

Serum Metabolomics Study and Eicosanoid Analysis of Childhood Atopic Dermatitis Based on Liquid Chromatography–Mass Spectrometry

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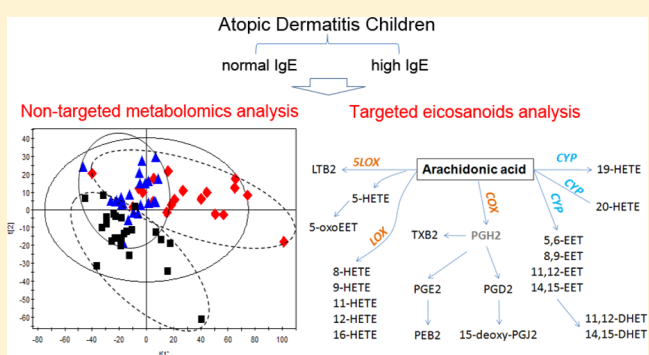
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S Supporting Information

ABSTRACT: Atopic dermatitis (AD) is the most common inflammatory skin disease in children. In the study, ultra high performance liquid chromatography–mass spectrometry was used to investigate serum metabolic abnormalities of AD children. Two batch fasting sera were collected from AD children and healthy control; one of them was for nontargeted metabolomics analysis, the other for targeted eicosanoids analysis. AD children were divided into high immunoglobulin E (IgE) group and normal IgE group. On the basis of the two analysis approaches, it was found that the differential metabolites of AD, leukotriene B₄, prostaglandins, conjugated bile acids, etc., were associated with inflammatory response and bile acids metabolism. Carnitines, free fatty acids, lactic acid, etc., increased in the AD group with high IgE, which revealed energy metabolism disorder. Amino acid metabolic abnormalities and increased levels of Cytochrome P450 epoxygenase metabolites were found in the AD group with normal IgE. The results provided a new perspective to understand the mechanism and find potential biomarkers of AD and may provide a new reference for personalized treatment.

KEYWORDS: Atopic dermatitis, metabolomics, eicosanoids, immunoglobulin E, LC–MS,



1. INTRODUCTION

Atopic dermatitis (AD) is the most common inflammatory skin disease in children. Particularly in industrialized countries, the prevalence of this disease is about 20%.¹ According to an epidemiological investigation in the Shanghai region, the prevalence of AD was 8.3% in children aged 3 to 6.² The etiology of AD is complex, including diet, pollution, microbial exposure, and interactions with genetic factors and the immune system.^{1,3} As the first manifestation of allergic diseases, AD is considered to associate with childhood asthma and rhinitis. The high levels of immunoglobulin E (IgE) is a major pathogenic factor of AD and other allergic diseases, such as asthma and rhinitis.⁴ IgE is in charge of most adaptive immune response and leads to intensive inflammation reactions.⁵ IgE increase in blood is also one of the diagnostic criteria of AD.⁶ However, there are many patients of AD, who do not show the increase of blood IgE. Pathogenic mechanism study of the patients with high IgE and normal IgE is valuable for different treatment.

Metabolomics is a powerful tool in disease phenotype investigation, which provides abundant information for

biomarker discovery, pathogenesis, and personalized treatment.^{7–9} A urinary metabolomics study was used to investigate the change of urine profiles in infants with AD by ¹H-nuclear magnetic resonance; the children with AD and healthy control were clearly separated by principal component analysis.¹⁰ Skin lipids were investigated by liquid chromatography–mass spectrometry; ceramides and free fatty acids were analyzed.^{11,12} However, the study on serum metabolic characteristics of AD patients was very few. Though the inflammation role of leukotrienes and prostaglandins was identified in AD patients,^{13,14} the serum levels of the eicosanoids were not well characterized.

In the study, we investigated serum samples of AD children with a nontargeted metabolomics approach and a targeted eicosanoids analysis approach based on ultra high performance liquid chromatography–mass spectrometry (UHPLC–MS). The study strategy is shown in Figure 1. Our aims are to

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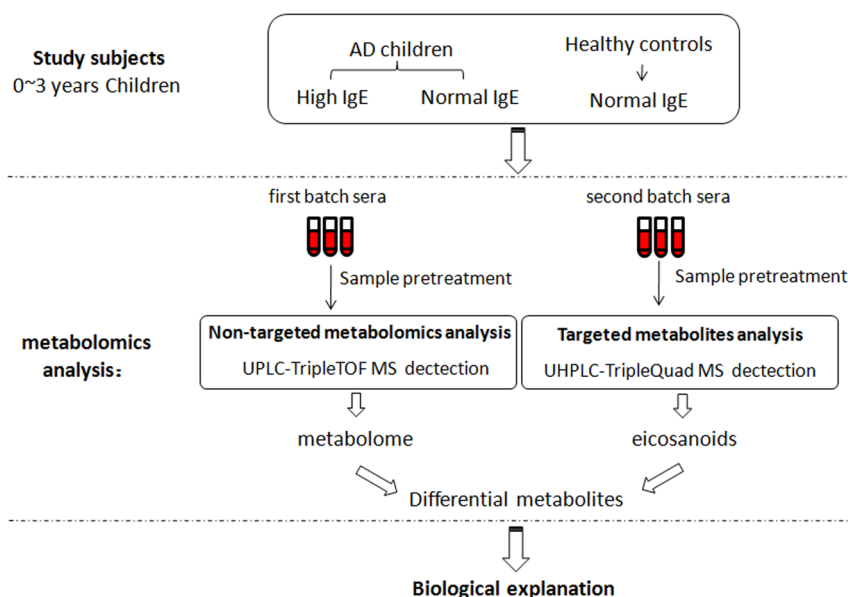


Figure 1. Flowchart of study strategy.

Table 1. Patient Information

		total IgE (IU/mL)	age (months)	gender
first batch	healthy controls (<i>n</i> = 23)	6.8 ± 7.2 (0.6–32.6)	16.2 ± 10.4	10 girls, 13 boys
	ADNIgE (<i>n</i> = 23)	12.0 ± 10.7 (0.8–35.6)	15.3 ± 9.5	10 girls, 13 boys
	ADH IgE (<i>n</i> = 19)	220.7 ± 159.0 ^{a,b} (52.3–541)	18.8 ± 11.0	5 girls, 14 boys
second batch	healthy controls (<i>n</i> = 22)	16.3 ± 13.4 (0.0–43.2)	19.4 ± 7.2	5 girls, 17 boys
	ADNIgE (<i>n</i> = 22)	16.8 ± 13.3 (0.0–40.2)	15.4 ± 9.5	11 girls, 11 boys
	ADH IgE (<i>n</i> = 19)	194.7 ± 112.2 ^{a,b} (51.4–454)	19.2 ± 8.6	7 girls, 12 boys

^a*p* < 0.05 compared with healthy controls. ^b*p* < 0.05 compared with ADNIgE group.

provide a new perspective to understand the mechanism and to find potential biomarkers of AD. The results may provide a new reference for the diagnosis and personalized treatment of AD.

