Sputum metabolomic profiling reveals metabolic pathways and signatures associated with inflammatory phenotypes in patients with asthma

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September 24, 2021

Abstract

Background: The molecular links between metabolism and inflammation that drive different inflammatory phenotypes in asthma are poorly understood. **Objectives:** To identify the metabolic signatures and underlying molecular pathways of different inflammatory asthma phenotypes. **Method:** In the discovery set (n=119), untargeted ultra-high performance liquid chromatography–mass spectrometry (UHPLC-MS) were applied to characterize the induced sputum metabolic profiles from asthmatic patients classified by different inflammatory phenotypes using orthogonal partial least-squares discriminant analysis (OPLS-DA) and pathway topology enrichment analysis. In the validation set (n = 114), differential metabolites were selected to perform targeted quantification. Correlations between targeted metabolites and clinical indexes in asthma patients were analyzed. Logistic and negative binomial regression models were established to assess the association between metabolites and severe asthma exacerbation. **Results:** 77 differential metabolites were identified in the discovery set. Pathway topology analysis uncovered that histidine metabolism, glycerophospholipid metabolism, nicotinate and nicotinamide metabolism, linoleic acid metabolism, phenylalanine, tyrosine and tryptophan biosynthesis were involved in the pathogenesis of different asthma phenotypes. In the validation set, 24 targeted quantification metabolites were significantly differentially expressed between asthma inflammatory phenotypes. Finally, adenosine 5'-monophosphate (RRadj = 1.000, 95%CI = [1.000, 1.000], $P = 0.050$), allantoin (RRadj = 1.000, 95%CI = [1.000, 1.000], $P = 0.043$) and nicotinamide (RRadj = 1.001, 95%CI = [1.000, 1.002], $P = 0.021$) were demonstrated to predict severe asthma exacerbation rate ratios. **Conclusions:** Different inflammatory asthma phenotypes have specific metabolic profiles in induced sputum. The potential metabolic signatures may serve as identification and therapeutic target in different inflammatory asthma phenotypes.

Introduction

Asthma is a serious global health problem of chronic inflammatory airway disease, which is characterized by airway hyperresponsiveness and reversible airway obstruction resulting in recurrent episodes of wheezing, shortness of breath, chest tightness, and cough¹. Asthma is a heterogeneous disease with different phenotypes and endotypes, within which the inflammatory phenotypes of asthma (eosinophilic asthma [EA], neutrophilic

asthma [NA], and paucigranulocytic asthma [PGA]) are widely recognized by differential proportions of induced sputum granulocytes². Although some guidelines recommend tailored management approaches for patients with different inflammatory phenotypes^{1,3,4}, treatment strategy for specific inflammatory phenotypes especially neutrophilic asthma is lacking and represents an unmet medical need in asthma^{5,6}. Meanwhile, the molecular mechanisms driving different inflammatory phenotypes asthma are poorly understood and are likely heterogeneous. Therefore, it is important to identify the specific biomarkers and to understand the molecular mechanism of different inflammatory phenotypes of asthma, which may lead to more personalized medicine⁷.

Metabolomics is the systematic identification and quantitation scientific study of chemical processes involving metabolites, small molecule substrates, intermediates and products of cell metabolism in a given organism or biological sample^{8,9}. Metabolomics represent an integrated pathophysiological profile encompassing genetic and environmental interactions, which can be instrumental in elucidating the biological mechanisms of asthma. There is growing evidence that metabolomic profiles in exhaled breath condensate (EBC), urine, and blood could distinguish asthma and asthma phenotypes¹⁰. Our previous study has also showed for the first time that the metabolite profiles in induced sputum better separate asthma phenotypes¹¹. Although some studies have shown that inflammatory asthma phenotypes can be discriminated by an electronic nose breath analyzer¹², there are no specific metabolic biomarkers and molecular pathways identified in terms of different inflammatory asthma phenotypes in the study. Therefore, it can be more justified with the use of induced sputum for studies that aimed at obtaining a close molecular mechanism of different inflammatory asthma phenotypes.

The primary aim of this study was to identify the metabolic signatures and underlying molecular pathways of different inflammatory asthma phenotypes. The secondary aim was to explore if these signatures are related to asthma control and exacerbation. We applied ultra-high performance liquid chromatography–mass spectrometry (UHPLC-MS) to characterize the induced sputum metabolic profiles from healthy controls and asthmatic patients classified by different inflammatory phenotypes as EA, NA and PGA in the discovery set. Following multivariate modelling, the concentrations of selected metabolites were confirmed with targeted mass spectrometry assays in the validation set.

