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Serum metabolomics reveals pathways and biomarkers associated with asthma pathogenesis

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Abstract

Background: Asthma is a chronic inflammatory disease caused by complex interactions of genetic, epigenetic, and environmental factors. For this reason, new approaches are required to clarify the pathogenesis of asthma by systemic review.

Objective: We applied a ^1H -NMR metabolomics approach to investigate the altered metabolic pattern in sera from patients with asthma and sought to identify the mechanism underlying asthma and potential biomarkers.

Method: A global profile of sera from patients with asthma ($n = 39$) and controls ($n = 26$) was generated using ^1H -NMR spectroscopy coupled with multivariate statistical analysis. Endogenous metabolites in serum were rapidly measured using the target-profiling procedure.

Results: Multivariate statistical analysis showed a clear distinction between patients with asthma and healthy subjects. Sera of asthma patients were characterized by increased levels of methionine, glutamine, and histidine and by decreased levels of formate, methanol, acetate, choline, O-phosphocholine, arginine, and glucose. The metabolites detected in the sera of patients with asthma are involved in hypermethylation, response to hypoxia, and immune reaction. Furthermore, the levels of serum metabolites from patients with asthma correlated with asthma severity; in particular, lipid metabolism was altered in patients with lower FEV_1 % predicted values. In addition, potential biomarkers showed strong predictive power in ROC analysis, and the presence of asthma in external validation models was predicted with high accuracy (90.9% for asthma and 100% for control subjects).

Conclusion & Clinical Relevance: These data showed that ^1H -NMR-based metabolite

profiling of serum may be useful for the effective diagnosis of asthma and a further understanding of its pathogenesis.

Key words: asthma, diagnosis, metabolite profiling, nuclear magnetic resonance

Introduction

Asthma has a 7–10% prevalence worldwide, which will likely increase with further environmental changes [1]. Although the incidence and prevalence of asthma are increasing, the mechanism(s) by which genetic, epigenetic, and environmental factors interact remain(s) poorly understood. Moreover, though airway inflammation is an essential part of asthma, several inflammatory patterns are involved, which may contribute to the variation in clinical phenotypes [2, 3]. Thus, “-omics” approaches involving various technological platforms are required to identify biomarkers associated with asthma and to understand the mechanism(s) underlying its pathogenesis.

Metabolomics, the multi-targeted analysis of endogenous metabolites from biological samples, is a recently developed technique that is useful for detecting metabolic profiles associated with genetic defects, distinguishing between experimental subject phenotypes, and understanding the pathophysiological processes associated with disease [4, 5]. Numerous metabolomic studies of lung disease have been applied as examples of diagnostic and toxicological assays using different fluids, such as exhaled breathing condensates (EBC) or urine [6–9], but a recent nuclear magnetic resonance (NMR)-based metabolomic study of EBC fluid raised doubts about the accuracy of the technique [10]. In addition, to-date, serum metabolic profiling of asthma patients has not been reported.

Tissue lesions, organ dysfunction, and pathological states can alter both the chemical and protein composition of blood serum, which is the primary carrier of small molecules around the body. In a previous proteomics study, Gomes–Alves *et al.* [11] revealed protein

signature in the peripheral blood that discriminated asthma patients from control subjects using SELDI-TOF MS. However, genomic and proteomic studies have provided extensive information about genotype, but only limited information about phenotype, which is the closest link to low-molecular-weight compounds such as metabolites.

Global metabolite profiles of serum samples were used to identify potential biomarkers, which can provide new and unexpected insights into biological processes [12]. In particular, a metabolomic approach using NMR spectroscopy coupled with pattern recognition has proven useful for generating high-density metabolite data and characterizing metabolic changes in biological samples [13]. In addition, NMR analysis has an advantage with regard to identification, because it allows for observation of specific quantum mechanical magnetic properties of the atomic nucleus, and performance of quantitative analysis without analyte standards or calibration curves is the most attractive feature of this technique [14].

The hypothesis of the present study was that NMR-based metabolomics was possible to detect the status of asthma by systemic review in a simpler way and provided that asthma alters the chemical composition of blood serum. Thus, we investigated the metabolic pattern in sera from patients with asthma and identified metabolic markers associated with asthma pathogenesis using NMR-based metabolic profiling.

Materials and Methods

Subjects

The study included 39 (13 males) patients with asthma (mean age, 44.77 years; range, 19-63) and 26 (10 males) healthy individuals (mean age, 44.73 years; range, 18-62). Patients with asthma were recruited from Ajou University Hospital in Suwon, Republic of Korea. Healthy controls of the same age range were recruited from volunteers (Table 1).

The diagnosis of asthma was confirmed by allergy specialists and was based on

clinical history, such as recurrent cough, shortness of breath, recurrent wheeze, chest tightness, sputum eosinophilia, evidence of airway obstruction that was reversible after short-acting bronchodilator inhalation (greater than 12% increase of FEV₁ % predicted values), and a PC20 methacholine level of <16 mg/mL.

Healthy controls, those with no history of allergic or any other chronic disease, were also enrolled. Enrolled asthmatic patients did not have a history of acute upper or lower airway infection for six weeks prior to the study. Of the 39 total asthma patients, 17 were atopic and had positive skin-prick tests or high serum IgE specific to common inhalant allergens.

Most asthmatic patients, all except nine, had used inhaled corticosteroid (ICS) as a maintenance medication and/or additional leukotriene antagonist or theophylline, and their symptoms were in a stable state. These patients stopped oral corticosteroid treatment for one month prior to enrollment. All asthmatic medications were stopped for at least three days before sample collection to minimize possible drug effects on plasma metabolite levels.

All study participants provided informed consent. The protocol was approved by the Institutional Review Board of Ajou University Hospital with the approval number AJIRB-GEN-GEN-09-140.

Study design

This is a cross-sectional study with serum samples from 39 asthma patients and 17 healthy controls and included 15 asthma patients who revisited with 1-2 years' intervals. In addition, 10 asthma patients which were not included in original modeling were enrolled during the study period for external validation test.

Measurements

NMR sample preparation and NMR spectroscopy

Serum samples were collected and stored at -80°C until NMR analysis. Before the NMR experiment, frozen serum samples were thawed at room temperature and vortexed. The 250- μL serum samples were mixed with 500 μL saline solution (10% D_2O for lock signal, 0.9% sodium azide, pH 7.0) to minimize variations in the pH of the samples. Samples were centrifuged at $12,000 \times g$ for 10 min, and 600- μL aliquots of supernatant were transferred into 5-mm NMR tubes for analysis.

^1H -NMR spectra were acquired using a VNMR-600MHz NMR spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA) at 298K using a triple resonance 5-mm HCN salt tolerant cold probe. The water-suppressed Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence (RD-90°- [τ -180°- τ] n-ACQ) with a total T2 relaxation time of 60 ms was used to attenuate broad signals from proteins and lipoproteins. For each sample, the ^1H -NMR spectrum was collected with 128 transients into 32 K data points using a spectral width of 6720.4 Hz with a relaxation delay of 2.0 s and an acquisition time of 2.381 s. The free induction decays were weighted by an exponential function with a 0.3-Hz line-broadening factor prior to Fourier transformation. All acquired NMR spectra were phase and baseline corrected, then referenced to the doublet of lactate at 1.32 ppm.

Analysis

NMR data processing and multivariate statistical analysis

One of the major challenges for automated data analysis and peak assignment for spectra of biofluids from metabolomics studies is the effect of shifting peaks. To reduce this, the spectra are integrated within small spectral regions; this is called “binning”. Then, NMR spectra data between 0.7–8.49 ppm were reduced to segments of 0.002 ppm. The water region (4.02–5.55 ppm) was removed prior to normalization and spectra alignment.

