



Metabolomic study for monitoring of biomarkers in mouse plasma with asthma by gas chromatography–mass spectrometry



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ABSTRACT

Asthma is a multifaceted chronic disease caused by an alteration of various genetic and environmental factors that is increasing in incidence worldwide. However, the biochemical mechanisms regarding asthma are not completely understood. Thus, we performed a metabolomic study for understanding of the biochemical events by monitoring of altered metabolism and biomarkers in asthma. In mice plasma, 27 amino acids (AAs), 24 fatty acids (FAs) and 17 organic acids (OAs) were determined by ethoxycarbonyl (EOC)/methoxime (MO)/*tert*-butyldimethylsilyl (TBDMS) derivatives with GC–MS. Their percentage composition normalized to the corresponding mean levels of control group. They then plotted as star symbol patterns for visual monitoring of altered metabolism, which were characteristic and readily distinguishable in control and asthma groups. The Mann-Whitney test revealed 25 metabolites, including eight AAs, nine FAs and eight OAs, which were significantly different ($p < 0.05$), and orthogonal partial least-squares-discriminant analysis revealed a clear separation of the two groups. In classification analysis, palmitic acid and methionine were the main metabolites for discrimination between asthma and the control followed by pipecolic, lactic, α -ketoglutaric, and linoleic acids for high classification accuracy as potential biomarkers. These explain the metabolic disturbance in asthma for AAs and FAs including intermediate OAs related to the energy metabolism in the TCA cycle.

1. Introduction

Asthma is a multifaceted chronic disease involving inflammation and airway obstruction, which is caused by an alteration of various genetic and environmental factors. Its prevalence has been increasing worldwide [1–3]. On the other hand, the biochemical mechanisms and various clinical symptoms regarding asthma are not completely understood. Therefore, studies for the treatment, diagnosis and prevention of asthma have become important tasks in the clinical fields.

Typically, an alteration of various metabolites in biological fluids reflects the physiological states of normal and diseases [4–7]. Recently, metabolomic studies of asthma in biological fluids were performed as new approaches [8–15]. Among the various metabolites, amino acids (AAs) and organic acids (OAs) including fatty acids (FAs) are closely related to the metabolic pathways. In previous studies, an alteration of

AAs, OAs, and FAs in asthma were reported in various biological fluids, such as urine and plasma including tissues from guinea pigs [8] and humans [9–11,14,15]. In a recent report, correlation between plasma arginine metabolites among AAs and airway dysfunction was reported in a murine model of allergic airway inflammation [16]. In previous results, metabolomic approaches in asthma were performed by nuclear magnetic resonance (NMR) spectroscopy [8,11,14,15], NMR and gas chromatography (GC) coupled with GC-time of flight mass spectrometry (MS) [15], GC–MS, and liquid chromatography–MS/MS (LC–MS/MS) [10,12,13].

Generally, profiling analysis of metabolites in various biological fluids has been performed using high-resolution capillary GC with a high resolving power combined with MS for positive peak confirmation. On the other hand, there are no reports of the simultaneous profiling of AAs, OAs, and FAs by GC–MS combined with star pattern analysis to

Abbreviations: AA, amino acid; OA, organic acid; FA, fatty acid; EOC, ethoxycarbonylation; MO, methoximation; TBDMS, *tert* butyldimethylsilylation; PCA, principal component analysis; OPLS-DA, orthogonal partial least-squares-discriminant analysis; LOO, leave-one-out; 3-NN, three-nearest neighbor; LDA, linear discriminant analysis; DLDA, diagonal linear discriminant analysis; SLDA, stabilized linear discriminant analysis; SVM, support vector machine; PLS-DA, partial least-squares-discriminant analysis; PUFA, polyunsaturated fatty acid; OVA, ovalbumin

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monitor the altered metabolism in asthma.

In previous studies, simultaneous profiling analysis of AAs, OAs, and FAs as ethoxycarbonylation (EOC), methoximation (MO) combined with *tert*-butyldimethylsilyl (TBDMS) derivatives was useful for monitoring the altered metabolism in the urine of patients with inherited metabolic disorder [17,18] and the plasma of aged mice [19] by using GC and GC–MS.

Furthermore, pattern recognition analysis is required to discriminate the normal and disease states from a complex profile of metabolites. In previous results, the star symbol plotting method was effective for visual monitoring and discrimination between normal and disease states [20–23]. Therefore, as new approaches in this study, a metabolomic study was performed to better understand the biochemical events by monitoring the biomarkers and altered metabolism in plasma from ovalbumin (OVA)-induced asthma mice by GC–MS profiling (AAs, OAs, and FAs) combined with star symbol plotting analyses.

2. Materials and methods

2.1. Chemicals and reagents

Ovalbumin (A5503) was purchased from Sigma-Aldrich (Louis, USA). Purified rat anti-mouse IgE (R35-72), IgG1 (A85-3) and IL-4 including IL-5, and biotin rat anti-mouse IgE (R35-118), IgG1 (A85-1) and IL-4 including IL-5 were purchased from BD Biosciences (San Diego, USA). AAs, OAs, FAs, methoxyamine hydrochloride, ethyl chloroformate (ECF), and triethylamine (TEA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA). Toluene, diethyl ether, ethyl acetate, dichloromethane, and sodium chloride of pesticide grade were supplied by Kanto Chemical (Tokyo, Japan). All other chemicals were of analytical grade and used as received.