Methods

Study design and subjects

This study included two parts with the first part as a cross-sectional study to identify the metabolic signatures and underlying molecular pathways of different inflammatory asthma phenotypes and the second part as a prospective cohort study to explore the association between those identified signatures in the first part and asthma exacerbation in the 12 month followed-up. This study included patients with asthma based on the Australasian Severe Asthma Network (ASAN)¹³. Asthma participants in the discovery set (n=119) of the cross-sectional study and in the validation set (n=114) of the prospective cohort study were recruited from the Asthma Clinic of West China Hospital, Sichuan University, and 20 healthy volunteers were recruited by advertisement between March 2014 and April 2019. All of patients were aged [?] 18 years with stable asthma (no respiratory tract infection and no exacerbation or systemic corticosteroid use in the previous 4 weeks). Asthma was diagnosed according to American Thoracic Society (ATS) guidelines¹⁴ and Global Initiative for Asthma (GINA)¹. The subjects were excluded if they were pregnant or breast feeding, had cognitive impairment, current solid organ malignancy. Asthma inflammatory phenotype was assigned based on a sputum eosinophil cutoff of greater than 3% and a sputum neutrophil cutoff of greater than 61% (Patients with a sputum proportion of < 61% neutrophils and < 3% eosinophils were classified as PGA, with [?] 61% neutrophils and < 3% eosinophils classified as NA, with < 61% neutrophils and [?] 3% eosinophils classified as EA, respectively)². This study was reviewed and approved by the institutional review board of West China Hospital, Sichuan University (Chengdu, China) (No.2014-30), and all subjects provided written informed consent.

Clinical assessment

Data on demographics and clinical characteristics were collected using standardized case report form and detailed were shown in the Online Data Supplement. Asthma Control Questionnaire (ACQ) score¹⁵, and Asthma Quality of Life Questionnaire (AQLQ) score¹⁶, were analyzed in all participants. In the validation set, asthma-related exacerbations in the 12 month follow-up were recorded according to the ATS/European Respiratory Society (ERS) guidelines¹⁷

Sputum induction, processing and airway inflammatory cytokine assay

Sputum induction and processing were performed based on standard methods as described in our previous studies^{18,19}. Additional details are described in the Online Data Supplement. In the validation set, sputum supernatant cytokines, regarded as airway inflammatory biomarkers, including IL-1 β , IL-5, IL-8, IL-13, IL-17A, IFN- γ , and TNF- α , were detected using a Luminex-based MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel Kit (EMD Millipore Corporation, Billerica, Mass) with the MILLIPLEX Analyst 5.1 software. Spiking experiments on cytokines in sputum supernatants showed that recovery ranged from 70% to 130% in all detectable analyses^{20,21}

Ultra high performance liquid chromatography–mass spectrometry method for metabolomics

In the discovery set, the collected sputum supernatant samples were thawed on ice, and metabolites were extracted with acetonitrile and methanol using previously described method^{22, 23}. Sputum supernatant samples were analyzed through using an UHPLC system (1290, Agilent Technologies) with a UPLC BEH Amide (2.1 mm $\times$ 100 mm, 1.7 μ m) coupled to TripleTOF 6550+6600 (Q-TOF, AB Sciex) via a previously described method with modifications²⁴. The detailed sputum preparation, spectrometry method and metabolite identification were described in the Online Data Supplement.

Univariate and multivariate analysis for metabolomic profiling

After high-throughput screening of the metabolic features using the above rules, a total of 772 (473 from positive ion mode and 299 from negative ion mode). For each metabolite peak reproducibly detected in whole samples, the unpaired two-tailed Wilcoxon rank-sum test (a nonparametric univariate method) was carried out to measure the significance of each metabolite among different groups and adjusted by false discovery rate (FDR) correction (Benjamini–Hochberg method). The merged UHPLC-MS data were log-transformed for subsequent analyses. The filtered and normalized data matrix was exported for multivariate statistical analysis using principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) with SIMCA 16.1 software package (Umetrics, Umea, Sweden). To further validate the model, the permutation tests ($n = 200$) was proceeded. By combining the univariate and multivariate statistical analysis, on the basis of a variable importance in the projection (VIP) threshold of 1 from the 10-fold cross-validated OPLS-DA model, fold change < 0.83 or > 1.2 , FDR < 0.05 , numbers of different metabolites among different groups were obtained.

Pathway analysis

Metabolomics Pathway Analysis (MetPA) had been performed by online tool MetaboAnalyst with hypergeometric test for over-representation analysis and relative-betweenness centrality for pathway topology analysis²⁵

Targeted quantification in the validation set

After untargeted metabolomics analysis, 40 differential metabolites were selected to perform quantification in patients in the validation set. Metabolites were assayed in multiple reaction monitoring (MRM) mode by LC-MS/MS using an EXION LC System (SCIEX) coupled with SCIEX 6500 QTRAP + Triple Quadrupole mass spectrometry (SCIEX). Details were described in the supplementary material.

Statistics analysis

Continuous variables were expressed as the mean $\pm$ standard deviation or median (quartiles [Q]1, [Q]3) depending on their distribution. Categorical variables are summarized as numbers and percentages. For continuous variables, the differences among different inflammatory asthma phenotypes and healthy were

analyzed using A student's t-test, or ANOVA if they were normally distributed, otherwise Wilcoxon rank-sum test were used to assess. Categorical data were compared using the chi-squared test or Fisher's test. Correlations between targeted metabolites and clinical indexes in asthma patients in the validation set were expressed using Spearman's correlation coefficients and adjusted by false discovery rate (FDR) correction (Benjamini–Hochberg method). Between two groups, targeted metabolites were examined by receiver operating characteristic (ROC) curves and the area under the curve (AUC) of each ROC was calculated to show their capability of discriminating different groups in validation set. Logistic and negative binomial regression models were established to assess the association between metabolites and severe asthma exacerbation during follow-up in the validation set. These analyses were conducted using SPSS version 25.0 (IBM Corp, Armonk, NY, USA). In all statistical analyses, a double tailed P -value of < 0.05 was considered statistically significant.