2. MATERIALS AND METHODS

2.1. Sample Collection

All children were recruited from Dalian Children's Hospital. The study was approved by the Ethics Committee of the hospital. Children from three months age to 36 months age were selected in the study. AD was diagnosed according to Hanifin and Rajka diagnostic criteria.⁶ The first batch of fasting sera for nontargeted metabolomics analysis was collected from 42 AD patients and 23 healthy controls during February to June, 2013. There were 19 AD patients with high IgE levels (ADHIgE group) and 23 AD patients with normal IgE levels (ADNIgE group). The second batch of fasting sera was collected from 41 AD patients and 22 healthy controls for targeted eicosanoids analysis during June to October, 2013. There were 19 AD patients with high IgE levels and 22 AD patients with normal IgE levels. Two batches of serum were needed because of the limited blood collected from each child. Sample information is summarized in Table 1.

2.2. Nontargeted Metabolomics Analysis

Two hundred microliters of serum was deproteinized with 4 volumes of acetonitrile containing 14 internal standards.¹⁵ The internal standards were carnitine C2:0-d3, carnitine C10:0-d3, carnitine C16:0-d3, cholic acid-d4, lysophosphatidylcholine (LPC) 12:0, LPC 19:0, phenylalanine-d5, tryptophan-d5, free

fatty acid (FFA) 16:0-d3, chenodeoxycholic acid-d4, leucine-enkephalin, lansoprazole, sphingomyelin (SM) d18:1/12:0, and phosphatidylethanolamine (PE) 34:0 according to our recent article.¹⁵ After centrifuged at 13 000g for 15 min, the supernatant was divided into two aliquots and dried in a vacuum centrifuge for following positive ion mode detection and negative ion mode detection. The aliquots were reconstituted in 100 μL of acetonitrile/water (2:8) and analyzed by a Waters ACQUITY ultra performance liquid chromatography system (UPLC) (Waters Corp, Milford, USA) coupled with an AB SCIEX TripleTOF 5600 System (AB SCIEX, Framingham, USA). The injection volume was 6 μL. The quality control (QC) samples were prepared by mixing 10 μL of each sample and were analyzed after each six serum samples.

A 2.1 × 100 mm ACQUITYTM 1.7 μm C8 BEH column (Waters, Ireland) was used for LC separation in positive ion mode. The mobile phases were (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The gradient elution was 95% A kept 1 min, then changed linearly to 100% B within 24 min, held for 4 min. For negative ion mode, the LC separation was performed with a 2.1 × 100 mm ACQUITYTM 1.8 μm T3 HSS column (Waters, Ireland). The mobile phases were (C) water with 6.5 mM NH₄HCO₃ and (D) 95% methanol/water with 6.5 mM NH₄HCO₃. The gradient elution was 2% D kept 1 min, then changed linearly to 100% D within 18 min, kept for 4 min. The flow rate was 0.35 mL/min. Column temperature was maintained at 50 °C.

Table 2. Mass Transitions, Collision Energy, Precision, and Linear Range of the Method

	retention time	parent ion	product ion	CE (eV)	precision RSD %	linear regression equation	correlation coefficient	linear range (nM)
6-ketePGF1a	1.49	369.5	163.1	20	23.6%	$y = 0.006x - 0.302$	$R^2 = 0.990$	0.02–16
TXB2	1.93	369.3	169.1	15	15.8%	$y = 0.152x - 6.916$	$R^2 = 0.999$	10–2500
PGD2	2.82	351.4	271.3	10	11.4%	$y = 0.112x - 17.449$	$R^2 = 0.995$	10–500
PGE2	3.19	351.4	271.3	10	17.7%	$y = 0.041x - 5.208$	$R^2 = 0.996$	0.2–30
5S,6R-LXA4	3.65	351.5	114.8	15	36.0%	$y = 3.839x - 60.24$	$R^2 = 0.994$	1.1–2800
PGB2	4.86	333.3	271.2	10	32.6%	$y = 0.004x - 7.734$	$R^2 = 0.974$	1–300
PGA1	5.52	335.2	317.1	10	18.3%	$y = 0.083x - 16.65$	$R^2 = 0.996$	3–300
LTB4	6.36	335.3	195.1	10	29.7%	$y = 0.229x - 18.09$	$R^2 = 0.997$	1.2–1800
9,10-DiHOME	7.09	313.5	313.5	10	25.2%	$y = 38.14x - 582.1$	$R^2 = 0.995$	12–30000
14,15DHET	7.47	337.5	207.0	10	31.5%	$y = 0.025x - 0.194$	$R^2 = 0.999$	0.1–300
11,12DHET	8.00	337.5	167.0	15	25.4%	$y = 32.77x + 30.86$	$R^2 = 0.996$	30–3000
15-deoxy-PGJ2	8.72	315.4	271.3	10	17.5%	$y = 0.008x - 0.582$	$R^2 = 0.998$	0.12–100
19HETE	8.76	319.3	274.0	10	18.5%	$y = 195.1x - 42.94$	$R^2 = 0.990$	1.2–3000
8,9DHET	8.92	337.3	319.1	10	25.4%	$y = 0.040x - 3.647$	$R^2 = 0.993$	0.1–300
20HETE	8.94	319.3	319.3	10	15.4%	$y = 0.018x - 14.22$	$R^2 = 0.997$	1.2–1800
5,6DHET	9.34	337.3	319.0	10	11.6%	$y = 28.16x + 21.97$	$R^2 = 0.984$	1.2–3000
16HETE	9.45	319.3	319.3	10	28.1%	$y = 0.012x - 6.497$	$R^2 = 0.998$	1.2–1800
13HODE	9.77	295.5	277.1	10	20.7%	$y = 0.030x + 0.905$	$R^2 = 0.999$	1.3–300
9HODE	9.92	295.2	171.2	10	28.8%	$y = 0.015x - 4.514$	$R^2 = 0.991$	0.15–300
11HETE	10.68	319.3	167.2	10	17.7%	$y = 0.037x - 17.25$	$R^2 = 0.996$	1.2–1800
12HETE	11.05	319.3	257.3	10	25.9%	$y = 0.086x - 6.146$	$R^2 = 0.998$	1.2–1000
8HETE	11.10	319.3	155.1	10	23.2%	$y = 0.100x - 9.592$	$R^2 = 0.996$	1.2–1000
9HETE	11.41	319.3	150.7	10	26.6%	$y = 0.956x - 23.28$	$R^2 = 0.992$	2.5–1800
5HETE	11.80	319.5	319.5	10	13.0%	$y = 2.998x - 40.43$	$R^2 = 0.991$	1.2–3000
12,13EpOME	12.39	295.5	295.5	10	12.2%	$y = 0.166x - 13.61$	$R^2 = 0.991$	1.3–1000
14,15EET	12.80	319.3	319.3	10	19.7%	$y = 0.094x - 16.39$	$R^2 = 0.995$	1.2–3000
5OxoETE	13.44	317.5	108.8	10	24.2%	$y = 5.873x - 4.38$	$R^2 = 0.999$	1.2–3000
11,12EET	13.51	319.3	167.2	10	12.2%	$y = 0.395x - 9.263$	$R^2 = 0.998$	1.2–3000
8,9EET	13.91	319.3	68.7	30	24.5%	$y = 1.471x - 1.183$	$R^2 = 0.999$	1.2–3000
5,6EET	14.68	319.3	319.3	10	15.9%	$y = 0.107x - 34.50$	$R^2 = 0.996$	1.2–5000