2.2. Induction of asthma by OVA and assessment of airway hyperresponsiveness (AHR), inflammatory cytokine, and immunoglobulin in OVA-induced asthmatic mice

Mice were sensitized and challenged with OVA as previously described [24,25]. C57BL/6 mice were sensitized by intraperitoneal injection of 10 mg of OVA and 1 mg of Imject Alum (Pierce) in 0.2 mL of saline on days 0 and 7. Control mice were also injected with an equal volume of PBS. On days 14–16, mice received 10 µg of OVA dissolved in 50 µL of saline intranasally under anesthesia with Zoletil and Rumpun. On day 17, blood was collected in retro orbital plexus and bronchoalveolar lavage (BAL) of mice was obtained from 2 mL of instilled saline. After final challenge, mice were weighed and anesthetized with an injection by the administration of Zoletil and Rumpun. The mice was then placed in a flow-type body plethysmograph and connected by the endotracheal cannula to a small-animal ventilator (Flexivent, SCIREQ Inc., Montreal, Canada). Doses of methacholine (MCh) were administered using a nebulizer (Aeroneb) and progressively doubling concentrations ranging from 0 to 50 mg/mL.

2.3. Preparation of standard solutions

Standard stock solutions were prepared individually at 10 µg/µL of AAs including norvaline as the internal standard (IS) in 0.1 M HCl, OAs including 3,4-dimethoxybenzoic acid as IS, and FAs including pentadecanoic acid as IS in methanol. Working solutions of 0.1 µg/µL were then prepared by diluting each stock solution with 0.1 M HCl for the AAs and methanol for the OAs and FAs, respectively. All standard solutions were stored at 4 °C.

2.4. Plasma samples from control and asthma mice

The control (7–8 weeks, $n = 9$) and OVA-induced asthma model (7–8 weeks, $n = 9$) in female C57BL/6N mice (ORIENT BIO, Seongnam, Republic of Korea) were used for the metabolomics study. The blood samples were obtained after all mice were sacrificed; the plasma was then isolated and frozen immediately at -70°C until analyzed. This study performed with the permission of the Institutional Animal Care and Use Committee (UACUC) in the Sunchon National University (Suncheon, Republic of Korea, SCNU IACUC-2015-04).

2.5. Gas chromatography–mass spectrometry

GC–MS profiling analysis in selected ion monitoring (SIM) mode was performed by an Agilent 7890N gas chromatograph interfaced with an 5975C mass-selective detector (70 eV, electron impact mode) and installed with an Ultra-2 (5% phenyl-95% methylpolysiloxane bonded phase; $25\text{ m} \times 0.20\text{ mm i.d.}$, $0.11\text{ }\mu\text{m}$ film thickness) cross-linked capillary column (Agilent Technologies, Atlanta, GA, USA). The temperatures of the injector, interface, and ion source were 260, 300, and 230°C , respectively. Helium was used as the carrier gas at a flow rate of 0.5 mL/min in constant flow mode. The samples were introduced in split-injection mode (10:1), and the oven temperature was initially maintained at 100°C for 2 min and then programmed to 300°C (10 min) at a rate of $5^{\circ}\text{C}/\text{min}$. The mass range was 50–750 μ at a rate of 0.43 scans/s in scan mode.

2.6. Sample preparation for simultaneous profiling analysis of AAs, OAs and FAs in plasma

Simultaneous profiling analysis of AAs, OAs, and FAs was attempted in mice plasma of the control and asthma as their EOC/MO/TBDMS derivatives according to our previously described methods [17,18]. Briefly, the proteins were removed by using acetonitrile to 0.1 mL of plasma containing 0.1 µg of norvaline, 3,4-dimethoxybenzoic acid and pentadecanoic acid as ISs. After centrifugation, 1 mL of distilled water added to the supernatant. Each aliquot solution adjusted to $\text{pH} \geq 12$ with 5.0 M sodium hydroxide was added to dichloromethane (1.0 mL) containing ECF (20 µL), which was converted to the EOC derivative. This was then converted to the MO derivative by a reaction with methoxyamine hydrochloride at 60°C for 30 min. The aqueous phase as sequential EOC/MO derivatives was acidified ($\text{pH} \leq 2.0$) with 10% sulfuric acid, saturated with sodium chloride, and extracted with diethyl ether ($3\text{ mL} \times 2$). The extracts were evaporated to dryness using a gentle nitrogen stream. Dry residues containing AAs, OAs, and FAs were reacted at 60°C for 30 min with TEA (5 µL), toluene (20 µL), and MTBSTFA (20 µL) to form TBDMS derivatives. Each calibration sample containing ISs and AAs, FAs and OAs at varied amounts of 0.1–5.0 µg/mL was subjected to the sequential EOC/MO/TBDMS derivatives for quantification analysis as described procedure. All samples were prepared individually in triplicate and examined directly by GC–MS in SIM mode.