Results

Demographics and Clinical characteristics

Characteristics of the participants are summarized in Table 1 and Table E1-2. There were 20 healthy control subjects and 119 asthma patients (40 patients with EA, 36 patients with NA and 43 patients with PGA) recruited in the discovery set and 114 asthma patients (35 patients with EA, 30 patients with NA and 49 patients with PGA) recruited in the validation set. Detail demographics and clinical characteristics were described in the Online Data Supplement.

In the validation set, sputum supernatant cytokines, regarded as airway inflammatory biomarkers were assessed. Amongst the asthma phenotypes, patients with EA had the highest level of IL-5 and the lowest levels of TNF- α , IL-1 β , IL-8 (all $P < 0.05$). However, patients with NA had the highest IL-1 β and the lowest IL-5 levels (all $P < 0.05$) (Table 1). In the 12 month follow-up, there were 3(10%) patients with NA and 3(6.1%) PGA who were loss to follow-up. Finally, there were 108 patients with asthma (EA, $n=35$; NA, $n=27$; PGA, $n=46$) were included in the analysis with asthma exacerbations. As a result, there were significant difference in proportions of patients experiencing severe asthma exacerbation (EA vs NA vs PGA: 17.1% vs 37% vs 6.5%, $P = 0.004$) and severe asthma exacerbation times (EA vs NA vs PGA: 0.29 ± 0.75 vs 0.70 ± 1.33 vs 0.09 ± 0.35 , $P = 0.004$) between these phenotypic groups (Table 1).

Untargeted metabolic profiles discriminate between different inflammatory asthma phenotypes and healthy subjects in the discovery set

The score plot of OPLS-DA analysis showed a distinct separation between different asthma phenotypes and the Asthma group was also clearly separated from the Healthy group (Figure 1). The parameters for the classification models from the software were showed in the Table E3, suggesting all of the model had a very good fitness and predictive capability. Furthermore, a leave-one-out cross-validation (LOOCV) was used to estimate the robustness and predictive ability of each model, and thus a 200 permutation test was applied. The low value of the Q^2 intercept in each model indicated the robustness of these model and thus showed a low risk of over-fitting in all of the comparison.

By combining the univariate and multivariate statistical analysis, on the basis of a variable importance in the projection (VIP) threshold of 1 from the 10-fold cross-validated OPLS-DA model, fold change < 0.83 or > 1.2 , FDR < 0.05 , a total of 77 differential metabolites were screened between different asthma phenotypes and healthy subjects as shown in the heatmap in the Figure 2.

Pathway topology analysis in the discovery set

To analyze the most relevant pathways after identifying differential metabolites, the metabolic pathway topology enrichment analysis was applied with the impact value to evaluate the importance of the pathways underlying the pathophysiology mechanism in different asthma phenotypes and healthy subjects (Figure 3). As a result, histidine metabolism ($P = 0.019$; impact value = 0.189) was identified between asthma patients and healthy subjects. Differential metabolites between EA and NA were involved in glycerophospholipid metabolism ($P = 0.015$; impact value = 0.121), nicotinate and nicotinamide metabolism ($P = 0.020$; impact

value = 0.332) and histidine metabolism ($P = 0.022$; impact value = 0.189). Differential metabolites between EA and PGA were involved in glycerophospholipid metabolism ($P = 0.001$; impact value = 0.186), linoleic acid metabolism ($P = 0.001$; impact value = 1.000), histidine metabolism ($P = 0.014$; impact value = 0.189) and phenylalanine, tyrosine and tryptophan biosynthesis ($P = 0.046$; impact value = 0.500). Differential metabolites between NA and PGA were involved in linoleic acid metabolism ($P = 0.002$; impact value = 1.000), glycerophospholipid metabolism ($P = 0.010$; impact value = 0.160) and nicotinate and nicotinamide metabolism ($P = 0.015$; impact value = 0.332).

Targeted quantification of identified metabolites between different inflammatory asthma phenotypes and healthy subjects in the validation set

To validate and determine the expression levels of 40 differentially expressed metabolites, which were selected to perform targeted quantification in patients in the validation set. Amongst which, 24 metabolites were significantly differentially expressed between asthma inflammatory phenotypes (Table 2), with 9 of these metabolites reaching a significant P value of .001 or less. Histamine expression was highest but levels of 5-L-glutamyl-L-alanine, nicotinamide, dihydrothymine, L-leucine, L-phenylalanine, alanyl-leucine, phenylalanyl-serine, phenylalanylphenylalanine were the lowest in patients with EA. Expression levels of adenosine 5'-monophosphate, glyceric acid, taurine, dihydrothymine, L-leucine, tyramine, L-glutamate, alanyl-leucine, phenylalanyl-serine, threoninyl-phenylalanine were highest but level of glycerophosphocholine was lowest in patients with NA. Expression levels of glycerophosphocholine, cyclohexylamine, heptadecanoic acid, oleic acid were highest but level of dodecanoic acid was lowest in patients with PGA (Table 2).