Spectral data were normalized to the total spectral area (which accounts for differences in the overall concentration of samples and is crucial because variation of overall concentrations obscures specific changes of metabolites) and converted to an ASCII format file. The ASCII files were imported into MATLAB (R2008a, Mathworks, Inc., 2008), and all spectra were aligned using the correlation optimized warping method, which shifted, stretched, and shrunk the segmented spectra to maximize the correlation coefficient between the segments of a spectrum and a target spectrum and is an alternative to reducing the spectrum to account for peak shifts [15].

The resulting datasets were then imported into SIMCA-P 12.0 (Umetrics, Umeå, Sweden) for multivariate statistical analyses. All imported data were Pareto-scaled for the multivariate analysis. First, principal components analysis (PCA) was conducted on the spectra of serum samples to discern the presence of inherent similarities in spectral profiles. Next, a partial least-squared discriminate analysis (PLS-DA) was used to maximize covariance between the measured data (peak intensities in NMR spectra, X matrix) and the response variable (predictive classifications, Y matrix), by searching for X variables that correlated with class membership, so that the response variable Y can be predicted from X.

Analysis of NMR spectral data was also accomplished using targeted profiling through the use of Chenomx NMR Suite 6.0 (Chenomx, Edmonton, Canada) to assign the metabolites, which is difficult using the binning data due to the reduced resolution of the spectra resulting from the integration process. The software contains a database of known metabolites with their referenced spectral resonant frequencies and signatures. These known resonant frequencies were matched to the observed resonant frequencies of the collected spectra. The ambiguous peaks caused by overlap or slight shifts were confirmed by spiking samples with the respective standard compounds and 2D experiments (Fig. S1 and S2)

PLS-DA was applied to the relative concentration data of assigned metabolites. The

regression coefficient and VIP plots were then used to identify which metabolites contributed most to clustering or trends observed in the data.

The regression coefficient plot shows all metabolites that contribute to the separation between studied groups by re-expressing the Y variables as multiple regression models of the X variables. The VIP parameters are essentially a measure of the degree to which a particular variable explains the Y variance.

In addition, color coding, depending on the FEV₁% predicted values, was used in the score plot of PCA of targeted profiles of sera from patients with asthma to easily understand the relationship between severity of asthma and metabolic patterns.

Statistical analysis and validation and reproducibility tests

A two-sided *t*-test, the Mann–Whitney *U*-test (non-Gaussian distribution) for the metabolite concentration, and Spearman's rank correlation test were conducted using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

To validate our models, permutation tests were repeated 30 times using SIMCA-P software. Additionally, an external validation test with randomized samples (11 asthma patients and 10 healthy controls) and a reproducibility test with 15 samples from revisited asthma patients were performed. The same criteria used in the training model were utilized to diagnose the patients with asthma and controls in the validation set. Correct classification was based on a predicted Y cutoff of 0.5 [16].

Results

¹H-NMR spectroscopy and pattern recognition analysis

Figure 1 shows representative 600-MHz ¹H NMR spectra of serum samples from the control (Fig. 1a) and asthma (Fig. 1b) groups. Initially, PCA, an unsupervised pattern recognition method, was performed to examine the intrinsic variation in ¹H-NMR data of sera

from the control and asthma groups. The PCA score plot (Fig. 2a) derived from the NMR spectra showed clear separation between the control and asthma groups ($R^2X = 0.695$, $Q^2 = 0.535$).

Next, PLS-DA was performed to reduce the possible contribution of intergroup variability and to further improve the separation between the two groups. The score plots (Fig. 2b) showed a clear differentiation between healthy individuals and asthma patients ($R^2Y = 0.971$, $Q^2 = 0.932$), and the reliability of the model was validated using 30-time repeated permutation tests (Fig. S3).

PLS-DA loading (Fig. 2c) was generated to identify the metabolites responsible for the variation in the score plots. Sera from patients with asthma was characterized by higher levels (VIP >1) of cholesterol-C18, VLDL/LDL (CH₂)_n, lactate, lipid CH₂CH₂C=C, glutamate, N-acetylglycoprotein, glutamine, methionine, succinate, trimethylamine n-oxide, and glycine and lower levels (VIP >1) of VLDL/LDL CH₃, isoleucine, leucine, valine, alanine, arginine, acetate, acetone, histidine, choline, glucose, O-phosphocholine, methanol, and formate.

Targeted metabolic profiling to identify novel markers

To minimize the effects of unknown materials and noise on the multivariate analysis of NMR spectra, PLS-DA was conducted with relative concentration data of 25 metabolites detected in the global profiling analysis (Table S1 and S2). The score plot ($R^2Y = 0.922$ and $Q^2 = 0.901$; Fig. 3a) showed good discrimination between the two groups, and the permutation plot showed a high value of predictability (Fig. S3).

To further investigate the significance of metabolite levels, a combination of statistical approaches was necessary [17]. Thus, we selected biomarkers using the *t*-test, as well as jack-knifing of coefficients and VIP (variable importance of projection) values.

A PLS-DA regression coefficient plot was generated to identify metabolites responsible for the difference in the score plots (Fig. 3b). This plot showed decreased levels of formate, choline, histidine, acetate, glucose, O-phosphocholine, arginine, methanol, leucine, valine, alanine, and cholesterol-C18 and increased levels of methionine, glutamine, lipid $\text{CH}_2\text{CH}_2\text{C}=\text{C}$, glutamate, succinate, lactate, VLDL/LDL $(\text{CH}_2)_n$, and N-acetylglycoprotein in sera of patients with asthma compared to the controls. In addition, all these metabolites exhibited statistically significant differences ($p < 0.05$; Table S2). Among the metabolites shown in the PLS-DA coefficient plot, 10 metabolites, including formate, choline, histidine, methionine, acetate, glucose, glutamine, O-phosphocholine, arginine, and methanol, showed high VIP values ($\text{VIP} > 1$; Fig. 3b).

External validation and reproducibility assessment

To validate the 10 selected metabolites, external validation and reproducibility tests were carried out in PLS-DA models using profiles of the 10 metabolites that were statistically significant among the identified biomarker candidates (Fig. 4). An external validation was performed with randomly selected asthma patients ($n = 11$) and healthy controls ($n = 10$) who were not originally part of the modeling exercise. All (100%) of the 11 asthma patients and 90% of the healthy controls (9 of 10) were correctly classified.

A reproducibility test with 15 samples from revisited asthma patients was also performed. All (100%; 15 of 15) asthma patients who were included in the original modeling were correctly classified again.

In addition, the diagnostic effectiveness of the PLS model was assessed using ROC curve analysis. The area under the curve (AUC) values of the training and external validation sets were 1 ($p < 0.001$) and 0.9771 ($p < 0.001$), respectively, indicating good diagnostic accuracy (> 0.8 of AUC [18]; Fig. S4).

Relationship between metabolites and clinical parameters

To understand the relationship between metabolite levels and clinical parameters, the relationship was tested statistically using a correlation analysis. Among targeted metabolites, three had significant correlations with FEV₁% predicted values, a marker of the degree of airflow obstruction and endorsed by the National Asthma Education and Prevention Program (NAEPP) [19, 20] (Fig. 5). In particular, among 10 significant metabolites, choline and arginine had relatively weak but significant correlations with FEV₁ % predicted values ($r = 0.386$ and 0.353 , $p = 0.029$ and 0.047 , respectively; Fig. 5a and 5b).

More interestingly, the score plot of PCA of serum metabolites from patients with asthma also showed that patients were located within the plot according to their FEV₁% predicted values, from the lower left to the upper right (Fig. 6a). A loading plot (Fig. 6b) revealed that levels of very low density lipoprotein (VLDL), low density lipoproteins (LDL), and lipid CH₂CH₂C=C were much higher in patients with lower FEV₁% predicted values compared to patients with normal FEV₁% predicted values. In addition, acetone showed a negative correlation with FEV₁ % predicted values ($r = -0.408$, $p = 0.021$; Fig. 5c).

