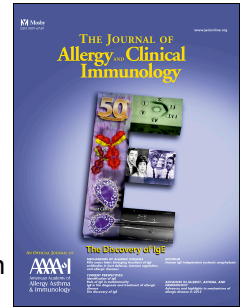


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**High resolution metabolomics to identify urine biomarkers in corticosteroid
resistant asthmatic children**

Running Head: Urine metabolomics in CS resistant asthmatic children

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Abstract

Background

Corticosteroid (CS) treatment has been established as the first anti-inflammatory treatment for adults and children with asthma. However, a subset of patients fails to respond to combined systemic and inhaled CS treatment.

Objective

This study is aimed at further understanding CS resistance among severe asthmatic children.

Methods

High resolution metabolomics (HRM) was performed on urine samples from CS-respondent (n=15) and CS-nonrespondent (n=15) children to determine possible urine biomarkers related to CS resistance. The metabolic phenotypes of CS-responders and CS-nonresponders were analyzed using bioinformatics including Manhattan plot with false discovery rate (FDR), hierarchical cluster analysis (HCA), Kyoto Encyclopedia Genes and Genomes (KEGG) and Mummichog pathway analysis.

Results

The two-way HCA study determined 30 metabolites showing significantly different levels between CS-responders and CS-nonresponders. The important metabolites annotated were 3,6-dihydronicotinic acid (126.05 m/z, RT: 106, $[M+H]^+$), 3-methoxy-4-hydroxyphenyl(ethylene)glycol (185.05 m/z, RT: 155, $[M+H]^+$), 3, 4-dihydroxy-phenylalanine (198.07 m/z, RT: 446, $[M+H]^+$), γ -glutamylcysteine (236.06 m/z, RT: 528, $[M+S(34)+H]^+$), Cys-Gly, (253.06 m/z, RT: 528, $[M-NH_3+H]^+$), reduced FMN (517.0794 m/z, RT: 533, $[M+NaCl]^+$). Tyrosine metabolism, degradation of aromatic

compounds and glutathione metabolism are suggested to be significant pathways relating to CS resistance.

Conclusion

High resolution metabolomics (HRM) is a promising approach in asthma research. Five candidate markers were identified to be related to CS resistant severe asthmatic children. These compounds, upon validation, may contribute further in the understanding of CS resistance among severe asthmatics through the use of urine.

Key Messages

- Five potential biomarkers were identified from severe asthmatic children with CS resistance.

Capsule Summary

The study uses high resolution liquid chromatography with mass spectrometry on urine samples to identify metabolic differences from severe asthmatic children with CS resistance.

Keywords: *metabolomics; LC-MS; pediatric asthma; corticosteroid resistance*

Abbreviations

CS – Corticosteroid

HRM – High Resolution Metabolomics

FDR – False Discovery Rate

FTMS – Fourier Transform Mass Spectrometry

CS-R – CS Responders

CS-NR – CS Non Responders

KEGG – Kyoto Encyclopedia of Genes and Genomes

GSH - Glutathione

Introduction

Asthma is the most common chronic airway disorder in children and is characterized by inflammation and hypersensitivity of the airways. Although asthma treatment currently involves a “one-size fits-all” approach with graded steps of bronchodilators and anti-inflammatory agents that balance effective therapy with minimal adverse side effects¹⁻³, asthma is considered a multifactorial disease^{4,5}, with diverse overlapping pathologies and phenotypes⁶⁻⁸. This contributes to significant heterogeneity in the clinical manifestations and treatment responses associated with the disorder.

Corticosteroids (CS) are the cornerstone of asthma treatment.⁹⁻¹¹ Inhaled CS are the first-line therapy for asthma while systemic CS are used for immediate treatment due to their rapid onset of effect and relatively easy administration. Despite their efficacy, CS-associated side effects are common. Moreover, a subset of children with severe asthma fails to respond to combined systemic and inhaled CS treatment despite very high doses and prolonged treatment^{12,13}. Although the prevalence of CS-resistance among children is very low, these children account for a disproportionate healthcare spending¹⁴.

There are a growing number of studies focused on molecular mechanisms of CS resistance in asthma¹⁵⁻¹⁸. Because the long-term prognosis for children with severe asthma may be directly related to CS responsiveness, early and reliable identification of CS responsiveness is important¹³. Therefore, there is a need to further understand the molecular features of CR among these children. A number of clinical tools, including the measurement of exhaled gases such as nitric oxide^{19,20}, as well as inflammatory markers, have been used to characterize airway inflammation, but the clinical utility of these techniques remains unproven^{21,22}.