Data were acquired with full scan mode from m/z 80–1000 with cycle time 275 ms. Mass spectrometry parameters were using ion spray voltage of 5500 V in positive ion mode and 4500 V in negative ion mode, curtain gas of 35 PSI, ion source gas 1 of 50 PSI, ion source gas 2 of 50 PSI, and an interface heater temperature of 500 °C.

2.3. Targeted Arachidonic Acid Metabolism Analysis

Four hundred microliters of serum was extracted with 1 mL of ethyl acetate containing 0.1% formic acid.¹⁶ The internal standards were PGF1 α -d4, 13-hydroxy octadecadienoic acid (13-HODE)-d4, and 15-hydroxy eicosatetraenoic acid (15-HETE)-d8. The extract was dried in a vacuum centrifuge. For analysis, samples were reconstituted in 40 μ L of methanol. The analysis was performed by an UHPLC (Agilent 1290 Infinity, USA) coupled to an Agilent 6400 Triple Quad mass spectrometry (Agilent, USA). The injection volume was 10 μ L. The pool quality control (QC) samples were analyzed after each six serum samples.

A 2.1 $\times$ 100 mm ACQUITYTM 1.7 μ m C18 BEH column (Waters, Ireland) was used for LC separation. The mobile phases were (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The gradient elution was 40% B, kept 2 min, changed linearly to 60% B at 8 min, then changed linearly to 65% B at 16 min, and changed linearly to 100% B at 18 min, kept 3 min. The flow rate was 0.30 mL/min. Column temperature was maintained at 40 °C.

Multiple reaction monitoring (MRM) in negative ion mode was used for eicosanoid detections by an Agilent 6400 Triple

Quad mass spectrometry. The mass transitions and collision energy are given in Table 2. Mass spectrometry parameters were set as follows: gas temperature at 300 °C, gas flow rate at 8 L/min, capillary voltage at 3500 V, and nozzle voltage at 400 V.

2.4. Data Collection and Data Analysis

Nontargeted metabolomics data were extracted and aligned using Markerview workstation (AB SCIEX, USA). Internal standards were selected to obtain the minimum RSD of the peaks in QC sample. After the intensity of each peak was calibrated with the suitable internal standard, nontargeted metabolomics data from positive ion mode and negative ion mode were integrated into a data set for further data analysis to achieve more metabolite information.

Targeted metabolomics data were extracted by MassHunter workstation (Agilent, USA). The concentrations of eicosanoids were calibrated with one of the internal standards, PGF1 α -d4, 13-HODE-d4, and 15-HETE-d8. Multivariate statistical analysis was performed by the SIMCA-P software (version 11.0; Umetrics, Umea, Sweden). After unit variance scaling, principal component analysis (PCA) or partial least-squares-discriminant analysis (PLS-DA) was applied to distinguish healthy controls and the children with AD. Orthogonal signal correction (OSC) PLS-DA with center scaling was used for the separations of ADNIgE or ADHIgE children with healthy controls. Permutation test was used to check the validity and the degree of overfit for the model. The metabolites with variable importance in the projection (VIP) values larger than 1 in

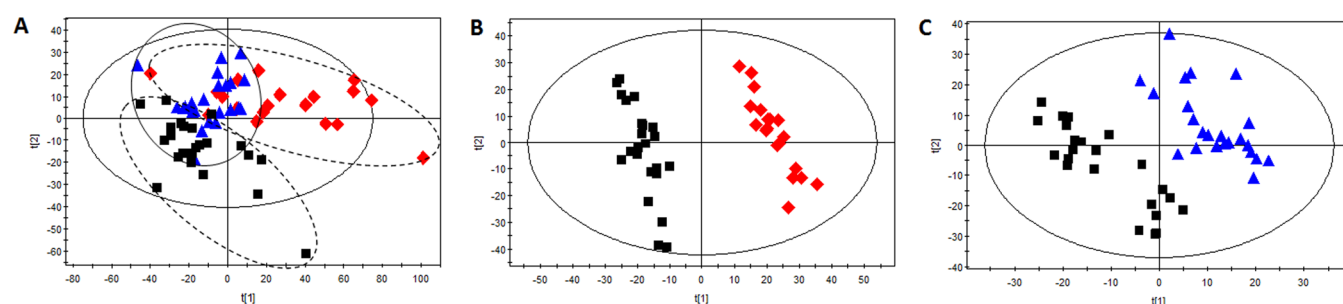


Figure 2. (A) Scores plots of PLS-DA model separating healthy control children, ADHIgE children, and ADNIgE children. $R^2Y = 0.575$, $Q^2 = 0.173$, and no overfitting was found according to the permutation validation in the PLS-DA model. (B) Scores plots of OSC PLS-DA model separating healthy control children and ADHIgE children. $R^2Y = 0.977$, $Q^2 = 0.894$, and no overfitting was found according to the permutation validation in the PLS-DA model. (C) Scores plots of OSC PLS-DA model separating healthy control children and ADNIgE children. $R^2Y = 0.889$, $Q^2 = 0.536$, and no overfitting was found according to the permutation validation in the PLS-DA model. Black square, healthy control; red diamond, ADHIgE group; blue triangle, ADNIgE group.