2.7. Star symbol plotting

After the levels of AA, OA and FA were determined based on their calibration curves, which is expressed as the percentage composition. Their levels were normalized to the corresponding mean values from control mice. Each normalized value was plotted as a line radiating from a common central point. The far ends of the lines (27 lines of AAs, 17 lines of OAs, 24 lines of FAs) were joined together to produce star symbol patterns using Microsoft Excel (Microsoft, Redmond, WA) [20–23].

2.8. Statistical analysis

A nonparametric Mann-Whitney test was used to detect the statistical differences in normalized metabolite between the OVA-induced asthma model and control group. Multivariate statistical data analysis and classification analysis were performed on the log₁₀-transformed, mean-centered and unit variance-scaled data. Principal component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were applied for multivariate analysis. For classification analysis, 17 statistically significant candidate metabolites were selected based on the results of the Mann-Whitney test and OPLS-DA after excluding the redundant metabolites showing a high correlation. Subsequently, a total 9483 combinations composed of 2–4 metabolites among 17 candidate metabolites were generated and the discriminatory performance of each combination was evaluated by applying the leave-one-out (LOO) cross validation method and nine supervised machine learning algorithms, including three-nearest neighbor (3-NN), linear discriminant analysis (LDA), diagonal linear discriminant analysis (DLDA), Random Forest, Naïve Bayes, neural network (NNET), stabilized linear discriminant analysis (SLDA), support vector machine (SVM), and partial least-squares-discriminant analysis (PLS-DA). All statistical analyses were performed using the R statistical system (<http://www.R-project.org/>).

3. Results and discussion

3.1. Assessments of AHR, cytokines, immunoglobulins, and BAL total cells in OVA treated with mice

The asthmatic airway inflammation contributes to the AHR. The AHR is developed by the excess accumulation of muscle mass and mechanical augmentation of luminal narrowing. To determine the effect of OVA on airway function, the mice were exposed to MCh aerosols. OVA group exhibited higher airway responsiveness (resistance, elastance, tissue damping, and dynamic elastance) to MCh than control group. Moreover, the total cells, IL-4, and IL-5 in BALF were increased in OVA group compared with control group. In the serum, the total IgE and OVA-specific IgG1 in OVA group were significantly increased compared with the control group. These results suggest that OVA induced the asthma.

3.2. Metabolite profiles of AAs, FAs and OAs in plasma samples of control and asthma mice

In the plasma metabolic profiling analysis from the control and asthma mice, 68 metabolites including 27 AAs (18 protein and 9 non-protein AAs), 17 OAs including seven OAs in the TCA cycle and 24 FAs including seven polyunsaturated FAs (PUFAs) were positively identified and determined as EOC/MO/TBDMS derivatives by GC–MS–SIM. Their levels as the percentage composition showed in Table 1. In the control and asthma groups, among the 27 AAs, glutamine was most abundant, followed by valine and alanine. AAs including alanine among 27 AAs were increased in asthma group except for four AAs of pyroglutamic acid, glutamic acid, glutamine, and lysine. And ornithine level was significantly increased in plasma from OVA-induced asthma mice by compare with that of control mice. This result showed similar pattern to previous result [16] although we did not measured arginine level and arginase activity. Among 24 FAs from two groups, linoleic acid was most abundant FA, palmitic acid and arachidonic acid were second and third most abundant, respectively. The percentage compositions of the six FAs were reduced in asthma group, while 18 FAs were increased. In the OA profile of the two groups, most abundant OA was lactic acid. In the control group, the second most abundant OA was 3-hydroxybutyric acid and citric acid was the third most abundant OA. In asthma group, however, the second and third most abundant OAs were citric acid and 3-hydroxybutyric acid, respectively. Among 17 OAs, 14 OAs were

reduced or identical in the asthma group except for three OAs of lactic, malonic and 4-hydroxyphenylacetic acids. In the asthma group, most of the AAs and FAs were increased, while the OAs were reduced compared to those of the control group. The alteration of metabolites reflected the disturbance of the FA metabolism, biosynthesis, and deamination of AAs including the energy metabolism in the TCA cycle from asthma. In particular, higher AAs and lower OAs than those of the control group are related to a reduction for the deamination of AAs in the metabolic pathway of asthma. Generally, ω -6 and ω -3 PUFAs are well known proinflammatory and anti-inflammatory mediators, respectively [26]. In this result, ω -6 was reduced and ω -3 PUFAs was elevated in the asthma group compared to the normal group, which may explain the altered FA metabolism associated with inflammation.

3.3. Star pattern recognition of control and asthma groups

The percentage composition (%) of 68 metabolites (27 AAs, 17 OAs, 24 FAs) were normalized to the corresponding control group mean value. These are listed in Table 1, which were informative in expressing the altered AA, FA and OA levels as multiple ranges of 0.8–2.1 (AAs), 0.6–1.3 (FAs), and 0.3–1.2 (OAs) in asthma group, respectively. As variables, their normalized levels were plotted in star graphs composed of 27 (AAs), 24 (FAs), and 17 (OAs) rays for a visual monitoring of the difference for each metabolite in each group (Fig. 1). The distorted star patterns of AAs (Fig. 1A), FAs (Fig. 1B), and OAs (Fig. 1C) in the asthma group were readily distinguishable and characteristic for the control group. This may explain the metabolic disturbance of AA, OA, and FA in the asthma group compared to those of the control group.

