine, aspartic acid, alanine, Creatinine and 3-Methylhistidine were detected and identified from BEH Amide column; the others were detected and identified from HSS T3 column.

reference standards by matching their retention time and accurate mass measurement. The concentrations of 19 biomarkers (nos. 1–19) were significantly increased and one (no. 20) decreased in the C4880 group, and 19 biomarkers (nos. 1–16, 25, 27 and 28) were significantly increased and eight (nos. 19, 21–24, 26, 29 and 30) decreased in the OVA group compared with the normal control group. The effect mechanism of the anaphylactoid reaction was explored using the 30 metabolites according to the KEGG database (**Figure 3**).

## Discussion

Acute allergic reaction mainly includes type I anaphylaxis and anaphylactoid reaction, and the latter is often caused by injections, such as *Gingko biloba* and Taxol injections. More and more attention has been paid to it recently. Previous studies found that BN rats are more suitable for anaphylactoid evaluation and OVA can induce anaphylactoid reactions. C4880 is well recognized to induce a mast cell-dependent, non-specific anaphylactoid reaction.[7] So, metabolomics were used to study the anaphylactoid mechanism, to find the potential biomarkers of anaphylactoid reactions.

Histamine, an early diagnostic marker belonging to the immediate phase medium which has been described as a "gold standard" for acute allergic monitoring, is a typical mediator which causes various pathophysiologic events in anaphylactoid reactions.[2,19] When the histamine level was ≤ 1 ng/mL, there were no symptoms; at 1–2 ng/mL, there was only skin response; at 3 ng/mL, there was a systemic reaction; at >100 ng/mL, there was a serious reaction (allergic shock).[20] The study showed that the histamine level was higher in the C4880 group than the OVA group, so serious systemic reactions were caused by C4880 but mild symptoms such as trembling were caused by OVA, revealing that the concentration of histamine is intimately correlated with nonallergic symptoms of animals. Besides that, according to the VIP, the histamine metabolites N-acetylhistamine (VIP = 1.82) and N(α)-γ-glutamylhistamine (VIP = 2.18) were upregulated in both groups. Research has shown that injecting mice with more than 60 μg N-acetylhistamine can lead to low temperature,[21] thus the trembling phenomenon of BN rats may be related to that. This also showed that an anaphylactoid reaction causes abnormality of histidine metabolism, and N-acetylhistamine and N(α)-γ-glutamylhistamine could be used as potential biomarkers of histidine metabolism for anaphylactoid reactions.

Leukotrienes, belonging to the new synthetic medium, are abnormal synthesis products of arachidonic acid through lipoxygenase pathway metabolism, and are also biomarkers of mast cell degranulation.[22,23] This study showed that arachidonic acid, LTA4, LTB4, 5-HPETE and 5-OxoETE present different levels after injection with C4880 and OVA, suggesting that the body exhibits different immune responses to different allergens.[24,25] Our results showed that anaphylactoid reactions cause arachidonic acid metabolism abnormality.

An in vitro study concluded that histamine release from mast cells consumes massive energy and the progress also facilitates oxygen uptake.[26] Such stress often promotes the TCA cycle to produce more ATP. The elevation of citrate, isocitrate, oxalosuccinate, fumarate and malate after injection with

C4880 and OVA further supports the conclusion. Moreover, oxidative stress is closely related to anaphylactoid reactions.[27] To overcome the reactive oxygen species produced by oxidative stress, glutathione peroxidase is activated.[28] It is therefore reasonable to observe the elevation of glutathione. Obviously, anaphylactoid reactions could cause the disorder of energy metabolism. Due to its high VIP, malate (VIP = 1.53) could be used as a potential biomarker of energy metabolism for anaphylactoid reactions.

Interestingly, the levels of adenosine, adenine and urate were upregulated in both groups. Urate is a compound released during severe physical or metabolic stress conditions and a high level of urate is indicative of a high rate of cell death or damage.[29] A study showed that the purine metabolic pathway is involved in the development of anaphylactoid reaction,[30] and adenosine (VIP = 2.30) could be used as a potential biomarker of purine metabolism for anaphylactoid reactions.

Correlation analysis and multiple linear regression analysis were also used to assay the symptom scores of individual BN rats with the metabolites. The results of correlation analysis indicated that histamine (R = 0.922, p = 0.000), adenosine (R = 0.849, p = 0.000), N(α)-γ-glutamylhistamine (R = 0.833, p = 0.000), xanthine (R = 0.824, p = 0.000) and arachidonic acid (R = −0.546, p = 0.006) were correlated with symptom scores. Moreover, multiple linear regression was used to confirm the result and the equation is described as follows:

$$\hat{Y}_{\text{symptom scores}} = 2.970 + 0.252X_{\text{Histamine}} + 0.948X_{\text{N(α)-γ-glutamylhistamine}} - 0.006X_{\text{Arachidonic acid}}$$

The results showed that histamine, N(α)-γ-glutamylhistamine and arachidonic acid were independently correlated with symptom scores (R = 0.650, p = 0.000; R = 0.267, p = 0.015; and R = 0.002, p = 0.023; respectively). Thus, histamine, N(α)-γ-glutamylhistamine and arachidonic acid are suggested to be used as potential biomarkers for evaluating anaphylactoid symptoms of animals.

As discussed above, the levels of a number of metabolites and proteins (F13b, Prdx2, Sc5b9 and C4d) were upregulated in both groups; F12, Cndp1, MYLPF and DCD levels were downregulated in both groups; C1qa and Gpx1 levels were downregulated in the C4880 group; the Kng1 level was upregulated in the C4880 group; and Serpind1, C6 and C2 levels were downregulated in the OVA group. The proteomics data shown previously 15 were identified and an in depth functional study was carried out. The integrated pathway analysis by MetaboAnalyst indicated that the TCA cycle, histidine metabolism and arachidonic acid metabolism are the main anaphylactoid reaction pathways according to topology and enrichment (**Figure 4**).

## Conclusion

To the best of our knowledge, this is the first study to analyze the anaphylactoid mechanism by the positive compounds through metabolomics in a preclinical setting.

Obviously, anaphylactoid reactions can cause the disorder of histidine metabolism, energy metabolism and arachidonic acid metabolism. Adenosine, histamine, N-acetylhistamine, N(α)-γ-glutamylhistamine, malate and xanthine (VIP = 2.30, 2.38, 1.82, 2.18, 1.53 and 2.13, respectively) are important factors in anaphylactoid reactions. Histamine, N(α)-γ-gluta-mylhistamine and arachidonic acid could be used as potential biomarkers for evaluating anaphylactoid symptoms.

## Acknowledgements

This project was supported by the funding of the National Natural Science Foundation of China (81373939).

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