Furthermore, those differentially expressed metabolites were examined by receiver operating characteristic (ROC) curves to discriminate different inflammatory asthma phenotypes (Table E4). As a result, taurine, alanyl-leucine, phenylalanyl-serine and threoninyl-phenylalanine could be used as a candidate metabolite marker to discriminate between NA and EA or PGA with the AUC was range from 0.816 to 0.975 (all $P < 0.05$). However, no single metabolite could satisfactorily discriminate between EA and PGA as all of the AUC < 0.7 .

Correlations of differential metabolites with clinical and inflammatory profiles in the validation set

Correlations analysis showed that differentially expressed metabolites correlated to lung function, asthma control and inflammatory profiles in all subjects with asthma (Figure 3 and Table E5) with the detail in the Online Data Supplement.

We explored the association between metabolites and severe asthma exacerbation during the 12 month follow-up using logistic regression and negative binomial regression models (Table E6). As a result, adenosine 5'-monophosphate was significantly associated with severe asthma exacerbation (Table 3). Adenosine 5'-monophosphate had increased rate ratios of proportions of patients experiencing severe asthma exacerbation (RRadj = 1.000, 95%CI = [1.000, 1.000], $P = 0.050$) and had increased rate ratios of severe asthma exacerbation times (RRadj = 1.000, 95%CI = [1.000, 1.000], $P = 0.008$) after adjusting for age, gender, BMI and FEV₁%. In addition, allantoin and nicotinamide both had increased rate ratios of severe asthma exacerbation times (RRadj = 1.000, 95%CI = [1.000, 1.000], $P = 0.043$, and RRadj = 1.001, 95%CI = [1.000, 1.002], $P = 0.021$, respectively).

Discussion

To our knowledge, this is the first study that establishes the metabolic signatures and underlying molecular pathways of different inflammatory asthma phenotypes in induced sputum sample by combing untargeted and targeted metabolomics. This study demonstrated that the unique metabolic profiles that existed between different inflammatory asthma phenotypes and healthy subjects with 77 differential metabolites identified. Pathway topology analysis uncovered that 5 pathways could be involved in the pathogenesis of different asthma phenotypes. Further, 24 targeted quantification metabolites were validated significantly differentially expressed between asthma inflammatory phenotypes, which significantly correlated to clinical and

inflammatory profiles. Finally, adenosine 5'-monophosphate, allantoin and nicotinamide were shown to predict rate ratios of severe asthma exacerbations. These findings indicated novel immunometabolic mechanisms in different asthma phenotypes, with providing more phenotypic and therapeutic implications.

There have been many metabolomics studies on the difference between asthmatics and healthy subjects or on distinct asthma severity have been performed extensively^{10, 26}. As for the metabolome research on inflammatory phenotype, some studies have shown that inflammatory asthma phenotypes can be discriminated by an electronic nose breath analyzer¹². Brinkman et al²⁷ further demonstrated that inflammatory phenotypes of severe asthma could be identified by unbiased clustering of exhaled breath profiles using eNose technology. However, eNose technology seems to be suitable for the noninvasive identification of asthma inflammatory phenotypes but is principally unable to identify the individual metabolic pathway and metabolites driving the distinction between the subgroups.

This current study unraveled that histidine metabolism, glycerophospholipid metabolism, nicotinate and nicotinamide metabolism, linoleic acid metabolism, and phenylalanine, tyrosine and tryptophan biosynthesis were involved in the pathogenesis of different asthma phenotypes. In fact, previous studies supported our results as histidine metabolism was identified as implicated pathway between asthma and healthy subjects²⁸, and glycerophospholipid metabolism was also participated in the pathogenesis of asthma, especially EA^{29, 30}. In addition, linoleic acid metabolism was the most significant pathway between NA and EA or PGA (all impact value =1, $P < 0.05$), which indicated that the important role of linoleic acid metabolism in the NA. Panda et al.³¹ had proved that linoleic acid metabolite leads to steroid resistant asthma features partially through NF- κ B. Therefore, it is speculated that the development of targeted treatment of linoleic acid metabolism has important implications for the individualized treatment of NA.

Notably, it found that differential metabolites correlated to clinical and inflammatory profiles. Adenosine-5'-monophosphate was related with poor asthma control and severe asthma exacerbation. The discovery that adenosine-5'-monophosphate levels are increased in the bronchoalveolar lavage fluid of patients with asthma and increase further after allergen challenge raises the possibility that the adenosine generated in asthmatic airways itself contributes to the pathogenesis of asthma^{32, 33}. It is possible that the recruitment and activation of inflammatory cells and subsequent smooth muscle contraction that occur during asthma exacerbation leads to an increase in oxygen and energy demand, thus leading to an increase in the level of adenosine in the airway in asthma³⁴. We also demonstrated for the first time that allantoin and nicotinamide were involved in severe asthma exacerbation. The roles of these metabolites in asthma pathogenesis and clinical outcomes of asthma remain unknown and further studies are required to their involvement in asthma.