Moreover, the pattern did not differ according to atopic status (Fig. 7a), level of ICS used (Fig. 7b) or any other type of asthma medication, including leukotriene receptor antagonist and theophylline, or any history of oral steroid use during asthma exacerbation (Fig. S5).

Discussion

In this study, we applied a ¹H NMR-based metabolic profiling approach to investigate metabolic changes in sera from asthma patients. Several metabolites, including formate, choline, histidine, methionine, acetate, glucose, glutamine, O-phosphocholine,

arginine, and methanol, were identified as biomarkers of asthma, and these metabolites could explain the pathogenesis of asthma by way of systemic review.

Enhanced methylation

Among the 10 identified metabolites, five, including formate, choline, methionine, O-phosphocholine, and methanol, were related to enhancement of the methyl transfer pathway [21–23], and increased arginine methylation has been described as a key process in asthma [24, 25]. Thus, it is possible that increased methylation may be related to pathogenesis of asthma. In addition, several studies have suggested that DNA methylation skews immune responses toward a Th2 phenotype and enhances the risk of airway inflammation [26, 27]. Additionally, Hollingsworth *et al.* reported that a maternal diet supplemented with methyl donors in C57BL/6J mice enhanced the severity of allergic airway disease in the offspring; this trait was inherited transgenerationally [28] and may be modified by drugs [29, 30]. Moreover, increased levels of methylarginine (asymmetric dimethylarginine [ADMA] and monomethylarginine) have been noted in patients with severe asthma compared to those with non-severe asthma [24]. In addition, arginine methylation regulates cytokine overexpression in allergic diseases and asthma [25]. In this study, we observed decreased levels of arginine in the sera of patients with asthma. Furthermore, a significant correlation was found between FEV₁% predicted values and arginine levels. Taken together, we propose that a novel epigenetic mechanism of systemic hypermethylation might be related to development and progression of asthma. Phosphocholine is a component of the endothelial cell barrier and a pulmonary surfactant. Therefore, markedly decreased phosphocholine levels in the sera of patients with asthma could indicate a lack of protection of the alveolar region and on the conducting airways [31].

Metabolites from hypoxia and immune responses

A well-known hypoxia-induced change was observed, with significantly lower levels of glucose and increased levels of lactate in the sera of patients with asthma [32], which is consistent with previous urine metabolomics data from patients with asthma [9]. Additionally, we also found higher levels of histidine, the precursor of histamine, which is an important protein involved in immune responses, contributing to an inflammatory response and constriction of smooth muscle in asthma patients [33].

Alteration of lipid metabolism in severe asthma patients

Current evidence indicates that lipids play an important role in asthma manifestation [34]. Furthermore, the presence of VLDL and products of VLDL hydrolysis have been found to activate the release of histamine, which is an effective mediator of vascular permeability and smooth muscle constriction in the airways [35, 36]. In this study, we also found that sera from asthma patients with lower FEV₁% predicted values were characterized by higher levels of VLDL/LDL products. In addition, breakdown of these increased lipids under conditions of insufficient glucose may lead to increased levels of acetone in patients with severe asthma; this also showed a correlation with FEV₁ % predicted values. Moreover, these results were in concordance with studies of volatile organic compounds (VOC) in exhaled breath using GC-MS [37–39], which also reported that lipid metabolism was closely related to the pathology and phenotype of asthma because inflammation is accompanied by oxidative stress and, subsequently, lipid peroxidation.

Limitations of this study include the low subject number, which were insufficient to evaluate clinical implications. Although no significant associations were found between atopy or sputum eosinophil count and serum metabolite levels, we found significant correlations

between FEV₁% predicted values and choline, arginine, and acetone levels. Moreover, these findings were confirmed by two validation studies using a different set of asthma patients as well as the same patient group re-analyzed in follow-up experiments. Second, the difference in metabolic patterns between asthma patients and healthy controls might have occurred due to medication effects (healthy subjects received no treatment). However, there have been many reports that suppression of methylation is an additional mechanism mediated by the anti-inflammatory effects of corticosteroids [40, 41]. In addition, there were no differences in metabolic patterns according to the level of ICS treatment or other type of asthma medication used. Therefore, the probability that enhanced methylation was due to medication use seems to be low. Also, many studies reported that only HDL was influenced by drugs, not VLDL or LDL [42, 43]. Third, this study demonstrated that ¹H-NMR-based metabolomics can be used to identify the metabolic difference in sera between patients with asthma and control subjects, although a prospective study with a larger cohort and non-asthmatic patients with airways disease will be needed to evaluate the possible application of this method in clinical practice. In addition, ¹H-NMR-based metabolic profiling could elucidate the mechanism underlying asthma severity.

In conclusion, global and targeted profiling using ¹H-NMR-based metabolomics has the potential to provide new insights into the pathogenic mechanism of asthma.

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Conflicts of Interest

The authors have no financial conflicts of interest.

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Figure legends

Fig. 1. Representative 600 MHz ^1H -NMR spectra of serum samples from healthy individuals (a) and patients with asthma (b). 1, Cholesterol-C18; 2, VLDL/LDL CH_3 ; 3, isoleucine; 4, leucine; 5, valine; 6, VLDL/LDL $(\text{CH}_2)_n$; 7, lactate; 8, alanine; 9, arginine; 10, lipid $\text{CH}_2\text{CH}_2\text{C}=\text{C}$; 11, acetate; 12, N-acetylglycoprotein; 13, glutamate; 14, glutamine; 15, methionine; 16, acetone; 17, pyruvate; 18, succinate; 19, citrate; 20, creatinine; 21, histidine; 22, tyrosine; 23, choline; 24, glucose; 25, trimethylamine n-oxide; 26, O-phosphocholine; 27, methanol; 28, glycine; 29, phenylalanine; 30, formate.

Fig. 2. PCA score plot (a) and PLS-DA score (b) and loading (c) plots derived from the ^1H -NMR spectra of sera obtained from control subjects (red squares) and patients with asthma (green circles). Global profiling by NMR spectra showed a clear discrimination between controls and patients with asthma. PCA showed intrinsic variation between controls and patients with asthma; target metabolites were selected from PLS-DA. The indicated metabolites of loading plots (c) represent the variables that had influence on predictive components and class discrimination ($\text{VIP} > 1$). 1, Cholesterol-C18; 2, VLDL/LDL CH_3 ; 3, isoleucine; 4, leucine; 5, valine; 6, VLDL/LDL $(\text{CH}_2)_n$; 7, lactate; 8, alanine; 9, arginine; 10, lipid $\text{CH}_2\text{CH}_2\text{C}=\text{C}$; 11, acetate; 12, N-acetylglycoprotein; 13, glutamate; 14, glutamine; 15, methionine; 16, acetone; 17, pyruvate; 18, succinate; 19, citrate; 20, creatinine; 21, histidine; 22, tyrosine; 23, choline; 24, glucose; 25, trimethylamine n-oxide; 26, O-phosphocholine; 27, methanol; 28, glycine; 29, phenylalanine; 30, formate.

Fig. 3. PLS-DA score plot (a) and coefficient plot with jack-knifed confidence intervals (b) derived from the metabolite concentrations obtained by targeted profiling of sera from controls (red squares) and patients with asthma (green circles). The asterisk (*) indicates which metabolites contributed significantly to the PLS-DA models ($\text{VIP} > 1$). Ten metabolites

showed significance through jack-knifing of the regression coefficient and VIP values.