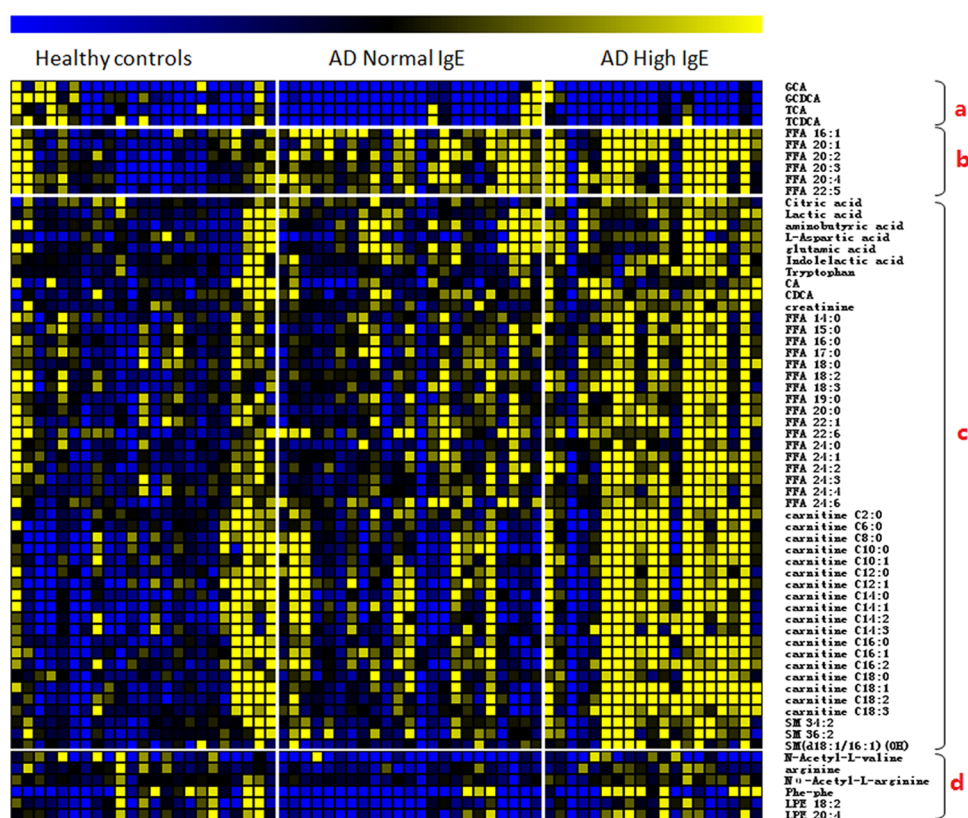


Figure 3. Heat map of differential metabolites found by metabolomics analysis, listed in Table S1, Supporting Information.

nontargeted metabolomics analysis and all metabolites in targeted metabolomics analysis were performed with the Wilcoxon Mann–Whitney test to identify significantly different metabolites, $p < 0.05$ was considered as significant, and false discovery rate (FDR) was used for multiple comparisons ($p < 0.10$). The ratios of different metabolites in the subject to the average of those in healthy control samples were calculated, and MeV version 4.5.1 software was used to illustrate the relationship between the different metabolites.¹⁷

3. RESULTS

3.1. Metabolomics Analysis of Children Sera by UPLC–MS

For nontargeted metabolomics analysis, sera were analyzed by UPLC–TripleTOF–MS with ESI positive ion mode and ESI negative ion mode. QC samples were inserted in every six

samples, which were used to evaluate the reproducibility of the analytical platform. After the intensity of each peak was calibrated with the suitable internal standards,¹⁵ 80% of ions had RSD% less than 20% among the 4248 ions acquired from QC samples in ESI positive ion mode, and 70% of ions had RSD% less than 20% among the 3287 ions acquired from QC samples in ESI negative ion mode (Supporting Information, Figure S1A,B). The result showed good reproducibility for metabolomics study. The metabolites were identified by accurate mass, fragmentation patterns, and retention time according to our published strategy,¹⁸ then available standard samples were used to confirm the identification.

Multivariate statistical analysis was performed for all metabolite ions acquired from both ESI positive ion mode and negative ion mode to investigate the changes of serum

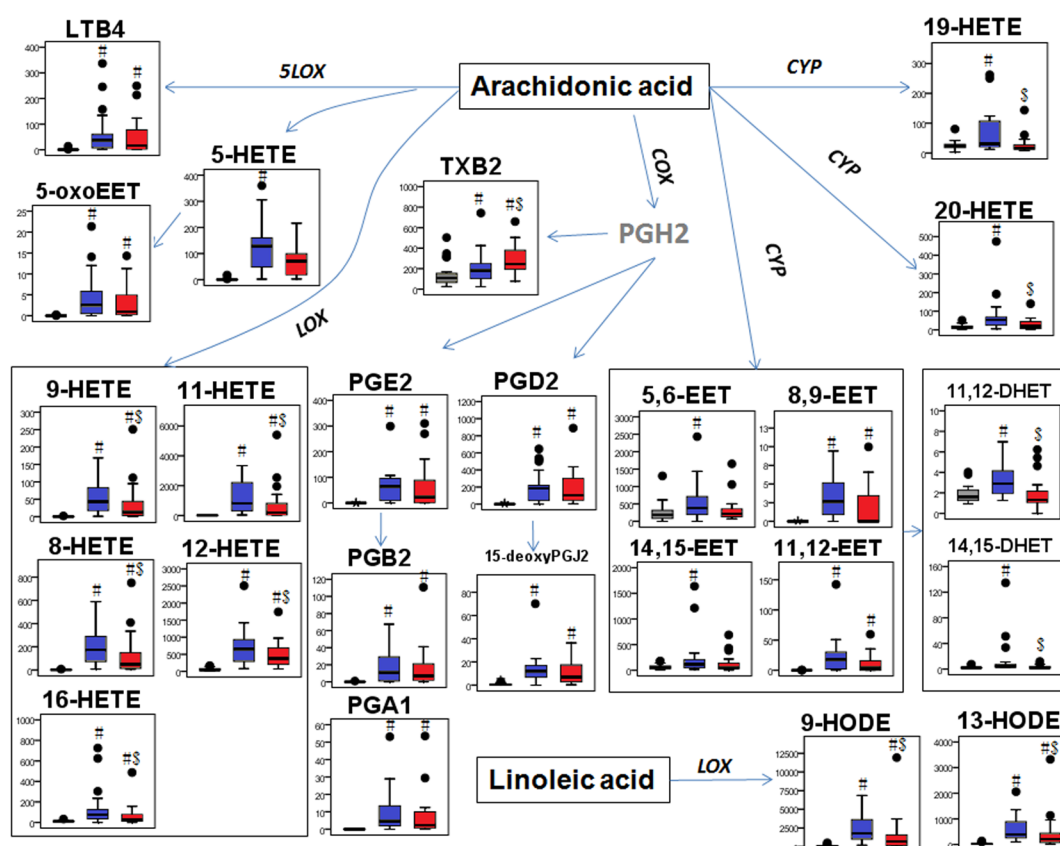


Figure 4. Comparison of the intensities of metabolites in arachidonic acid metabolism pathway. Gray column, healthy control; blue column, ADNIgE group; red column, ADHIgE group. # $p < 0.05$ compared with healthy control; \$ $p < 0.05$ ADHIgE compared with ADNIgE.

metabolome in AD children. On the basis of the PLS-DA model, ADHIgE children and healthy controls were clearly separated, while ADNIgE children were located in the middle between ADHIgE children and healthy children (Figure 2A). To investigate metabolic differences of each group, OSC PLS-DA was performed between every two groups. A clearly separation was shown in ADHIgE children and healthy children (Figure 2B) and was also shown in ADNIgE children and healthy children (Figure 2C). All PLS-DA models had no overfitting by the permutation test, which showed the models were reliable. The metabolites with VIP values larger than 1 were selected, which were the most relevant for explaining the separations.