116 Additionally, bronchoscopy is invasive and often impractical in very young children²³.
117 Therefore, more sensitive and practical diagnostic methods are needed. The identification
118 of non-invasive biomarkers in the urine of asthmatic patients would be extremely
119 beneficial not only for early identification of CS resistance, but also for future
120 development of novel treatments.

121 High Resolution Metabolomics (HRM) offers an important advantage in
122 analyzing highly complex metabolite mixtures such as biological extracts because the
123 detection of mass-to-charge ratios (m/z) with 5 ppm or better mass resolution and mass
124 accuracy substantially decreases the demand for physical separation prior to detection²⁴,
125 ²⁵. Minimal extraction avoids the perturbation of the relative abundance of the
126 metabolites in the sample. HRM is often used on urine samples because of the high
127 sensitivity and selectivity of mass spectrometry techniques; these techniques have
128 become the methods of choice in characterizing the human metabolome^{26, 27}.

129 This study aimed to use HRM to describe metabolomics patterns exhibited by CS
130 resistance among severe asthmatic children. The study examined differences in metabolic
131 profiles of urine and identified biomarkers which differed between CS-resistant and CS-
132 responsive severely asthmatic children through the use of high-throughput mass
133 spectrometry.

134 **Materials and Methods**

135 Chemicals

136 LC-MS grade water (Tedia, NJ, USA), acetonitrile (Burdick & Jackson, MI, USA) and
137 formic acid (Fluka, MO, USA) were used for the mobile phase in this study.

Characteristics of the study population.

The study was approved by the Emory University Institutional Review Board. Informed consent was obtained from all participating children and their legal guardians. Children 6 to 17 years with physician-diagnosed asthma treated with high-dose ICS and a second controller medication were recruited from an outpatient severe asthma clinic in Atlanta, Georgia. All children met published criteria for severe asthma including adherence to ICS evidenced by 10 or more monthly electronic prescription refills over the previous 12 months²⁸. Each participant had a history of either $\geq 12\%$ reversibility in the forced expiratory volume in one second (**FEV₁**) after bronchodilator administration or airway hyperresponsiveness to methacholine, evidenced by a provocative concentration of methacholine ≤ 16 mg/mL. Exclusion criteria included premature birth before 35 weeks gestation, aspiration disorders, vocal cord dysfunction, avascular necrosis, diabetes mellitus, historical or current bronchopulmonary aspergillosis, treatment with non-steroidal anti-inflammatory drugs or omalizumab, chronic bone disorders, or bone fractures within the previous 6 months. All participants were stable at the time of baseline characterization with no signs of acute respiratory illnesses. If a recent exacerbation was reported, the first visit was conducted 4 weeks after completion of an oral or injectable systemic corticosteroid burst. Permission to proceed with this study was granted by the Emory University Institutional Review Board. Informed written consent was obtained from the legally authorized representatives of eligible children and assent was also obtained from participants 6 years of age and older. After consent was obtained, participants completed two research characterization visits separated by 14 days. Intramuscular triamcinolone (1 mg/kg, 60 mg maximum dose) was administered in the

gluteal muscle at the completion of the first visit to all participating children. Spirometry (KoKo® PDS, Ferraris, Louisville, CO) was performed at baseline and the best of three vital capacity maneuvers was interpreted. Participants were asked to withhold bronchodilator medication prior to the study visits (i.e., ≥ 4 hours for SABAs and ≥ 12 hours for long-acting beta agonists (**LABAs**)).

Urine samples were collected from asthmatic children (n=30) enrolled in a previous study focused on the metabolic phenotypes accompanying severe asthma²⁹.

Children with severe asthma (n=30), by definition, were treated with higher doses of ICS (median, 1000 vs 250 μg fluticasone equivalent/day, $P < .001$), yet they required greater health care utilization for asthma as well as having increased airflow limitation, more impaired asthma control and a compromised quality of life. In addition, no child with severe asthma displayed complete corticosteroid responsiveness per our composite definition (Table 1). Corticosteroid resistance was defined by persistently poor asthma control with symptoms greater than twice weekly, in addition to $< 15\%$ improvement in the forced expiratory volume in one second, two weeks after systemic triamcinolone administration.

In this regard, 15 children had only partial responsiveness while 15 children had improved clinical responses to systemic triamcinolone administration (Figure 1).