Fig. 4. PLS-DA prediction scores for each validation set using profiles of 10 significant metabolites. Nine of 10 serum samples from blinded healthy controls, 11 of 11 serum samples from blinded asthma patients, and 15 of 15 serum samples from revisited asthma patients were correctly classified using a predicted Y cut off of 0.5.

Fig. 5. Correlation between FEV₁ % predicted values and metabolites. Choline (a), arginine (b), and acetone (c) were significantly associated with FEV₁ % predicted values.

Fig. 6. Score (a) and loading (b) plots of PCA of serum metabolites from patients with asthma showed FEV₁% predicted level-dependent changes. Patients are colored depending on their FEV₁ % predicted values. Asthma patients with lower FEV₁ % predicted values, who suffered from mild or moderate obstruction of the airways, showed significantly higher levels of VLDL and LDL metabolites.

Fig. 7. Scores plots of PCA of serum metabolites from patients with asthma, depending on the presence of atopy (a; green squares, non-atopic asthma patients; red triangles, atopic asthma patients) and the level of inhaled corticosteroid (ICS) used (b; red dot, no-use group; black squares, medium-dose ICS used; green squares, high-dose ICS used).

Table 1. Characteristics of the study participants

	Control	Asthma	Range	<i>p</i>
Age (mean, range)	44.73 (18–62)	44.77 (19–63)		0.9889
Sex (male/female), n	10/16	13/26		0.7941
Total IgE, KU/L	-	304.2±350.9*	24- 1383	
Sputum neutrophil (%)	-	44.97±35.31*	1-70	
Sputum eosinophil (%)	-	32.41±40.27*	15-95	
FEV ₁ % predicted	-	83.18±17.62*	57.4-109.1	
FVC% predicted	-	93.19±13.05*	75.3-120.1	

*: Mean ± SD, -: not measured.

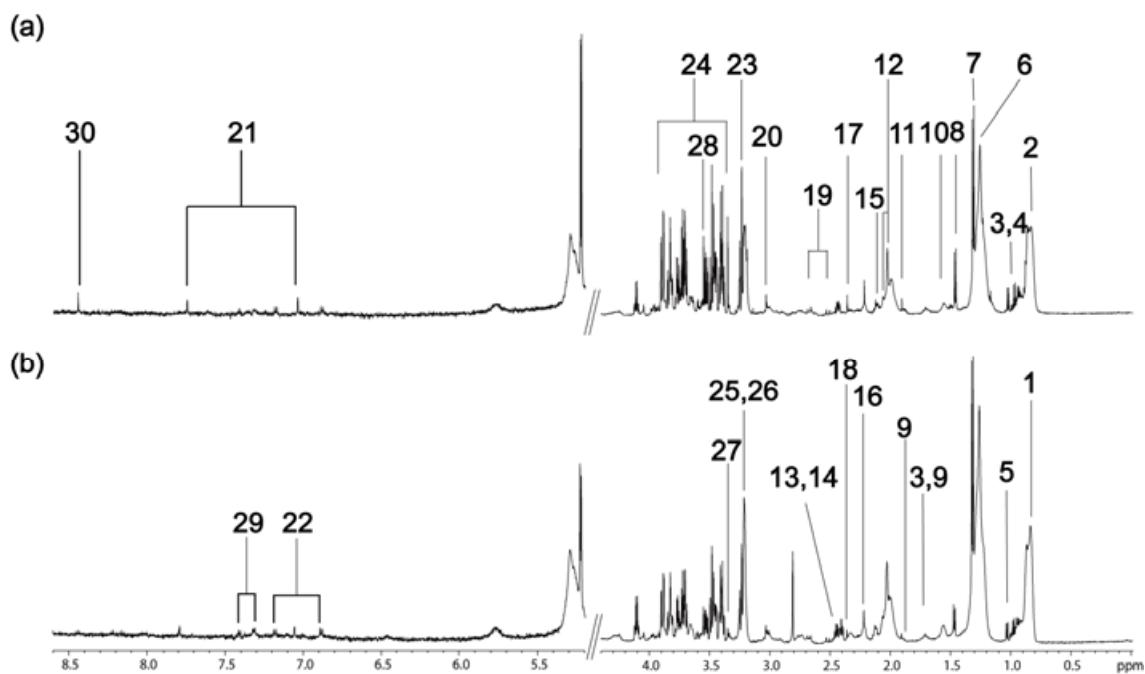
Fig. 1.

Fig. 2.

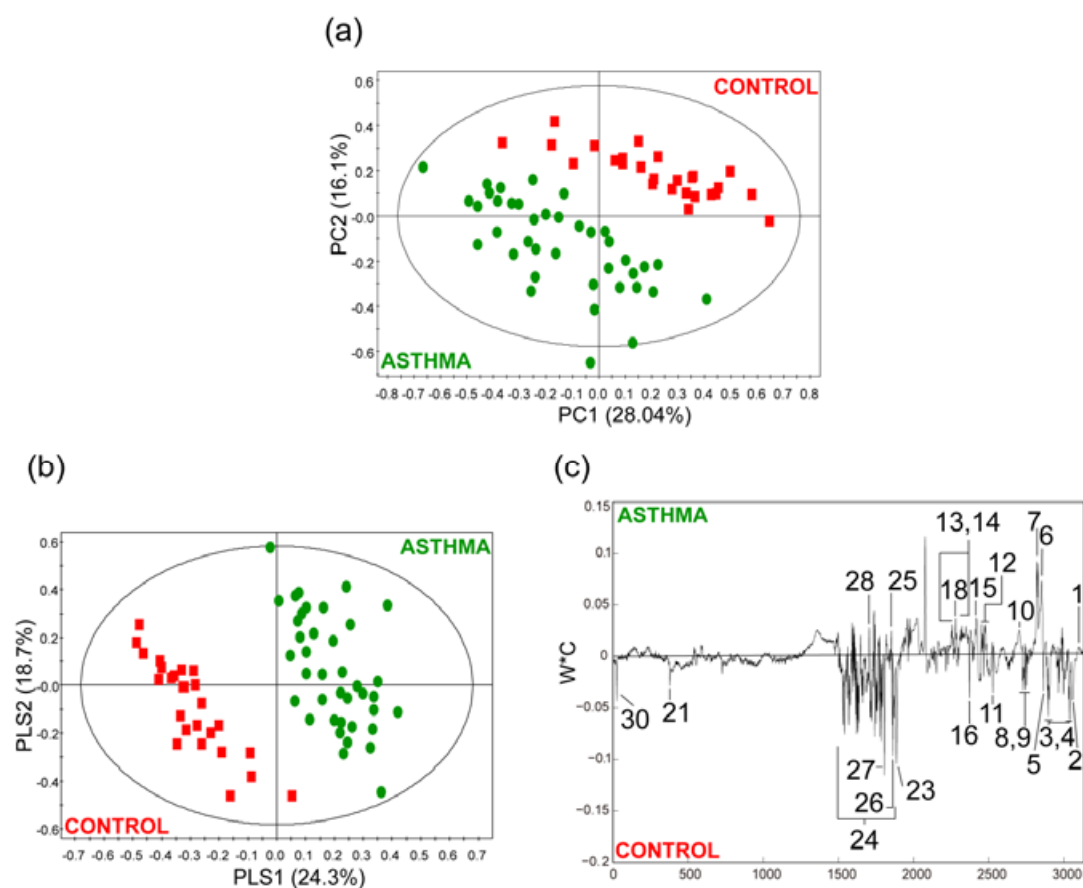


Fig. 3.