The Wilcoxon Mann–Whitney test was used to determine the significant differences of metabolites in the three groups, and FDR was used for multiple comparisons. The differential metabolites are shown in heat map (Figure 3 and Supporting Information (Table S1)). They mainly have four kinds of tendencies. In the upper part of the heat map, several conjugated bile acids were decreased in AD groups (Figure 3, part a), while some unsaturated fatty acids showed increasing serum levels in AD groups (Figure 3, part b). Next, the serum levels of some metabolites were only increased in ADHIgE group, but there were no significant differences between ADNIgE group and control group, including carnitines, some free fatty acids, sphingomyelins (SMs), lactic acid, citric acid, etc. (Figure 3, part c). In the bottom part of the heat map, the metabolites were only decreased in ADNIgE group, including some amino acids and lysophosphatidylethanolamines (LPEs) (Figure 3, part d).

3.2. Eicosanoids Analysis of Children Serum by UHPLC–MS/MS

Eicosanoids are synthesized from arachidonic acid, which has many important functions in vivo, such as mediation of inflammation, immunity, and as messengers of nervous system.¹⁹ In the study, serum levels of eicosanoids were investigated by a targeted UHPLC–Triple Quad–MS approach. The 30 eicosanoids were detected in serum (shown in Table 2). A pool serum QC sample was used to evaluate the precision of the method. Most eicosanoids had RSD% less than 30%, while 14,15-dihydroxy-eicosatrienoic acid (DHET), prostaglandin B2 (PGB2), and 5S,6R-leukotriene A4 (5S,6R-LXA4) had RSD% more than 30% owing to the low serum concentration. The precision and linear range of the method are given in Table 2. Multivariate statistical analysis was also performed. On the basis of eicosanoids data, healthy controls were clearly separated from ADHIgE children and ADNIgE children. The results were given in Supporting Information Figure S2.

The comparisons of significantly different eicosanoids in three groups are shown in Figure 4. Leukotriene B4 (LTB4), thromboxane 2 (TXB2), prostaglandins, hydroxyl octadecadienoic acids (HODEs), and most hydroxy eicosatetraenoic acids (HETEs), which were in lipoxygenase (LOX) and cyclooxygenase (COX) pathways, were significantly increased in two AD groups. Among them, LTB4 and prostaglandins, like prostaglandin D2 (PGD2), prostaglandin B2 (PGB2), prostaglandin E2 (PGE2), 11-oxo-5Z,9,12E,14E-prostatetraenoic acid (15-deoxy-PGJ2) did not show significant difference between ADNIgE group and ADHIgE group. While the serum

level of TXB2 was higher in ADHlgE group children than in ADNlgE children, and the serum levels of HETEs and HODEs, like 5-HETE, 8-HETE, 9-HETE, 11-HETE, 16-HETE, 9-HODE, and 13-HODE, were lower in ADHlgE group than in ADNlgE group. Different from other HETEs, 19-HETE and 20-HETE, which were synthesized by Cytochrome P450 epoxygenase (CYP), had a significant increase only in the ADNlgE group, but they did not show significant differences between the control group and ADHlgE group. Analogously, epoxyeicosatrienoic acids (EETs) and DHETs, which were also in CYP pathway, mainly showed a significant increase in ADNlgE group.

4. DISCUSSION

4.1. Metabolic Characteristics of AD Children

On the basis of metabolomics analysis, glycine and taurine conjugated bile acids were decreased in both ADNlgE group and ADHlgE group (Figure 3, part a), while primary bile acids (cholic acid and chenodeoxycholic acid) were increased in ADHlgE group (Figure 3, part c). Primary bile acids are synthesized from cholesterol by cytochrome P450 in liver cell, then the bile acids are conjugated with glycine or taurine.²⁰ Conjugated bile acids have higher water solubility, which provides much more ability in fats emulsification and absorption.²¹ Here, the decreasing of conjugated bile acids would be associated with absorption of fats and sterols in AD groups.

On the contrary, some unsaturated fatty acids, including FFA 16:1, FFA 20:1, FFA 20:2, FFA 20:3, FFA 20:4, FFA 22:5, etc. (Figure 3, part b), were increased in AD groups. Unsaturated fatty acids are a class of important metabolites in the body, especially arachidonic acid, which is involved in many metabolic pathways. As a precursor of eicosanoids, arachidonic acid plays many important roles in inflammation, immunity, and nervous system.¹⁹ The increase of unsaturated fatty acids may be related to the metabolic change of eicosanoids, and a further study focused on eicosanoids was performed by a targeted UPLC–Triple Quad–MS analysis.

On the basis of eicosanoid analysis, the serum levels of LTB4 were significantly increased in AD groups. LTB4 is synthesized by 5-LOX from arachidonic acid. It is a mediator of inflammatory and immunological reactions. The importance of leukotrienes had been defined in human allergic diseases like asthma, allergic rhinitis, and also AD.^{22,23} Accumulation of LTB4 level was found in skin and blood in AD patients,^{24,25} which causes aggregation and activation of neutrophils, macrophages, eosinophils, and lymphocytes, and plays a key role in the pathogenesis of AD.^{26,27}

Similar to LTB4, prostaglandins were also mediators of inflammatory reactions. Prostaglandins are synthesized by the COX pathway. PGD2, PGB2, PGE2, etc., were found significantly increased in AD groups. Functions of prostaglandins include aggregation or disaggregation of platelets and constriction or dilation in vascular smooth muscle cells, etc.²⁸ Recent studies showed that prostaglandins are closely related with AD by a new type prostaglandin receptor CRTH2.²⁹

In addition, 8-HETE, 9-HETE, 11-HETE, 12-HETE, 13-HODE, 9-HODE, etc., which are synthesized by LOX, were also found increased in AD groups. According to the above results, the metabolites in COX pathway and LOX pathway were accumulated in AD groups. The COX pathway and LOX pathway are most important in arachidonic acid metabolism,

which is well-known to play an important role in inflammatory diseases including cardiovascular disease,³⁰ arthritis,³¹ and also AD.¹⁴ Inhibitors of LOX and COX were used in treatment of AD.¹⁴ The significant increase in AD groups reflected inflammatory response in both ADHlgE group and ADNlgE group.