3.4. Statistical analysis

Table 1 showed the descriptive statistics of 68 metabolites including 27 AAs, 24 FAs, and 17 OAs in the plasma in the control and asthma groups. The PCA analysis, one of the powerful unsupervised pattern recognition methods, was performed to examine the intrinsic variations in the metabolite levels (Fig. 2A). The PCA score plot with the first two components showed good separation between asthma and control group explaining 55% of the total variances with the two components. To obtain better differentiation, OPLS-DA analysis was conducted with six-fold internal cross-validation. The score plot showed a clear separation of the two groups (Fig. 2B), R^2Y and Q^2 were 0.937 and 0.749, respectively. To identify the significant metabolites responsible for the discrimination between asthma group and control group, p -values of the Mann-Whitney test and regression coefficients of OPLS-DA were determined for a total of 68 metabolites (Table 1). The Mann-Whitney test showed that the levels of the 25 metabolites were significantly different between the asthma group and control group ($p < 0.05$), including eight AAs (methionine, pipecolic acid, proline, ornithine, phenylalanine, glutamine, alanine, and histidine), nine FAs (γ -linolenic, palmitic, eicosanoic, eicosenoic, beheniclauric, erucic, linoleic and docosahexaenoic acids), and eight OAs (lactic, 3-hydroxybutyric, succinic, α -ketoglutaric, 4-hydroxyphenylacetic, malic, 2-hydroxyglutaric, and citric acids) (Fig. 3A). Among the 25 significantly different metabolites, 16 metabolites were increased and nine metabolites were decreased in the OVA-induced asthma mice compared to the controls (Fig. 3A). In particular, elevated lactic acid levels [9,14], reduction for intermediate OAs in TCA cycle [9,15], and elevated methionine and histidine levels [14] in asthma were similar to previous results in patients with asthma. In addition, reduced γ -linolenic and linoleic acids as ω -6 PUFAs are related to the proinflammatory agent, including elevated docosahexaenoic acid as ω -3PUFA with an antioxidant effect among FAs may explain the regulation of metabolic disturbance and protection of inflammation in asthma. On the other hand, other 25 top-ranked metabolites were detected according to the regression coefficients of the variation by OPLS-DA, which included an almost identical set of metabolites to the metabolite list obtained from the Mann-Whitney test