As for the inflammation markers, histamine was significantly related to FeNO and IL-5. Histamine has been shown to play a key role in the pathogenesis of a variety of allergic diseases, including allergic asthma³⁵. Except for the partial expression of histamine by immune cells such as basophil³⁶, mast cells are the main source of histamine expression and express a large number of different receptors on their cell surface, such as Fc ϵ R1, Fc γ R1, complement receptors (C3AR and C5AR), and ligand receptors such as neurotrophic factor, substance P, vasoactive intestinal peptide, and adenosine phosphate³⁷⁻⁴⁰. These ligand and allergens can activate mast cells to release the pro-inflammatory mediators including histamine. Furthermore, histamine has been shown to promote T_H cell differentiation and the release of type 2 inflammatory factors such as IL-5. Consistently, antihistamine has been demonstrated to significantly reduce the risk of emergency visit and hospitalization for asthma, especially in asthmatic patients with other allergic diseases, such as allergic rhinitis^{41, 42}. Therefore, antihistamine therapy may be considered as an additive treatment for eosinophilic asthma, as it is often associated with allergic diseases such as allergic rhinitis, as also found in current study.

Various amino acids and amino acid dipeptides such as taurine, alanyl-leucine were significantly correlated to TNF- α , IL-1 β , IL-8 and IL-17, which indicated that these differential metabolites may play a very important role in the development of NA. For example, Taurine, a β -amino acid that is not integrated into proteins, is highly expressed in the intracellular chambers of most tissues. Taurine has been reported to be increased in bronchoalveolar lavage fluid in asthmatic patients^{43, 44}. It has been shown that plasma taurine is formed with arachidonic acid in asthmatic patients⁴⁵. Animal studies have also shown that branching-chain amino

acids valine, leucine and isoleucine mediated asthma-related airway inflammation through lipid oxidation pathways⁴⁶. Notably, this study also found a number of amino dipeptides that had not been reported in previous studies, and their differential expression were closely related to neutrophilic asthma. Because induced sputum specimens are biological specimens of open airway, they do not simply reflect the changes of human metabolism, but also may reflect the changes of local airway microenvironment, including local microbiota and human host. In fact, recent studies have shown a significant correlation between neutrophilic asthma and airway microbiology⁴⁷. Therefore, this study illustrated important differences of these amino acid dipeptides may reflect the metabolic characteristics of airway microorganisms of NA, and the analysis combined with microbiology may provide us with further scientific evidence.

This study has several limitations to be addressed. First, an external validation in a new cohort was lacking. However, the differential metabolites were validated by exploring the relationships of differential metabolites with clinical and inflammation profiles of asthma in an independent cohort population would strengthen the results. Second, as for the unavailability of reference standards, some of the identified metabolites in the discovery set are undetected, which somewhat limits the exploring of the potential metabolic signature.

In conclusion, different inflammatory asthma phenotypes have specific metabolic profiles with 77 differential metabolic signatures and 5 underlying molecular pathways identified. These metabolic pathways identified involve the histidine metabolism, glycerophospholipid metabolism, nicotinate and nicotinamide metabolism, linoleic acid metabolism, phenylalanine, tyrosine and tryptophan biosynthesis. Differential metabolites identified correlate to clinical and inflammatory profiles of asthma, which may serve as potential therapeutic target in different inflammatory asthma phenotypes.

ACKNOWLEDGMENTS

The authors are grateful to Ms Michelle Gleeson (Hunter Medical Research Institute, University of Newcastle, Australia), Ms Dan Wang (West China Hospital, Sichuan University, China), and Ms Zhi Lin (West China Hospital, Sichuan University, China) for their blood and sputum processing, and to all patients who volunteered for this study.