Using some metabolites of COX pathway and LOX pathway, like LTB4 and PGE2, AD children can be well distinguished from healthy controls. Receiver operating characteristic (ROC) curve shows that the area under the curve (AUC) was 0.979 for LTB4 and 0.984 for PGE2 (Supporting Information Figure S3A,B). The results suggested that inflammatory reactions are one of most important metabolic characteristics of AD, and which implied the potential application of LTB4 and PGE2 in auxiliary diagnosis. Furthermore, on the basis of nontargeted metabolomics analysis data, a combinational marker containing GCDCA, FFA 16:1, and FFA 20:4, which were defined by binary logistic regression model, showed good sensitivity and specificity; the AUC was 0.883 (Supporting Information Figure S3C).

4.2. Metabolic Differences between ADHlgE Group and ADNlgE Group

Except for the above-mentioned unsaturated fatty acids, most of the free fatty acids were shown higher serum levels only in ADHlgE group (Figure 3, part c). Similar to the fatty acids, the serum levels of carnitines were also significantly increased in ADHlgE group. Fatty acids are important sources of fuel, which undergoes β -oxidation to produce ATP using carnitine as a medium in mitochondria. Seino et al. suggested that AD cause the suppression of fatty acid β -oxidation.³² Some studies showed that mitochondrial dysfunction contributed to immune system disorders and was associated with elevated serum IgE.^{33,34} Mitochondrial dysfunction and/or reduced mitochondrial content decreased fatty acid oxidation.³⁵ In this study, the higher levels of carnitines and fatty acids prompted the decrease of fatty acid β -oxidation in ADHlgE group children.

In additional, citric acid and lactic acid were increased in children of the ADHlgE group. Citric acid is an important intermediate in the citric acid cycle, which is a key metabolic pathway to generate energy through the oxidation of carbohydrates, lipids, and amino acids in the matrix of the mitochondrion. Generally, the concentration of lactic acid rises when tissue does not get enough oxygen or not fast enough to utilize the oxygen. Once mitochondrial dysfunction happens, pyruvate cannot effectively be used by glycolysis, and excess pyruvate is converted to lactic acid, which leads to an increase of serum lactic acid.³⁶ Though some evidence has proved the relationship between mitochondrial function and elevated serum IgE,^{33,34} it has no direct evidence showing that mitochondrial dysfunction existed in ADHlgE group children. In this study, the increasing of fatty acids, carnitines, lactic acid, and citric acid proposes that energy metabolism disorder exists in AD children with higher IgE, which may be associated with mitochondrial function.

Some SMs were also increased in children of ADHlgE group. SM is an important component of cell membranes, and it has many significant functions in signal transduction, cell apoptosis, and lipid rafts.³⁷ Sphingomyelinase breaks down SM into ceramide, which was found in a decreased level in the stratum corneum of AD.^{11,38} Repression of sphingomyelinase activity has been investigated in AD.^{39,40}

Some amino acids were found decreased only in ADNIgE group, including arginine, *N*-acetyl-L-valine, *N* α -acetyl-L-arginine, etc. (Figure 3, part d). *N*-Acetyl-L-valine is a derivative of valine, which is a branched-chain amino acid (BCAA) and essential amino acid. BCAA supplementation has an important role in human health, involved in energy metabolism, immune response, and protein synthesis.^{41–43} Arginine is also an essential amino acid. Infants are unable to effectively synthesize arginine. Deficiency of arginine in preterm infants causes hyperammonemia and intestinal dysfunction.⁴⁴ Furthermore, arginine is a precursor of nitric oxide (NO), which is a signaling molecule in neurotransmission, inflammation, and immune response.⁴⁵ It has been suggested that NO is playing an important role in AD, food allergy, and intestinal inflammation.^{46–49} We hypothesized that the reduction of amino acids could be related to the absorption of nutrients in children of ADNIgE group.

Besides amino acids, LPEs were significantly decreased in ADNIgE group. Lysophospholipids are breakdown products of phospholipids by phospholipase A2, which release arachidonic acid particularly from sn-2 acyl bond. The decrease of serum LPEs and increase of serum unsaturated fatty acids in children of the ADNIgE group may relate to the activity of phospholipase A2. The activity of phospholipase A2 was reported to be involved in inflammatory diseases of the skin^{50,51} and has become a new therapeutic target of AD.^{52,53}

EETs, DHETs, 19-HETE, and 20-HETE, which are formed from arachidonic acid by CYP, showed significant increases in ADNIgE group, while the levels in ADHIgE group were close to those in the control group. EETs are produced by CYP, then they are converted to DHETs. EETs were found to play many biological effects, for example, calcium releasing from intracellular stores, decreased COX activity, increased cell proliferation, etc.⁵⁴ EETs had been confirmed to produce vasorelaxation and play anti-inflammatory effects in cardiovascular and kidney.^{55,56} EETs were significantly increased in plasma of cardiovascular patients, which were suggested a compensation mechanism for protection.⁵⁷ The relationship between EETs, DHETs, and AD was not well studied. We suppose that it perhaps starts an anti-inflammatory compensation mechanism in ADNIgE group.

The combinational markers were investigated to distinguish ADNIgE group and ADHIgE group by using binary logistic regression. Carnitine 18:2 and LPE 18:2 from nontargeted metabolomics analysis data and TXB2 and 11, 12-DHET from targeted eicosanoids analysis data showed good sensitivity and specificity. The AUC were 0.906 and 0.799, respectively (Supporting Information Figure S3D,E). The combinational markers provided a possibility to identify different phenotypes of AD and provided a reference for personalized treatment.

5. CONCLUSIONS

A nontargeted metabolomics approach and a targeted eicosanoids analysis approach were used to investigate serum metabolic characteristics of AD children. Metabolic abnormalities of AD children were revealed as an acceleration of COX and LOX pathways and decreasing of conjugated bile acids. The AD children with higher IgE levels were intimately related to energy metabolism disorder and amino acids metabolic abnormalities, while increased levels of CYP metabolites were found in the AD children with normal IgE levels. The results suggest that the LC-MS approach provides a new perspective to understand the mechanism of AD and the differential

metabolites implying the potential application to distinguish disease and the different phenotypes. Further study may provide a new reference for personalized treatment.