Table 1

68 Metabolites levels of 27 amino acids, 24 fatty acids and 17 organic acids, Mann-Whitney test and OPLS-DA analysis.

Class	No.	Metabolite	Percentage (%) in plasma (mean $\pm$ SD)		Normalized value ^a	difference of means Asthma – Control	Mann–Whitney test <i>p</i> -value	OPLS-DA Coefficient of variation
			Control (n = 9)	Asthma (n = 9)				
Amino acid	1	Alanine	3.360 $\pm$ 1.045	4.807 $\pm$ 1.689	1.4	1.447	0.03998	0.033
	2	Glycine	0.450 $\pm$ 0.096	0.605 $\pm$ 0.186	1.3	0.155	0.07701	0.034
	3	α -Aminobutyric acid	0.062 $\pm$ 0.012	0.055 $\pm$ 0.019	0.8	–0.007	0.29733	–0.020
	4	Valine	3.916 $\pm$ 1.068	5.228 $\pm$ 2.106	1.3	1.312	0.25808	0.026
	5	β -Aminoisobutyric acid	0.009 $\pm$ 0.001	0.012 $\pm$ 0.004	1.2	0.003	0.14491	0.029
	6	Leucine	1.720 $\pm$ 0.406	2.356 $\pm$ 0.976	1.3	0.636	0.16150	0.028
	7	Isoleucine	1.475 $\pm$ 0.337	2.037 $\pm$ 0.889	1.3	0.562	0.22242	0.028
	8	Threonine	1.092 $\pm$ 0.394	1.498 $\pm$ 0.624	1.3	0.406	0.17088	0.026
	9	Serine	0.868 $\pm$ 0.263	0.913 $\pm$ 0.504	1.0	0.045	1.00000	–0.005
	10	Proline	1.270 $\pm$ 0.409	2.435 $\pm$ 1.124	1.8	1.165	0.00276	0.045
	11	γ -Aminobutyric acid	0.136 $\pm$ 0.019	0.181 $\pm$ 0.073	1.3	0.045	0.34011	0.027
	12	Pipecolic acid	0.048 $\pm$ 0.006	0.072 $\pm$ 0.023	1.4	0.024	0.00048	0.047
	13	Pyroglutamic acid	0.277 $\pm$ 0.073	0.266 $\pm$ 0.181	0.9	–0.011	0.16150	–0.014
	14	4-Hydroxyproline	1.150 $\pm$ 0.282	1.574 $\pm$ 0.777	1.3	0.424	0.48943	0.023
	15	Methionine	0.539 $\pm$ 0.183	1.177 $\pm$ 0.399	2.1	0.638	0.00004	0.053
	16	Phenylalanine	0.629 $\pm$ 0.139	0.857 $\pm$ 0.264	1.3	0.228	0.01419	0.037
	17	Aspartic acid	0.087 $\pm$ 0.015	0.103 $\pm$ 0.038	1.2	0.016	0.66647	0.017
	18	N-Methyl-DL-aspartic acid	0.016 $\pm$ 0.002	0.022 $\pm$ 0.008	1.3	0.006	0.07724	0.031
	19	Glutamic acid	0.526 $\pm$ 0.125	0.491 $\pm$ 0.178	0.9	–0.035	0.48943	–0.011
	20	Asparagine	0.250 $\pm$ 0.104	0.441 $\pm$ 0.379	1.7	0.191	0.22242	0.024
	21	Ornithine	0.752 $\pm$ 0.161	0.965 $\pm$ 0.156	1.3	0.213	0.00777	0.043
	22	α -Aminoadipic acid	0.126 $\pm$ 0.016	0.149 $\pm$ 0.047	1.1	0.023	0.65851	0.020
	23	Glutamine	75.51 $\pm$ 5.332	65.75 $\pm$ 11.05	0.9	–9.760	0.01876	–0.035
	24	Lysine	2.843 $\pm$ 0.415	2.833 $\pm$ 0.336	1.0	–0.010	0.86331	0.000
	25	Histidine	1.720 $\pm$ 0.696	3.750 $\pm$ 2.706	2.1	2.030	0.03998	0.034
	26	Tyrosine	1.058 $\pm$ 0.273	1.288 $\pm$ 0.232	1.2	0.230	0.09391	0.031
	27	Tryptophan	0.109 $\pm$ 0.016	0.134 $\pm$ 0.037	1.2	0.025	0.19025	0.029
Fatty acid	28	Caproic acid	0.123 $\pm$ 0.057	0.153 $\pm$ 0.065	1.2	0.030	0.25808	0.014
	29	Caprylic acid	0.023 $\pm$ 0.012	0.020 $\pm$ 0.014	0.9	–0.003	0.79617	–0.017
	30	Capric acid	0.024 $\pm$ 0.005	0.025 $\pm$ 0.005	1.0	0.001	0.72380	0.006
	31	Lauric acid	0.005 $\pm$ 0.001	0.006 $\pm$ 0.001	1.2	0.001	0.02108	0.038
	32	Myristoleic acid	0.035 $\pm$ 0.013	0.036 $\pm$ 0.006	1.0	0.001	0.54570	0.007
	33	Myristic acid	0.405 $\pm$ 0.097	0.375 $\pm$ 0.072	0.9	–0.030	0.54570	–0.011
	34	Palmitoleic acid	1.891 $\pm$ 0.569	1.797 $\pm$ 0.335	1.0	–0.094	0.93143	–0.004
	35	Palmitic acid	15.74 $\pm$ 0.840	17.75 $\pm$ 1.636	1.1	2.010	0.00564	0.045
	36	γ -Linolenic acid	0.504 $\pm$ 0.090	0.320 $\pm$ 0.085	0.6	–0.184	0.00185	–0.053
	37	Linoleic acid	40.28 $\pm$ 2.521	36.52 $\pm$ 2.456	0.9	–3.760	0.03998	–0.044
	38	Oleic acid	11.30 $\pm$ 1.350	10.57 $\pm$ 0.881	0.9	–0.730	0.11349	–0.021
	39	α -Linolenic acid	1.997 $\pm$ 0.250	2.080 $\pm$ 0.333	1.0	0.083	0.43628	0.008
	40	Stearic acid	9.126 $\pm$ 1.023	9.615 $\pm$ 1.074	1.1	0.489	0.29733	0.018
	41	Arachidonic acid	14.19 $\pm$ 2.995	15.57 $\pm$ 2.950	1.1	1.380	0.29733	0.017
	42	Eicosapentaenoic acid	1.936 $\pm$ 0.423	2.076 $\pm$ 0.363	1.1	0.140	0.66647	0.014
	43	Eicosenoic acid	0.516 $\pm$ 0.119	0.663 $\pm$ 0.133	1.3	0.147	0.01419	0.038
	44	Eicosanoic acid	0.121 $\pm$ 0.020	0.156 $\pm$ 0.026	1.3	0.035	0.01061	0.043
	45	Docosahexaenoic acid	0.651 $\pm$ 0.122	0.809 $\pm$ 0.167	1.2	0.158	0.04215	0.035
	46	Docosapentaenoic acid	0.887 $\pm$ 0.153	1.171 $\pm$ 0.283	1.3	0.284	0.05031	0.038
	47	Eruic acid	0.039 $\pm$ 0.006	0.050 $\pm$ 0.009	1.3	0.011	0.02162	0.042
	48	Behenic acid	0.108 $\pm$ 0.017	0.135 $\pm$ 0.026	1.3	0.027	0.01419	0.040
	49	Nervonic acid	0.031 $\pm$ 0.007	0.037 $\pm$ 0.007	1.2	0.006	0.16150	0.030
	50	Lignoceric acid	0.035 $\pm$ 0.006	0.043 $\pm$ 0.009	1.2	0.008	0.06253	0.034
	51	Hexacosanoic acid	0.026 $\pm$ 0.005	0.032 $\pm$ 0.006	1.2	0.006	0.06355	0.034
Organic acid	52	3-Hydroxybutyric acid	6.351 $\pm$ 2.293	2.701 $\pm$ 1.028	0.4	–3.650	0.00049	–0.054
	53	Pyruvic acid	3.044 $\pm$ 0.573	2.410 $\pm$ 1.094	0.8	–0.634	0.09391	–0.028
	54	Acetoacetic acid	0.530 $\pm$ 0.358	0.447 $\pm$ 0.087	0.9	–0.083	0.60481	0.007
	55	Lactic acid	74.42 $\pm$ 4.464	83.44 $\pm$ 2.435	1.1	9.020	0.00004	0.055
	56	Glycolic acid	2.284 $\pm$ 3.073	0.745 $\pm$ 0.340	0.3	–1.539	0.16150	–0.025
	57	2-Hydroxybutyric acid	0.112 $\pm$ 0.043	0.088 $\pm$ 0.050	0.7	–0.024	0.29733	–0.020
	58	Malonic acid	0.025 $\pm$ 0.006	0.031 $\pm$ 0.021	1.2	0.006	0.96477	0.008
	59	Succinic acid	1.647 $\pm$ 0.403	1.151 $\pm$ 0.447	0.7	–0.496	0.01876	–0.039
	60	Fumaric acid	0.569 $\pm$ 0.114	0.465 $\pm$ 0.152	0.8	–0.104	0.13591	–0.029
	61	α -Ketoglutaric acid	3.662 $\pm$ 0.878	2.676 $\pm$ 0.736	0.7	–0.986	0.01876	–0.039
	62	4-Hydroxyphenylacetic acid	0.030 $\pm$ 0.007	0.038 $\pm$ 0.007	1.2	0.008	0.03147	0.039
	63	Malic acid	1.947 $\pm$ 0.336	1.450 $\pm$ 0.407	0.7	–0.497	0.03147	–0.040
	64	2-Hydroxyglutaric acid	0.184 $\pm$ 0.034	0.147 $\pm$ 0.028	0.8	–0.037	0.03147	–0.037
	65	cis-Aconitic acid	0.153 $\pm$ 0.027	0.153 $\pm$ 0.040	1.0	0.000	0.79098	–0.004
	66	4-Hydroxyphenyllactic acid	0.184 $\pm$ 0.033	0.188 $\pm$ 0.040	1.0	0.004	0.73044	0.002
	67	Citric acid	4.382 $\pm$ 0.824	3.395 $\pm$ 0.674	0.8	–0.987	0.03147	–0.039
	68	Isocitric acid	0.478 $\pm$ 0.067	0.473 $\pm$ 0.116	0.9	–0.005	0.79617	–0.005