Author's contributions

GW and YL conceived the study, performed the data interpretation and manuscript revision, and took accountability for all aspects of the work. YL, XZ, LZ, HGW, ZPL planned the work, carried out the data analysis and interpretation. YL drafted the manuscript. YL, XZ, LZ and GW conducted the participant recruitment. BGO, ZHC, LW, ACYH, MX, VM, WML, DL, FML, HJW interpreted the results and contributed to the manuscript revision. All authors approved the final manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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Table 1. Clinical characteristics grouped by airway inflammation phenotypes in the validation set	Table 1. Clinical characteristics grouped by airway inflammation phenotypes in the validation set	Table 1. Clinical characteristics grouped by airway inflammation phenotypes in the validation set	Table 1. Clinical characteristics grouped by airway inflammation phenotypes in the validation set	Table 1. Clinical characteristics grouped by airway inflammation phenotypes in the validation set	Table 1. Clinical characteristics grouped by airway inflammation phenotypes in the validation set
Variables	EA	NA	PGA	$\chi^2/\Phi/Z$	<i>P</i> value
n	35	30	49		
Female, n (%)	24(68.6)	19(63.3)	24(49)	3.584	0.167
Age, yrs	45.37±10.58	51.94±13.34 ^b	44.10±12.08 ^d	4.220	0.017
BMI, kg/m ²	23.68±2.86	23.28±3.78	24.63±4.13	1.404	0.250
Family history, n (%)	10(28.6)	14(46.7)	15(30.6)	2.844	0.241
Age onset, yrs	34.00(8.00,42.00)	39.00(22.50, 50.75)	33.00(16.50, 39.50)	3.050	0.218
Asthma duration, yrs	11.62(3.31, 35.26)	10.92(4.61, 22.22)	10.21(4.06, 20.42)	0.661	0.719
Severe asthma, n (%)	3(8.6)	5(16.7)	5(10.2)	1.170	0.557
BDP, ug	200(80, 400)	400(400, 1000)	400(0, 800)	12.516	0.002
LTRA, n (%)	15(42.9)	10(33.3)	18(36.7)	0.659	0.719
Theophylline, n (%)	3(8.6)	10(33.3)	11(22.4)	6.060	0.048
Exacerbation in previous 12 months,n(%)	14(40)	11(36.7)	15(30.6)	0.835	0.659
Atopic comorbidity					
Anaphylactic rhinitis, n(%)	23(65.7)	9(31)	25(51)	7.642	0.022
Eczema, n(%)	7(20)	8(26.7)	8(16.3)	1.236	0.539
SPT, n (%)	16(45.7)	12(40)	23(46.9)	7.790	0.100
ACQ	0.50(0.17, 1.83)	0.83(0.33, 1.33)	0.33(0.00, 1.17)	4.533	0.103
AQLQ	5.63(4.87, 6.42)	5.90(5.28, 6.42)	6.13(5.58, 6.59)	3.471	0.176
Pre- bronchodilator	r				
FEV ₁ , L	2.02±0.61	1.74±0.57	2.45±0.98 ^{b,c}	8.370	<0.001
FEV ₁ , %pred	69.69±19.41	63.73±20.05	77.39±20.64 ^c	4.477	0.013
FVC, L	3.19±0.73	2.83±0.63	3.46±1.10 ^c	4.730	0.011
FVC, %	91.77±17.75	85.07±12.451	91.88±17.02	1.930	0.150
FEV ₁ /FVC, %	63.00±11.02	61.57±15.43	70.07±12.90 ^{b,c}	4.972	0.009

Post-bronchodilator					
FEV ₁ , L	2.29±0.61	1.89±0.64b	2.61±0.88c	8.236	<0.001
FEV ₁ , %	73.79±18.85	68.44±19.37b	84.30±17.05c	6.749	0.002
FVC, L	3.39±0.70	3.01±0.67	3.56±0.98c	3.811	0.025
FVC, %	99.16±15.99	89.36±10.56a	95.99±13.62d	3.995	0.021
BDR					
Δ FEV ₁ , L	0.29(0.16, 0.46)	0.17(0.05, 0.25)a	0.15(0.04, 0.32)a	10.118	0.006
Δ FEV ₁ , %	18.06(7.30, 29.06)	10.68(3.47, 18.25)b	6.80(1.27, 17.08)a	9.364	0.009
FeNO, ppb	53.50(38.50, 79.75)	26.50(17.75, 49.25)b	29.50(14.25, 49.75)b	14.470	0.001
IgE, IU/mL	119.71(46.80, 378.21)	88.76(26.16, 391.25)	82.83(45.47, 347.16)	0.871	0.647
Sputum cytology					
Total cell	0.30(0.4, 0.42)	0.68(0.42, 1.23)	0.26(0.13, 0.54)	24.495	<0.001
Eosinophil, %	14.63(7.44, 38.75)	0.00(0.00, 0.25)a	0.00(0.00, 0.38)a	78.829	<0.001
Eosinophil, ×10 ⁴ /mL	4.24(1.50, 10.66)	0.00(0.00, 0.16)	0.00(0.00, 0.08)	74.370	<0.001
Neutrophil, %	26.00(13.00, 43.00)	90.25(78.50, 98.31)a	17.75(6.75, 35.25)b,c	67.844	<0.001
Neutrophil, ×10 ⁴ /mL	5.55(2.97, 12.83)	57.33(32.13, 107.30)	3.80(1.32, 11.19)	57.524	<0.001
Lymphocyte, %	1.00(0.50, 1.50)	0.50(0.25, 0.75)b	0.75(0.25, 2.00)d	6.245	0.044
Lymphocyte, ×10 ⁴ /mL	0.24(0.05, 0.41)	0.30(0.10, 0.53)	0.25(0.06, 0.65)	0.510	0.775
Macrophage, %	48.13(28.75, 66.63)	8.63(1.38, 19.31)a	80.50(63.75, 92.00)a,c	76.359	<0.001
Macrophage, ×10 ⁴ /mL	11.04(8.01, 19.08)	5.85(1.05, 12.57)	21.60(8.32, 39.80)	23.856	<0.001
Sputum cytokines					
TNF-α, pg/mL	6.13(2.67, 13.12)	32.58(5.53, 58.27)a	12.24(5.07, 26.46)a	13.398	<0.001
IFN-γ, pg/mL	2.01(1.80, 2.77)	2.18(1.90, 2.44)	2.01(1.66, 2.631)	0.631	0.729
IL-1β, pg/mL	8.28(4.44, 18.38)	64.30(21.35, 286.80)a	18.14(5.63, 57.70)b,c	20.533	<0.001
IL-4, pg/mL	43.75(21.99, 67.96)	52.19(35.78, 59.16)	43.75(10.30, 65.42)	1.543	0.462
IL-5, pg/mL	2.49(1.40, 7.21)	0.83(0.72, 1.26)a	1.23(0.87, 1.85)a,c	25.185	<0.001
IL-6, pg/mL	10.92(3.74, 19.94)	22.76(2.76, 46.12)	19.57(7.26, 46.62)	4.320	0.115
IL-8, pg/mL	953.15(426.06, 1517.75)	1307.50(701.92, 3757.75)a	1209.0(732.36, 1976.50)b	9.165	0.010