■ ASSOCIATED CONTENT

§ Supporting Information

Table S1, Differential metabolites of AD. Figure S1, Method reproducibility in the positive and negative ion modes. Figure S2, Scores plots of PCA model separating healthy control children, ADHIgE children, and ADNIgE children; scores plots of OSC PLS-DA model separating healthy control children and ADHIgE children; scores plots of OSC PLS-DA model separating healthy control children and ADNIgE children. Figure S3, ROC curves of metabolic biomarker. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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Supplementary materials

Table S1 Differential metabolites of AD

metabolite	class	pathway
decrease in AD groups compared with control group		
GCA *	conjugated bile acids	Bile Acid metabolism
GCDCA *	conjugated bile acids	Bile Acid metabolism
TCA *	conjugated bile acids	Bile Acid metabolism
TCDCA *	conjugated bile acids	Bile Acid metabolism
increase in AD groups compared with control group		
FFA 16:1 *	fatty acids	lipid metabolism
FFA 20:1	fatty acids	lipid metabolism
FFA 20:2	fatty acids	lipid metabolism
FFA 20:3	fatty acids	lipid metabolism
FFA 20:4 *	fatty acids	lipid metabolism
FFA 22:5	fatty acids	lipid metabolism
increase in ADHlgE group compared with control group		
Citric acid *	Organic Acids	Citric Acid Cycle
Lactic acid *	Organic Acids	Pyruvate Metabolism
aminobutyric acid	Organic Acids	Alanine, aspartate and glutamate metabolism
L-aspartic acid *	amino acids	Alanine, aspartate and glutamate metabolism
glutamic acid *	amino acids	Alanine, aspartate and glutamate metabolism
Indolelactic acid *	amino acids	Tryptophan Metabolism
Tryptophan *	amino acids	Tryptophan Metabolism
CA *	unconjugated bile acids	Bile Acid metabolism
CDCA *	unconjugated bile acids	Bile Acid metabolism
Creatinine *	Carboxylic Acid Amide	Arginine and proline metabolism
FFA 14:0	fatty acids	lipid metabolism
FFA 15:0	fatty acids	lipid metabolism
FFA 16:0 *	fatty acids	lipid metabolism
FFA 17:0	fatty acids	lipid metabolism
FFA 18:0 *	fatty acids	lipid metabolism
FFA 18:2 *	fatty acids	lipid metabolism
FFA 18:3	fatty acids	lipid metabolism
FFA 19:0	fatty acids	lipid metabolism
FFA 20:0	fatty acids	lipid metabolism
FFA 22:1	fatty acids	lipid metabolism
FFA 22:6 *	fatty acids	lipid metabolism
FFA 24:0	fatty acids	lipid metabolism
FFA 24:1	fatty acids	lipid metabolism
FFA 24:2	fatty acids	lipid metabolism
FFA 24:3	fatty acids	lipid metabolism
FFA 24:4	fatty acids	lipid metabolism
FFA 24:6	fatty acids	lipid metabolism

carnitine C2:0*	carnitines	beta oxidation of fatty Acids
carnitine C6:0*	carnitines	beta oxidation of fatty Acids
carnitine C8:0*	carnitines	beta oxidation of fatty Acids
carnitine C10:0*	carnitines	beta oxidation of fatty Acids
carnitine C10:1	carnitines	beta oxidation of fatty Acids
carnitine C12:0*	carnitines	beta oxidation of fatty Acids
carnitine C12:1	carnitines	beta oxidation of fatty Acids
carnitine C14:0	carnitines	beta oxidation of fatty Acids
carnitine C14:1	carnitines	beta oxidation of fatty Acids
carnitine C14:2	carnitines	beta oxidation of fatty Acids
carnitine C14:3	carnitines	beta oxidation of fatty Acids
carnitine C16:0*	carnitines	beta oxidation of fatty Acids
carnitine C16:1	carnitines	beta oxidation of fatty Acids
carnitine C16:2	carnitines	beta oxidation of fatty Acids
carnitine C18:0*	carnitines	beta oxidation of fatty Acids
carnitine C18:1	carnitines	beta oxidation of fatty Acids
carnitine C18:2	carnitines	beta oxidation of fatty Acids
carnitine C18:3	carnitines	beta oxidation of fatty Acids
SM 34:2	sphingomyelins	lipid metabolism
SM 36:2	sphingomyelins	lipid metabolism
SM(d18:1/16:1)(OH)	sphingomyelins	lipid metabolism
decrease in ADNIgE group compared with control group		
N-Acetyl-L-valine	branched-chain amino acids	Valine, leucine and isoleucine biosynthesis
Arginine*	amino acids	Arginine and proline metabolism
N α -Acetyl-L-arginine	amino acids	Arginine and Proline Metabolism
Phe-phe*	amino acids	Phenylalanine metabolism
LPE 18:2	lysophosphatidylethanolamine	lipid metabolism
LPE 20:4	lysophosphatidylethanolamine	lipid metabolism

* Validated with standard sample.

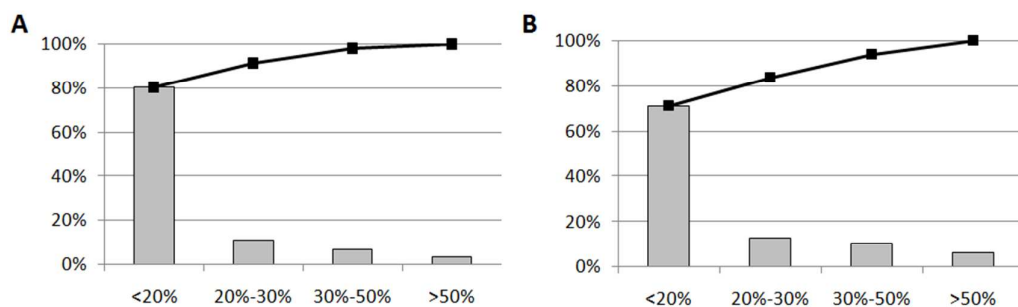


Fig. S1 (A) Percentages of ions at four different ranges of RSD among 4248 ions in the positive ion mode. (B) Percentages of ions at four different ranges of RSD among 3287 ions in negative ion mode. Columns represent the percentage of the peak number within the specified RSD% range. Lines show the cumulative percentages of the peak number within the specified RSD% range.