^a Values normalized to the corresponding control composition values.

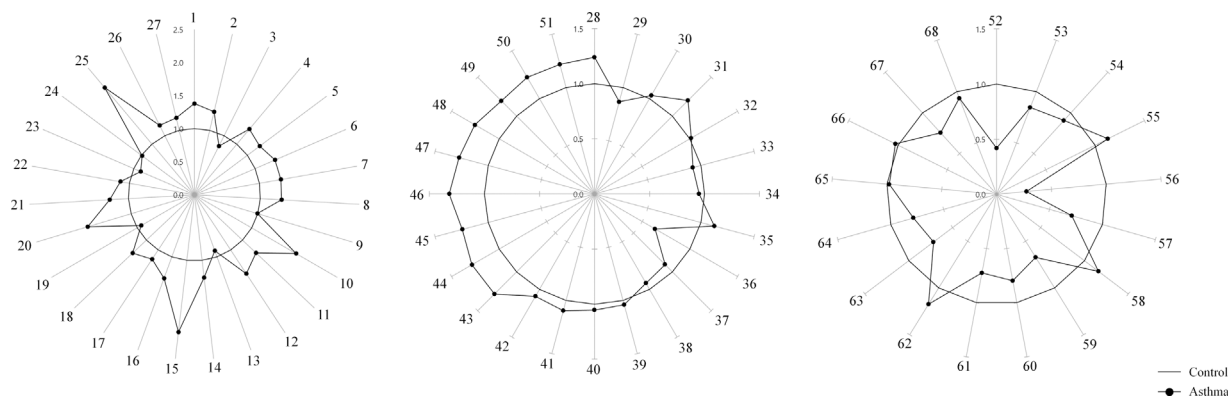


Fig. 1. Star symbol plots of the mean data from control and asthma mice drawn based on the AA (A), FA (B) and OA (C) levels after normalization to the corresponding mean values of the control mice. The numbers on the rays correspond to those in Table 1.