IL-13, pg/mL	3.33(1.95, 8.69)	3.39(2.14, 4.50)	3.62(2.44, 5.64)	1.687	0.430
IL-17A, pg/mL	2.08(1.58, 3.18)	3.56(2.11, 5.08) ^a	2.66(1.72, 4.18)	8.764	0.013
MDC, pg/mL	41.09(20.57, 75.15)	38.58(24.70, 64.29)	46.01(26.46, 75.14)	2.487	0.288
Exacerbation in the 12-month follow-up					
Withdraw, n (%)	0(0)	3(10)	3(6.1)	3.367	0.186
Severe exacerbation, n (%)	6(17.1)	10(37)	3(6.5) ^c	10.935	0.004
Severe exacerbation times	0.29±0.75	0.70±1.33	0.09±0.35 ^c	10.965	0.004
Emergency Visit, n (%)	3(8.6)	2(7.4)	2(4.3)	0.636	0.728
Emergency Visit times	0.11±0.40	0.07±0.27	0.09±0.41	0.552	0.759
Hospitalization, n (%)	5(14.3)	5(18.5)	3(6.5)	2.560	0.278
Hospitalization times	0.26±0.74	0.26±0.66	0.09±0.35	2.513	0.285

EA, eosinophilic asthma; NA, neutrophilic asthma; PGA, paucigranulo- cytic asthma; BMI, body mass index; BDP, be- clomethasone dipropionate; LTRA, leukotriene receptor antagonist; SPT, skin prick test; BDR, bronchodilator responsiveness; ACQ, asthma control questionnaire; AQLQ, asthma quality of life questionnaire; FeNO, fractional exhaled nitric oxide; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MDC, macrophage- derived chemokines; Vs. EA, ^a <i>P</i> < 0.01, ^b <i>P</i> < 0.05; Vs. NA, ^c <i>P</i> < 0.01, ^d <i>P</i> < 0.05	EA, eosinophilic asthma; NA, neutrophilic asthma; PGA, paucigranulo- cytic asthma; BMI, body mass index; BDP, be- clomethasone dipropionate; LTRA, leukotriene receptor antagonist; SPT, skin prick test; BDR, bronchodilator responsiveness; ACQ, asthma control questionnaire; AQLQ, asthma quality of life questionnaire; FeNO, fractional exhaled nitric oxide; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MDC, macrophage- derived chemokines; Vs. EA, ^a <i>P</i> < 0.01, ^b <i>P</i> < 0.05; Vs. NA, ^c <i>P</i> < 0.01, ^d <i>P</i> < 0.05	EA, eosinophilic asthma; NA, neutrophilic asthma; PGA, paucigranulo- cytic asthma; BMI, body mass index; BDP, be- clomethasone dipropionate; LTRA, leukotriene receptor antagonist; SPT, skin prick test; BDR, bronchodilator responsiveness; ACQ, asthma control questionnaire; AQLQ, asthma quality of life questionnaire; FeNO, fractional exhaled nitric oxide; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MDC, macrophage- derived chemokines; Vs. EA, ^a <i>P</i> < 0.01, ^b <i>P</i> < 0.05; Vs. NA, ^c <i>P</i> < 0.01, ^d <i>P</i> < 0.05	EA, eosinophilic asthma; NA, neutrophilic asthma; PGA, paucigranulo- cytic asthma; BMI, body mass index; BDP, be- clomethasone dipropionate; LTRA, leukotriene receptor antagonist; SPT, skin prick test; BDR, bronchodilator responsiveness; ACQ, asthma control questionnaire; AQLQ, asthma quality of life questionnaire; FeNO, fractional exhaled nitric oxide; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MDC, macrophage- derived chemokines; Vs. EA, ^a <i>P</i> < 0.01, ^b <i>P</i> < 0.05; Vs. NA, ^c <i>P</i> < 0.01, ^d <i>P</i> < 0.05	EA, eosinophilic asthma; NA, neutrophilic asthma; PGA, paucigranulo- cytic asthma; BMI, body mass index; BDP, be- clomethasone dipropionate; LTRA, leukotriene receptor antagonist; SPT, skin prick test; BDR, bronchodilator responsiveness; ACQ, asthma control questionnaire; AQLQ, asthma quality of life questionnaire; FeNO, fractional exhaled nitric oxide; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MDC, macrophage- derived chemokines; Vs. EA, ^a <i>P</i> < 0.01, ^b <i>P</i> < 0.05; Vs. NA, ^c <i>P</i> < 0.01, ^d <i>P</i> < 0.05	EA, eosinophilic asthma; NA, neutrophilic asthma; PGA, paucigranulo- cytic asthma; BMI, body mass index; BDP, be- clomethasone dipropionate; LTRA, leukotriene receptor antagonist; SPT, skin prick test; BDR, bronchodilator responsiveness; ACQ, asthma control questionnaire; AQLQ, asthma quality of life questionnaire; FeNO, fractional exhaled nitric oxide; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MDC, macrophage- derived chemokines; Vs. EA, ^a <i>P</i> < 0.01, ^b <i>P</i> < 0.05; Vs. NA, ^c <i>P</i> < 0.01, ^d <i>P</i> < 0.05
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Table 2. targeted Metabolites grouped by airway inflammation phenotypes in the validation set
Variables
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Diethanolamine, nmol/L
 Histamine, nmol/L
 Creatinine, nmol/L
 Adenine, nmol/L
 Acetylcholine, nmol/L
 Phosphorylcholine, nmol/L
 Glycerophosphocholine, nmol/L
 Adenosine 5'-monophosphate, nmol/L
 Pyruvate, nmol/L
 Lactate, nmol/L
 Glyceric acid, nmol/L
 3-Hydroxyisovaleric acid, nmol/L
 Taurine, nmol/L
 Barbituric acid, nmol/L
 Allantoin, nmol/L
 5-L-Glutamyl-L-alanine, nmol/L
 Arginyl-Serine, nmol/L
 N-Acetylneuraminic acid, nmol/L
 Cyclohexylamine, nmol/L
 Nicotinamide, nmol/L
 Dihydrothymine, nmol/L
 L-Leucine, nmol/L
 Tyramine, nmol/L
 L-Glutamate, nmol/L
 Xanthine, nmol/L
 L-Phenylalanine, nmol/L
 Alanyl-Leucine, nmol/L
 Allocystathionine, nmol/L
 Salbutamol, nmol/L
 Phenylalanyl-Serine, nmol/L
 Threoninyl-Phenylalanine, nmol/L
 Phenylalanylphenylalanine, nmol/L
 Indolelactic acid, nmol/L
 Dodecanoic acid, µg/mL
 cis-9-Palmitoleic acid, µg/mL
 Heptadecanoic acid, µg/mL
 Stearic acid, µg/mL
 Oleic acid, µg/mL
 Linoleic acid, µg/mL
 EA, eosinophilic asthma; NA, neutrophilic asthma; PGA, paucigranulocytic asthma; Vs. EA, ^a*P* < 0.01, ^b*P* < 0.05; Vs. NA, ^c*P* < 0.01, ^d*P* < 0.05; Vs. PGA, ^e*P* < 0.01, ^f*P* < 0.05.