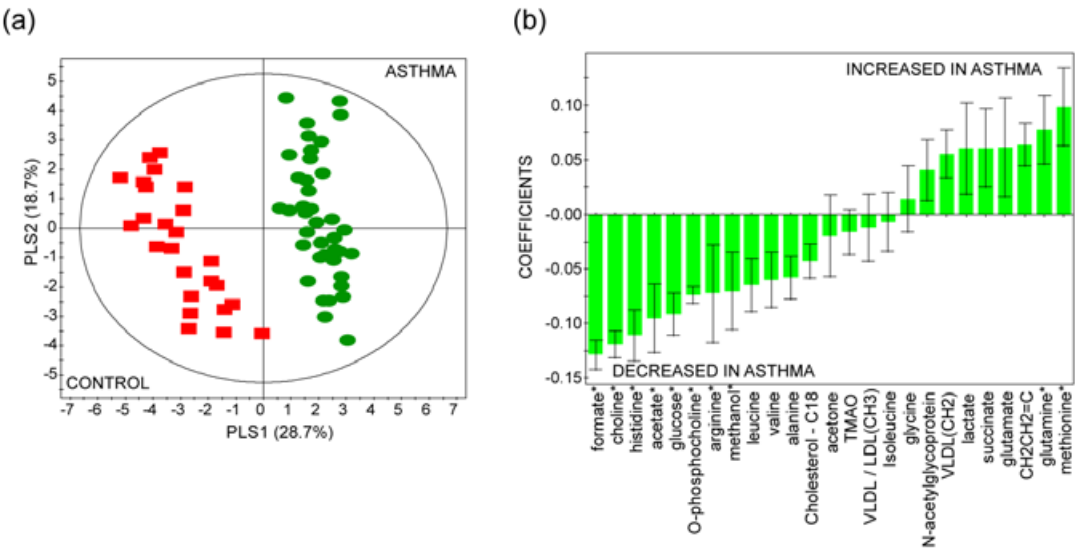


Fig. 4.

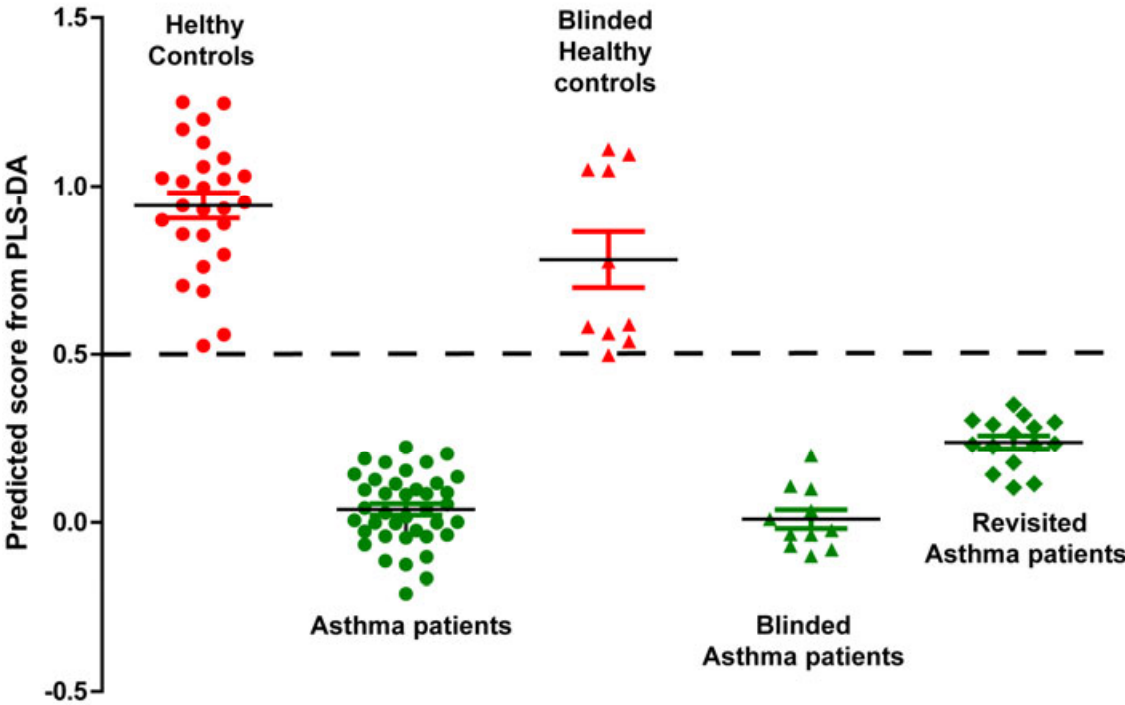


Fig. 5.

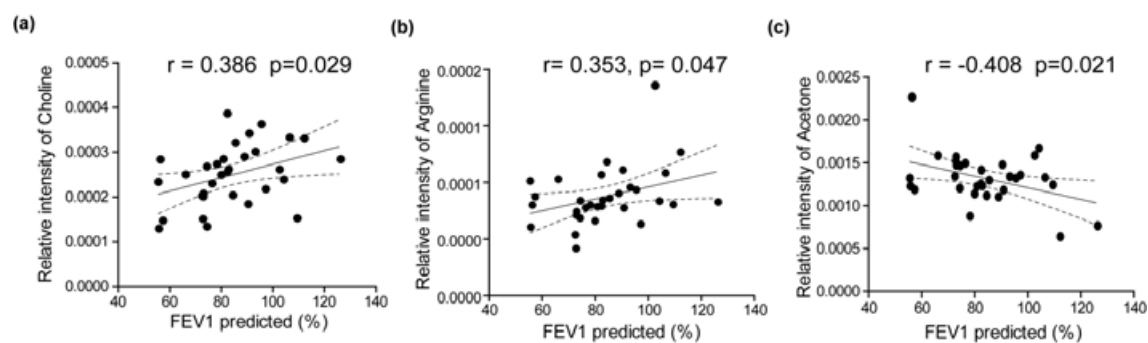


Fig. 6.

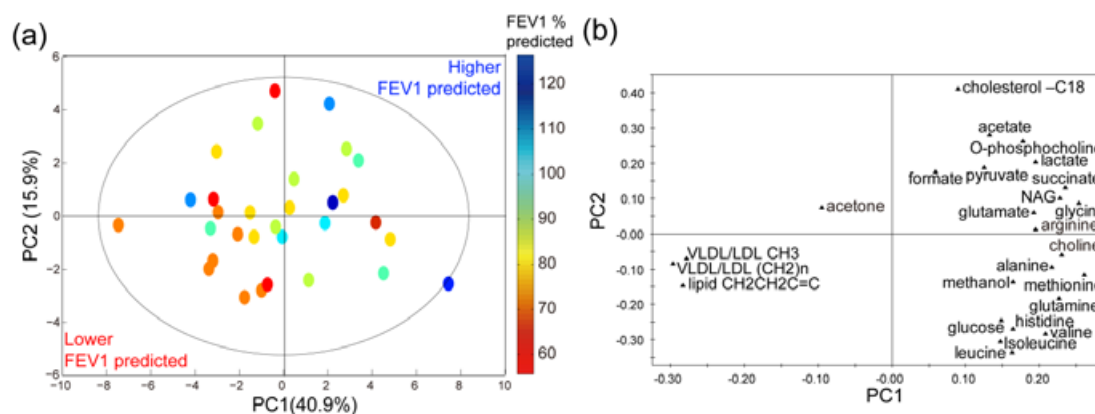
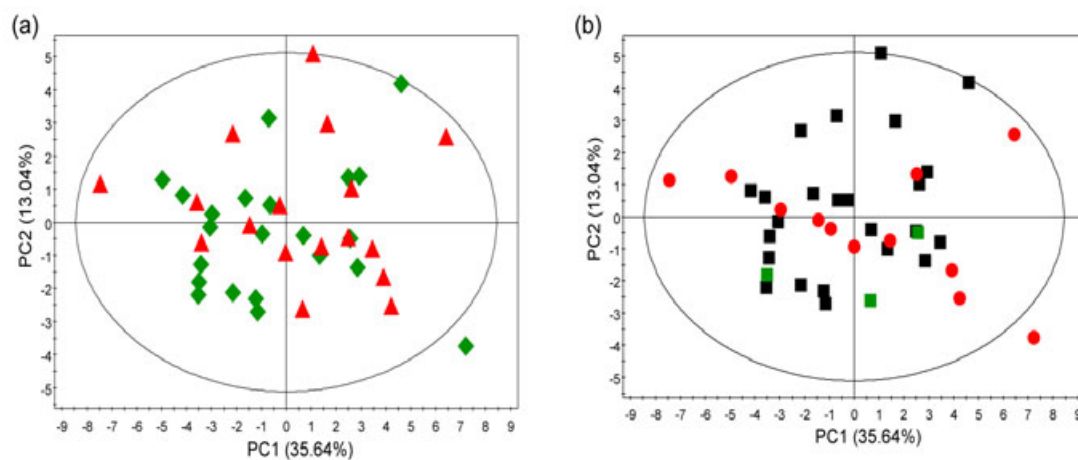


Fig. 7.