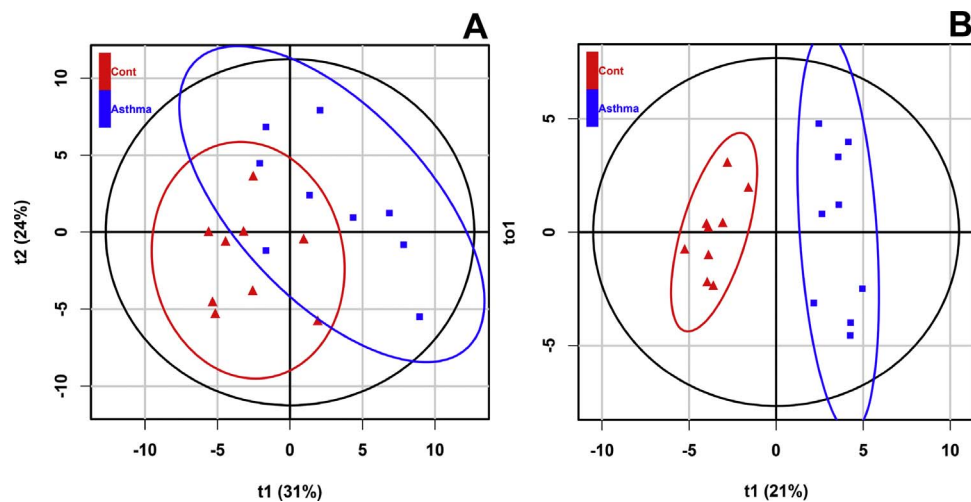


Fig. 2. PCA score plot (A) and OPLS-DA score plot (B) of OVA-induced asthma and control mice. Triangles (▲) and squares (■) indicate control and OVA-induced asthma mice, respectively.

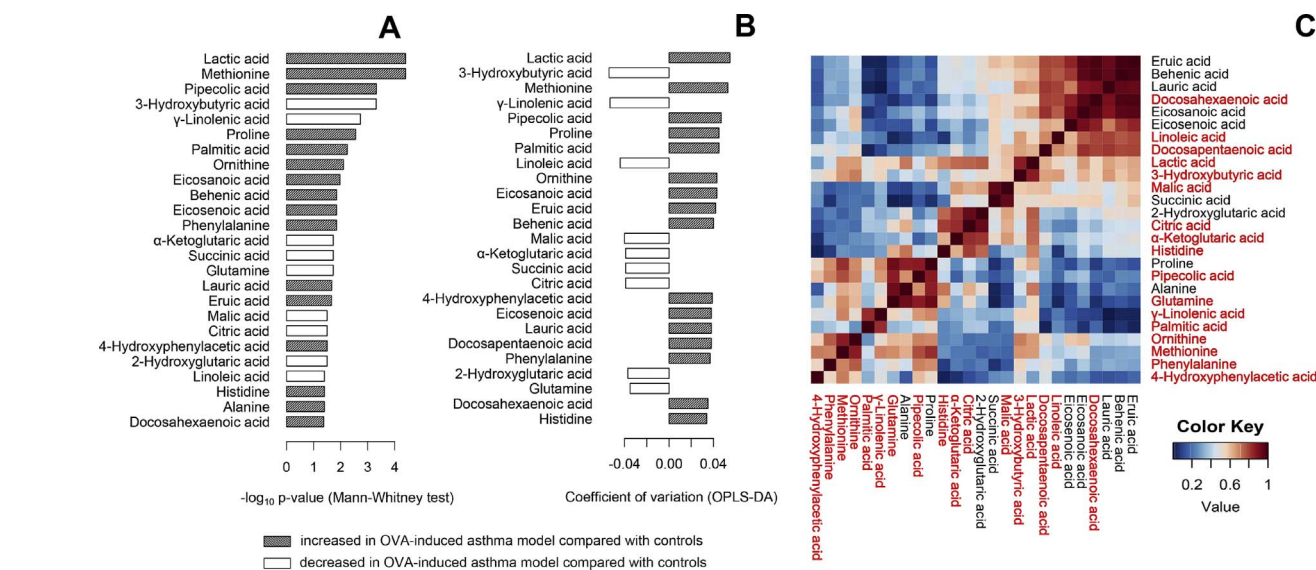


Fig. 3. Bar plot of $-\log_{10}$ p -values of 25 top metabolites ranked by the statistical significance of the Mann-Whitney test (A), bar plot of regression coefficients of variation of 25 top metabolites ranked by result of OPLS-DA (B), and heat map of the Pearson's correlation coefficients of 26 candidate metabolites of union of two sets of 25 top-ranked metabolites obtained by Mann-Whitney test and OPLS-DA (C). The metabolites in red indicate the final 17 metabolites used for further classification analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

except for only one metabolite difference, docosapentaenoic acid in OPLS-DA instead of alanine in the Mann-Whitney test (Fig. 3A and B). Consequently, a total of 26 metabolites of the union of the two sets of

25 top-ranked metabolites obtained by the Mann-Whitney test and OPLS-DA were considered as the candidate metabolites for the separation of OVA-induced asthma model from control group. Further

Table 2

Classification performance using metabolite combinations for discrimination between asthma and control group.