Table 3. metabolites predict asthma exacerbation(Adjusted Model)

Metabolites(nmol/L)	Proportions of patients experiencing severe asthma exacerbation OR(95%CI)	Proportions of patients experiencing severe asthma exacerbation <i>P</i> value
Adenine	0.997(0.994, 1.001)	0.146
Phosphorylcholine	1.030(0.986, 1.076)	0.181
Adenosine 5'-monophosphate	1.000(1.000, 1.000)	0.050
Pyruvate	1.000(1.000, 1.000)	0.350
3-Hydroxyisovaleric acid	0.996(0.992, 1.001)	0.108
Allantoin	1.000(1.000, 1.000)	0.136

N-Acetylneuraminic acid	1.000(1.000, 1.000)	0.125
Nicotinamide	1.001(0.999,1.003)	0.248
Tyramine	1.015(0.955, 1.080)	0.626
Allocystathionine	1.014(0.929, 1.106)	0.756
Threonyl-Phenylalanine	0.999(0.998, 1.001)	0.558
Adjusted for age, gender, BMI, FEV ₁	Adjusted for age, gender, BMI, FEV ₁	Adjusted for age

Figure legends:

Figure 1. Untargeted metabolic profiles discriminate in different inflammatory asthma phenotypes and healthy subjects in the discovery set. (A-D) Score plot of PCA model obtained from different inflammatory asthma phenotypes and healthy subjects retrospectively; (E-H) Score plot of OPLS-DA model obtained from different inflammatory asthma phenotypes and healthy subjects retrospectively; The labels t[1] and t[2] along the axes represent the scores (the first 2 partial least-squares components) of the model, which are sufficient to build a satisfactory classification model. (I-L) Permutation Test of the OPLS-DA model obtained from different inflammatory asthma phenotypes and healthy subjects retrospectively; Two hundred permutations were performed, and the resulting R² and Q² values were plotted.

Figure 2. Heatmap of identified differential metabolites in sputum samples in different inflammatory asthma phenotypes and healthy subjects in the discovery set

Figure 3. MetaboAnalyst Pathway Impact based on selected and more representative metabolites responsible for the class separation in sputum samples in different asthma phenotypes and healthy subjects. (A) Asthma and Healthy; (B) EA and NA; (C) EA and PGA; (D) NA and PGA. Circles represent metabolic pathways potentially involved in class separation. EA, eosinophilic asthma; NA, neutrophilic asthma; PGA, paucigranulocytic asthma.

Figure 4. The receiver operating characteristic (ROC) curves of metabolites to discriminate between NA and EA (A) or between NA and PGA (B). EA, eosinophilic asthma; NA, neutrophilic asthma; PGA, paucigranulocytic asthma.

Figure 5. Heatmap of correlations of differential metabolites with clinical and inflammatory profiles in all subjects with asthma in the validation set