Liquid chromatography coupled with Fourier-Transform Mass Spectrometry (FTMS)

All the samples were run in triplicate. One hundred μl aliquots of supernatant samples were treated with acetonitrile (2:1, v/v), spiked with a 2.5 μl internal standard mix, and centrifuged at 14,000 $\times g$ for 5 minutes at 4°C to remove protein, as described

previously³⁰. Analyses were done using MS mode scanning from an m/z range of 85-850 in the FT detector at a resolution of 50,000 with the wide range scan mode and 3 million ions per scan using a Thermo LTQ-FT mass spectrometer (Thermo Fisher Scientific, San Jose, CA). HRM was suitable for this purpose and detected 8570 total metabolites in urine. The triplicated runs were averaged, log 2 transformed and quantile normalized for the subsequent multivariate analysis.

Data collection, processing, and analysis

Data was preprocessed using the R package apLCMS, from which the total features of the samples were determined³¹. HRM was suitable for this purpose and detected 8570 total metabolites in urine. The triplicated runs were averaged, log 2 transformed and quantile normalized for the subsequent multivariate analysis. Baseline differences between children with CS-responders (CS-R) and CS-non-responders (CS-NR) in urine samples were assessed at Visit 2 and were evaluated using the false discovery rate (FDR). Furthermore, the metabolic profiles were discriminated using Limma-hierarchical cluster analysis to separate two groups in association with metabolites. Limma is originally a package of linear models for microarray to analyze the gene expression data arising from microarray or RNA-Seq technologies from Bioconductor³². Pathway analysis for the m/z of metabolites utilized the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Mummichog³³⁻³⁵, which showed that less than 10% of metabolites were redundant with others in terms of elemental composition. In addition, identified metabolites were annotated using the METLIN Mass Spectrometry Database³⁶. For such metabolites, quantification relative to stable isotope internal standards had coefficients of variation (5-

10%) and sensitivity similar to other methods, thus being sufficient to allow for targeted analyses of selected chemicals within the context of an information-rich non-targeted profiling of all m/z detected within biological samples.

Results

Manhattan plot with FDR

A Manhattan plot (Figure 2A) combines multiple testing correction which enables visual identification of statistically significant data points (metabolites) that display large-magnitude changes. Multiple testing corrections like FDR adjust p-values (q-values) derived from multiple statistical tests to correct for the occurrence of false positives. The Y axis represents the $-\log_{10}$ adjusted value of the raw p-value between CS-R and CS-NR. The X axis indicates m/z ranging 85-850 m/z. The dotted line shown is the FDR significant level; therefore, any m/z above this line were significantly different between the two groups at FDR $q=0.05$. The total number of significant features was 30 out of the 8570 detected. The FDR $q=0.05$, $1.7 [-\log (FDR q=0.05)]$ appears as a green dotted line (Figure 2A).

Two-way HCA on 30 significant features

A two-way HCA was performed: the top panel displayed sample classification, and the left panel visualized metabolites clustering to identify which metabolites are the most important for sample grouping. In this study, HCA was performed using 30 significant metabolites at FDR $q=0.05$, which were the key components to separate two groups

(Figure 2A). HCA resulted in good separation between CS-R (red bar) and CS-NR (green bar) except for two CS-R subjects who were grouped with CS-NR.

Annotation with METLIN data base and KEGG Pathway analysis using 30 significant metabolites

Significantly altered m/z features between CS-R and CS-NR were annotated using the METLIN database and mapped the features distribution on the KEGG human metabolic pathways. The mapped significant features between CS-R and CS-NR are shown as black dots on the pathway map in Figure 3. The important pathways influenced by significantly altered m/z are listed as tyrosine metabolism, degradation of aromatic compounds and glutathione metabolism in Table 2.

The relative concentrations of urine features altered significantly

Figure 4 shows the two metabolites involved in glutathione metabolism. The concentration of γ -glutamylcysteine in CS-R was 110029 ± 39764 and that in CS-NR was 27583 ± 15864 . Furthermore, cysteine-glycine was 11829 ± 5166 in CS-R and 78771 ± 27023 in CS-NR. The relative concentrations of 3,6-dihydronicotinic acid, 3,4-dihydro-L-phenylalanine and 1,2-dihydronaphthalene-1,2-diol increased in CS-NR compared to CS-R, as shown in Figure 5.

Mummichog pathway analysis

Mummichog analysis identified that tyrosine and xenobiotics metabolism were affected at p-value= 0.01 and 0.03 respectively by CS resistance. The important module (p=0.0002) which appeared in tyrosine metabolism is shown in Figure 6. The

concentration of 3,4-dihydroxy-L-phenylalanine and 3-methoxy-4-hydroxyphenyl(ethylene)glycol increased significantly, (FDR $q=0.05$), in CS-NR compared to CS-R. However, S-methyl homocysteine and S-methyl methionine decreased in CS-NR, but not to a statistically significant level.