Online supplementary data

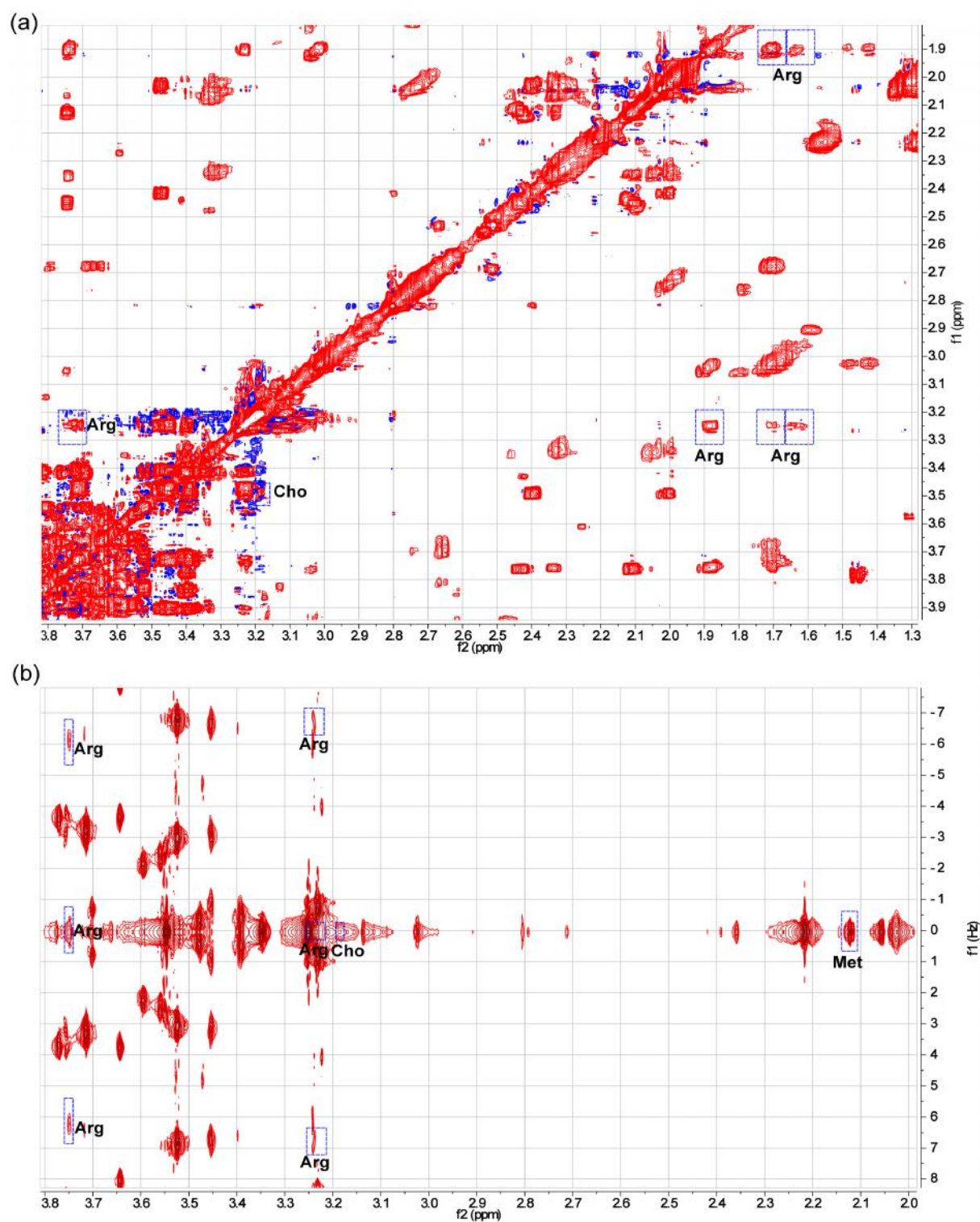


Figure S1. 2D NMR spectra of arginine, methionine and choline which were ambiguous metabolites in 1D NMR spectra. (a) TOCSY (Total correlation spectroscopy), (b) J-resolved NMR spectroscopy; Arg, arginine; Cho, choline; Met, Methionine