Number of metabolites	Combination of metabolites	Average of classification accuracy (%) ^a			Algorithm-specific classification accuracy (%)			
		Overall	Control	Asthma	Algorithm	Overall	Control	Asthma
4	Methionine, Palmitic acid, Linoleic acid, Lactic acid	100	100	100	All	100	100	100
4	Pipecolic acid, Palmitic acid, Linoleic acid, Lactic acid	100	100	100	All	100	100	100
4	Pipecolic acid, Methionine, Palmitic acid, α -Ketoglutaric acid	100	100	100	All	100	100	100
3	Methionine, Palmitic acid, α -Ketoglutaric acid	98.8	98.8	98.8	3-NN	100	100	100
					LDA	100	100	100
					DLDA	100	100	100
					Random Forest	100	100	100
					Naïve Bayes	100	100	100
					NNET	100	100	100
					SLDA	100	100	100
					SVM	94.4	88.9	100
					PLS-DA	94.4	100	88.9
3	Methionine, Palmitic acid, Docosahexaenoic acid	98.8	98.8	98.8	3-NN	100	100	100
					LDA	100	100	100
					DLDA	100	100	100
					Random Forest	94.4	100	88.9
					Naïve Bayes	100	100	100
					NNET	100	100	100
					SLDA	100	100	100
					SVM	94.4	88.9	100
					PLS-DA	100	100	100
2	Methionine, α -Ketoglutaric acid	94.4	96.3	92.6	3-NN	94.4	100	88.9
					LDA	94.4	88.9	100
					DLDA	94.4	88.9	100
					Random Forest	94.4	100	88.9
					Naïve Bayes	100	100	100
					NNET	94.4	100	88.9
					SLDA	94.4	100	88.9
					SVM	88.9	88.9	88.9
					PLS-DA	94.4	100	88.9
2	Palmitic acid, 3-Hydroxybutyric acid	94.4	95.1	93.8	3-NN	94.4	100	88.9
					LDA	94.4	88.9	100
					DLDA	94.4	88.9	100
					Random Forest	94.4	88.9	100
					Naïve Bayes	94.4	88.9	100
					NNET	88.9	100	77.8
					SLDA	100	100	100
					SVM	100	100	100
					PLS-DA	88.9	100	77.8
2	Pipecolic acid, Lactic acid	94.4	90.1	98.8	3-NN	83.3	77.8	88.9
					LDA	94.4	88.9	100
					DLDA	94.4	88.9	100
					Random Forest	94.4	88.9	100
					Naïve Bayes	100	100	100
					NNET	100	100	100
					SLDA	94.4	88.9	100
					SVM	88.9	77.8	100
					PLS-DA	100	100	100

^a Averaged classification accuracy was determined by averaging of eight overall classification accuracies calculated with eight different machine learning techniques.

calculations of the pairwise Pearson's correlation coefficients with log10 transformed, mean-centered and unit variance-scaled concentration of the 26 candidate metabolites showed that the concentrations of several metabolites were highly correlated. Accordingly, when the metabolites showed high correlations (Pearson's correlation coefficient ≥ 0.90), only one representative metabolite with the highest percentage in the plasma was left in the final list of the candidate metabolites. Finally, a total 17 metabolites were selected as the candidate metabolites (Fig. 3C), which were used for further classification analysis. To find the most parsimonious set of metabolites showing the highest discrimination power for the separation of OVA-induced asthma from the control group, a total of 9483 combinations comprised of 2–4 metabolites out of the 17 metabolites were generated. Subsequently, the discriminatory performance for each combination of metabolites was evaluated by applying the LOO cross validation method based on

nine supervised machine learning algorithms. Table 2 showed the classification accuracies of top-ranked combinations of metabolites. Perfect classification performance (100%) was observed in the three combinations composed of four metabolites (Table 2). All three combinations with perfect classification included palmitic acid commonly among the candidate FAs. In addition, two out of the three combinations were composed of linoleic acid, which was one of ω -6 FAs and detected using the highest percentage level in plasma among the FA metabolites. On the other hand, methionine or/and pipecolic acid were recognized as the AA metabolites for perfect classification accuracy, and lactic acid or α -ketoglutaric acid as OA metabolites. Considering all the top-ranked combinations of the metabolites with 2–4 metabolites, palmitic acid and methionine appeared to be the most responsible metabolites for the discrimination of the OVA-induced murine asthma model from the control group, which was followed by the metabolites,

such as pipecolic, lactic, α -ketoglutaric, and linoleic acids for high classification accuracy as potential biomarkers (Table 2). This may explain the alteration of the AAs and FAs as energy sources and anti-oxidant including intermediate OAs related to the TCA cycle and energy metabolism in asthma.

4. Conclusions

Metabolic profiling of AAs and FAs including OAs as EOC/MO/TBDSMS derivatives by GC–MS combined with star pattern analysis performed in the plasma samples of control and OVA-induced asthma mice. A total 68 metabolites of 27 AAs, 24 FAs and 17 OAs were determined in plasma samples of control and asthma mice. In star graphic pattern analysis, the metabolic patterns of AAs, FAs and OAs were characteristic and readily distinguishable for the control and asthma groups. Among the 25 significantly different metabolites ($p < 0.05$), 16 metabolites were increased and nine metabolites were decreased in the asthma mice compared to the control mice. In OPLS-DA, two groups were clearly separated. In classification analysis for the two groups, palmitic acid and methionine were the most common metabolites, followed by pipecolic, lactic, α -ketoglutaric, and linoleic acids as potential biomarkers. This finding may explain the disturbance of AA, FA, and OA metabolism in asthma. Therefore, the present metabolic profiling and star pattern analyses will be useful for understanding the biochemical events and monitoring the altered metabolism in asthma.

Conflict of interest

The authors declare there is no conflict of interest.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee in the Sunchon National University.

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