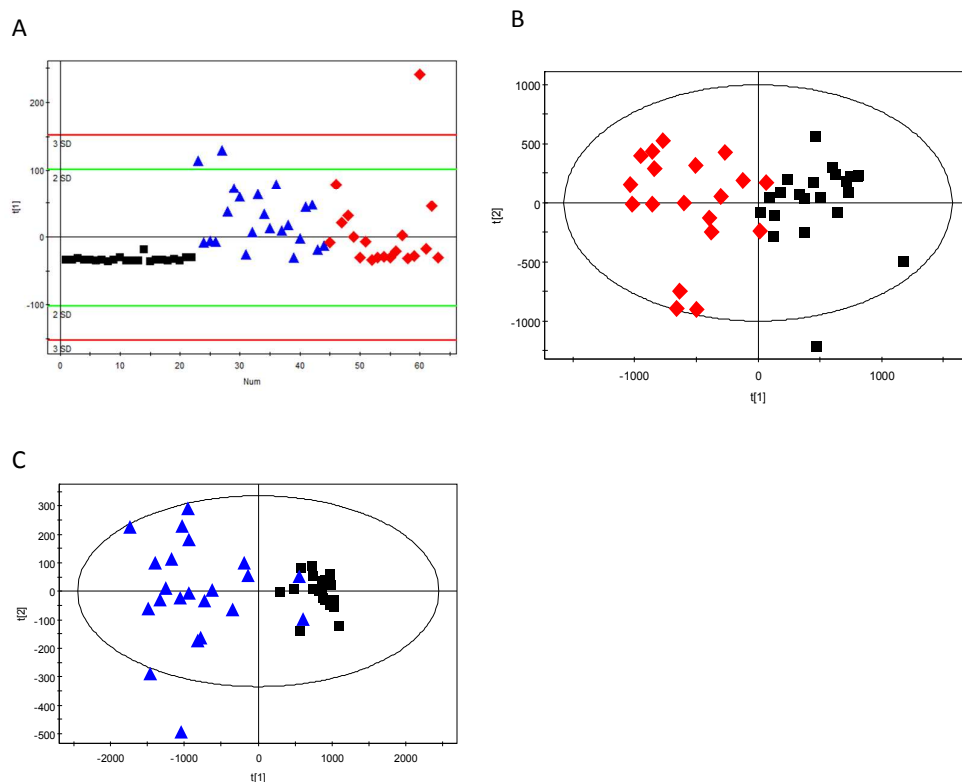


Fig. S2 (A) Scores plots of PCA model separating healthy control children, ADHIgE children and ADNIgE children. $R^2X=0.662$, $Q^2=0.572$. (B) Scores plots of OSC PLS-DA model separating healthy control children and ADHIgE children. $R^2Y=0.747$, $Q^2=0.726$, no over-flitting was found according to the permutation validation in the PLS-DA model. (C) Scores plots of OSC PLS-DA model separating healthy control children and ADNIgE children. $R^2Y=0.778$, $Q^2=0.748$, no over-flitting was found according to the permutation validation in the PLS-DA model. Black square: healthy control; red diamond: ADHIgE group; blue triangle: ADNIgE group.

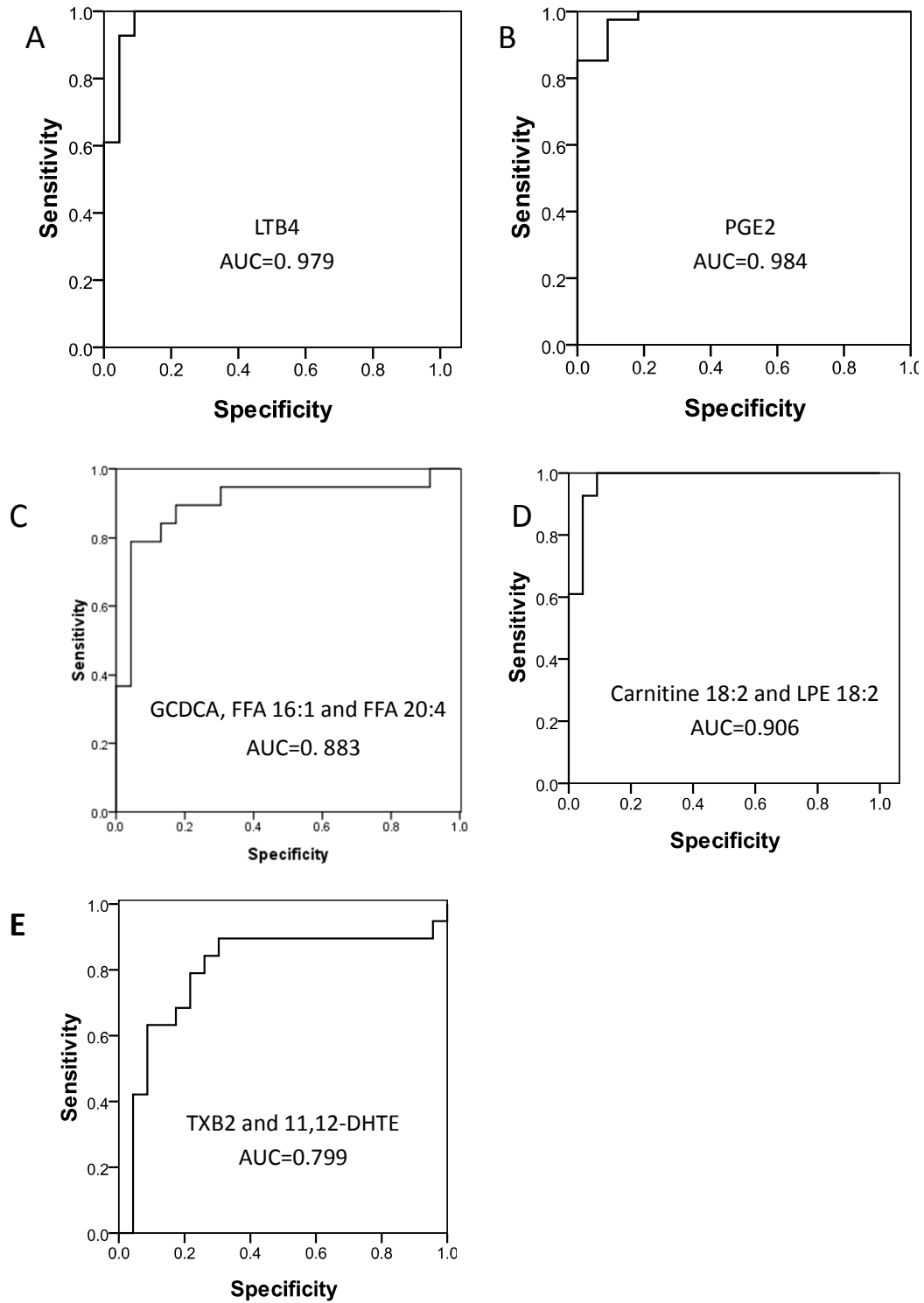


Fig. S3 ROC curves of metabolic biomarker. (A) ~ (C) distinguished ADHIgE group from healthy control; (D) (E) distinguished ADHIgE group from ADHIgE group.