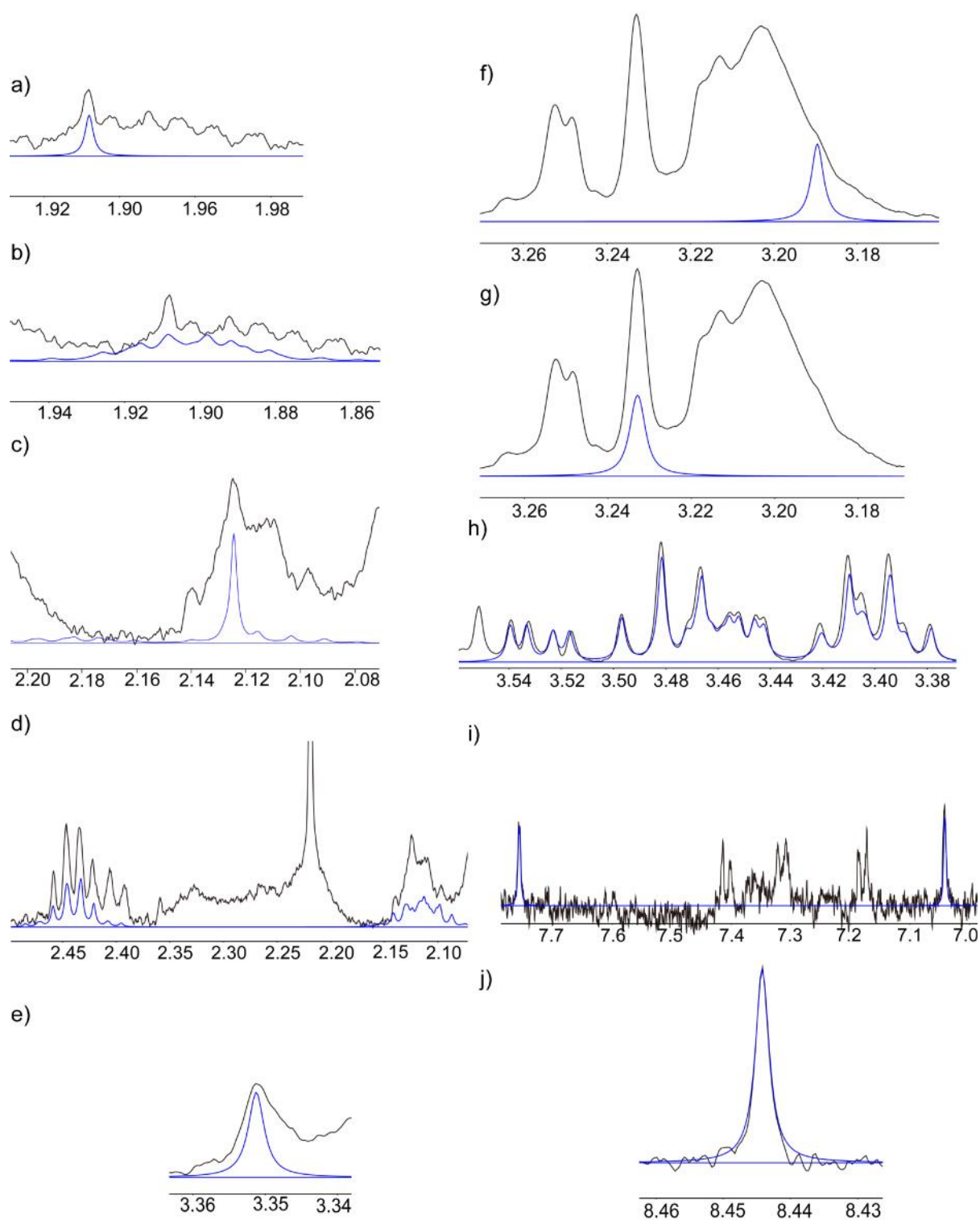


Figure S2. Spectra of 10 key metabolites. Black lines are original spectra of serum and blue lines indicate spectra of acetate (a), arginine (b), methionine (c), glutamine (d), methanol (e), choline (f), O-phosphocholine (g), glucose (h), histidine (i), formate (j).

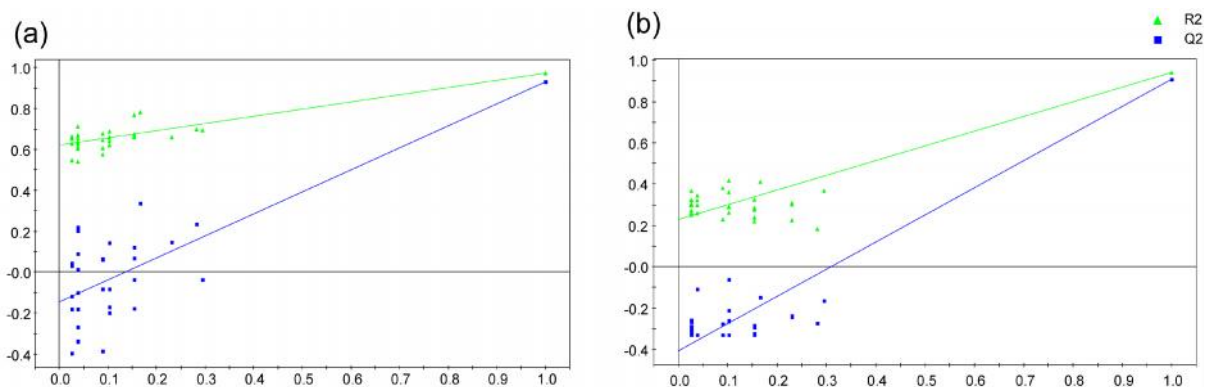


Figure S3. Permutation test (repeated 30 times) of global (a) and targeted (b) profiling. Thirty random permutations of the Y variable resulted in a substantial decrease in R^2 and Q^2 . Furthermore, intercept values larger than 0.05 are indicative of overfitted models. Therefore, these analyses showed that our models were statistically valid and that the high values of predictability did not arise from overfitting.

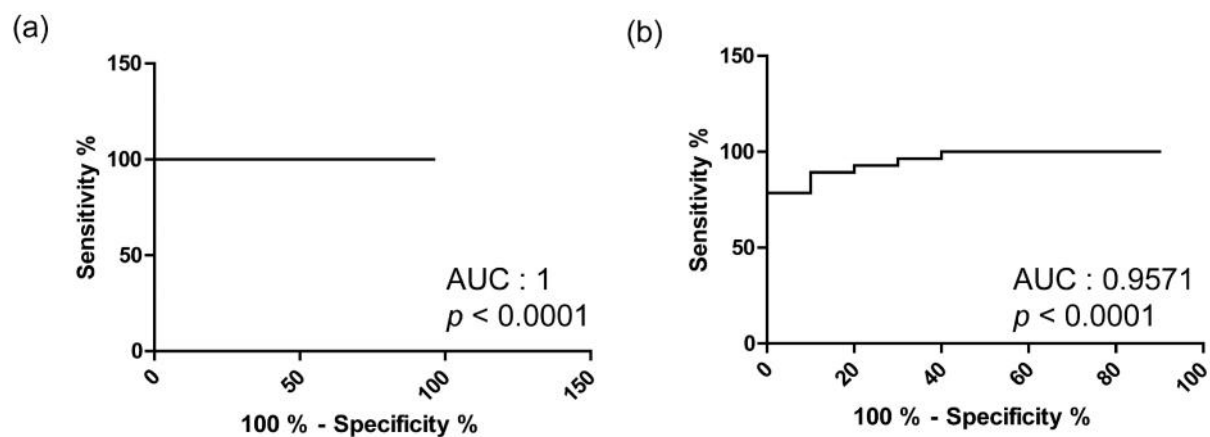


Figure S4. ROC curves for training (a) and external validation (b) sets using the PLS-DA model. AUC values of training and external validation sets were above 0.8, indicating good diagnostic accuracy.

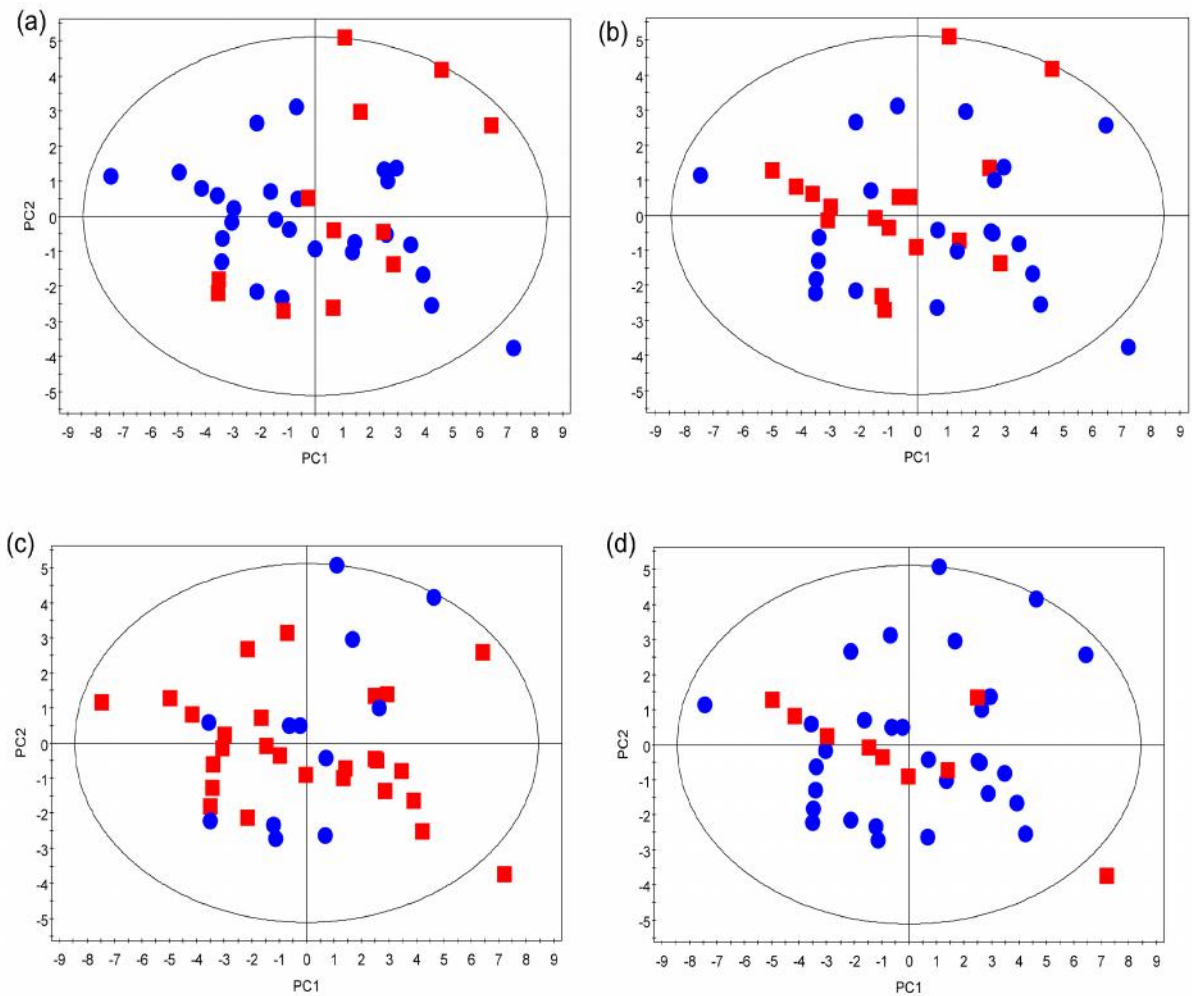


Figure S5. Scores plots of PCA of serum metabolites from patients with asthma, depending on treatment (blue dots, treatment group; red squares, non-treatment group) with a history of oral corticosteroid use during asthma exacerbation for 1 year prior to enrollment (a). Leukotriene receptor antagonist used (b), theophylline used (c), and other drugs used intermittently, such as anti-tussive drugs (d).

Table S1. Chemical shifts and regions used for quantification of serum metabolites.

Metabolite	Chemical shift (multiplicity)	Region used for quantification
Cholesterol-C18	0.83 (m)	0.832–0.836
VLDL/LDL CH ₃	0.36 (m), 0.86 (m)	0.862–0.866
Isoleucine	0.92 (t), 0.99 (d)	0.998–1.006
Leucine	0.94 (t), 1.70 (m), 3.72 (m)	0.944–0.95
Valine	0.97 (d), 1.02 (d), 2.15 (m), 3.60 (d)	1.032–1.035
VLDL (CH ₂) _n	1.25 (m)	1.254–1.26
Lactate	1.3 (d), 4.11 (q)	1.318–1.324
Alanine	1.47 (d), 3.77 (q)	1.468–1.474
Arginine	1.67 (m), 1.91 (m), 3.24 (t), 3.76 (t)	1.914–1.918
Lipid CH ₂ CH ₂ C=C	1.55 (br)	1.552–1.558
Acetate	1.91 (s)	1.902–1.91
N-acetylated glycoprotein	2.04 (br)	2.406–2.412
Glutamate	2.06 (m), 2.34 (m), 3.75 (m)	2.338–2.344
Glutamine	2.12 (m), 2.44 (m), 3.76 (m)	2.444–2.45
Methionine	2.12 (s), 2.11 (m), 2.63 (t), 3.85 (m)	2.118–2.124
Acetone	2.21 (s)	2.214–2.22
Succinate	2.41 (s)	2.388–2.394
Histidine	3.13 (dd), 3.24 (dd), 7.73 (s), 7.09 (s), 3.98 (dd)	7.736–7.73
Choline	3.19 (s), 3.50 (m), 4.07 (m)	3.186–3.19
Glucose	3.24 (dd), 3.40 (m), 3.45 (m), 3.53 (dd), 3.72 (m), 3.83 (m), 3.89 (dd), 4.64 (d), 5.23 (d)	3.51–3.516
Trimethyl-N-oxide	3.25 (s)	3.248–3.254
O-phosphocholine	3.2 (s), 4.15 (t), 3.58 (t)	3.214–3.22
Methanol	3.35 (s)	3.344–3.35
Glycine	3.55 (s)	3.544–3.55
Formate	8.44 (s)	8.438–8.444

Table S2. Serum metabolites.

Metabolite	Chemical shift (multiplicity)	Asthma vs. Healthy		VIP rank
		↑/↓ ^a	<i>p</i> value	
Cholesterol–C18	0.83(m)	↓	0.0164 [*]	19
VLDL/LDL CH ₃	0.36 (m), 0.86 (m)	– ^b	0.5040	24
Isoleucine	0.92 (t), 0.99 (d)	–	0.7066	25
Leucine	0.94 (t), 1.70 (m), 3.72 (m)	↓	0.0002 [*]	11
Valine	0.97 (d), 1.02 (d), 2.15 (m), 3.60 (d)	↓	0.0006 [*]	16
VLDL (CH ₂) _n	1.25 (m)	↑	0.0015 [*]	18
Lactate	1.3 (d), 4.11 (q)	↑	0.0005 ^{†*}	15
Alanine	1.47 (d), 3.77 (q)	↓	0.0009 [*]	17
Arginine	1.67 (m), 1.91 (m), 3.24 (t), 3.76 (t)	↓	<0.0008 ^{†*}	9
Lipid CH ₂ CH ₂ C=C	1.55 (br)	↑	0.0002 [*]	12
Acetate	1.91 (s)	↓	<0.0001 [*]	5
N-acetylated glycoprotein	2.04 (br)	↑	0.0067 ^{†*}	20
Glutamate	2.06 (m), 2.34 (m), 3.75 (m)	↑	<0.0001 ^{†*}	13
Glutamine	2.12 (m), 2.44 (m), 3.76 (m)	↑	<0.0001 ^{†*}	7
Methionine	2.12 (s), 2.11 (m), 2.63 (t), 3.85 (m)	↑	<0.0001 ^{†*}	4
Acetone	2.21 (s)	↓	<0.0001 ^{†*}	21
Succinate	2.41 (s)	↑	0.0004 [*]	14
Histidine	3.13 (dd), 3.24 (dd), 7.73 (s), 7.09 (s), 3.98 (dd)	↓	<0.0001 [*]	3
Choline	3.19 (s), 3.50 (m), 4.07 (m)	↓	<0.0001 ^{†*}	2
Glucose	3.24 (dd), 3.40 (m), 3.45 (m), 3.53 (dd), 3.72 (m), 3.83 (m), 3.89 (dd), 4.64 (d), 5.23 (d)	↓	<0.0001 ^{†*}	6
Trimethyl-N-oxide	3.25 (s)	–	0.1236 [†]	22
O-phosphocholine	3.2 (s), 4.15 (t), 3.58 (t)	↓	<0.0001 [*]	8
Methanol	3.35 (s)	↓	<0.0001 ^{†*}	10
Glycine	3.55 (s)	–	0.4862 [†]	23
Formate	8.44 (s)	↓	<0.0001 ^{†*}	1

^a Arrows (↓ and ↑) indicate a decrease or increase in metabolite levels in asthma patients compared with healthy individuals. ^b No change in levels. The levels were estimated from relative intensities of ¹H-NMR spectra of serum a following spectral normalization. s, singlet; d, doublet; q, quartet; t, triplet, m, multiplet; br, broad; *, *p* < 0.05; †, Mann–Whitney *U* test.

Table S3.LOD and LOQ of ten significant metabolites.

	LOD	LOQ
acetate	0.00076	0.00230
argine	0.29793	0.90283
choline	1.19470	3.62031
formate	0.14587	0.44203
glucose	0.94984	2.87832
histidine	0.04987	0.15113
methanol	0.33516	1.01563
phosphocholine	0.00716	0.02169
methionine	0.60670	1.83848
glutamine	0.27624	0.83708