An analysis between mild-to-moderate and severe asthmatics was also conducted to see the specificity of the selected biomarkers (Figure E1-E4). The findings showed that although there was a high separation between the mild and severe cases, the 5 selected compounds were not found significant. This shows the specificity of the selected compounds towards CS resistance among the severe asthmatics.

Discussion

The identification of predictive biomarkers in urine as a surrogate tissue for monitoring changes of metabolic profile occurring in lung airway tissue requires highly sensitive instruments to ensure measurement of the fmol-pmol quantities of metabolites in blood. This surrogate urine sample is expected to contain a much lower concentration of metabolites compared to the local tissue in asthmatics. Thus, HRM with mass spectrometry proves to be the best method for metabolic profiling in this study since HRM's high sensitivity and selectivity enables it to identify putative biomarkers in urine. In this study, HRM selected 30 significant features between CS-R and CS-NR in severe asthma sufferers. Among those, the two metabolites from glutathione (GSH) metabolism, γ -glutamylcysteine and cysteine-glycine, were changed significantly between two groups. Figure 4 describes γ -glutamylcysteine as low in CS-NR, suggesting a decrease in GSH

synthesis. Furthermore, cysteine-glycine increased in CS-NR, meaning a higher degradation in CS-NR³⁷. GSH is the one of the most important antioxidants for preventing oxidative stress³⁸⁻⁴⁰. This result demonstrated that the GSH pathway would be the target for CS resistance. Interestingly, dihydronicotinic acid was higher in CS-NR compared to CS-R, which led to smoking as an important factor for CS resistance. Other evidence linked to smoking was that the level of 1,2-dihydronaphthalene-1,2-diol increased in CS-NR compared to CS-R. This aromatic chemical might suggest an association with smoking since naphthalene is present in cigarette smoke⁴¹. Tyrosine metabolism may be affected by CS resistance since 3,4-dihydro-L-phenylalanine increased in CS-NR (Figure 6). This chemical is involved in catecholamine biosynthesis such as noradrenaline and adrenaline, causing bronchoconstriction. The five detected putative biomarkers provide solid evidence of the association with CS resistance.

The need for personalized medicine has been addressed in many areas such as airway inflammation, cancer, diabetes, and heart disease. The development and refinement of personalized approaches for asthma are particularly relevant due to multiple phenotypes. Identification and treatment of children with severe asthma in the clinical setting remains difficult, and the results from this study will be important given the significant cost and morbidity associated with the disorder and may aid in the development of personalized treatment regimes in the future. However, replication of the results in other populations is ultimately needed.

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All the authors made equal contributions in data gathering and analysis. YHP and CDM prepared this manuscript. All authors read and approved the final manuscript.

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Conflict of Interest Statement:

The authors declare that they have no competing interests.

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- 425

Tables

Table 1. Demographics of study subjects separated into corticosteroid responders (CS-R) and non-responders (CS-NR) two weeks after receiving 1 mg/kg (60 mg maximum) of intramuscular triamcinolone acetonide. Corticosteroid insensitivity was defined as <15% improvement in the forced expiratory volume in one second, two weeks after triamcinolone administration.

	CS-R (n=15)	CS-NR (n=15)	p-value
Age	12.8±2.7	13.9±3.3	0.1047
Number (male/female)	15(11/4)	15(10/5)	
Body mass index (kg/m ²)	26.9±6.9	26.0±6.7	0.5625
White/Nonwhite	4/11	2/13	
Asthma duration (y)	11.0(7-14)	12.9(7-20)	0.0723
Asthma medical history in previous year			
Number of subjects who visited emergency room	10	13	
Number of subjects hospitalized	3	6	
Number of subjects intubated	6	11	
Dose of inhaled corticosteroids	750	820	0.4539
Asthma control questionnaire score	0.50	1.69	0.0015
Asthma quality of life questionnaire score	5.96	5.14	0.0838
Exhaled nitric oxide	14.9±3.8	29.7±7.6	0.0203
Blood eosinophils	2.0±0.5	2.2±0.6	0.9041
FVC (% predicted)	109±28	97±25	0.0385
FEV1 (% predicted)	99±26	82±21	0.0241
FEV1/FVC	91±24	84±22	0.2509

439 Table 2. KEGG Pathway Analysis

Pathways	Number of hitting metabolites
Metabolic Pathways	21
Tyrosine metabolism Diiodo-L-tyrosine Dihydroxy-L-phenylalanine 3-Methoxy-4-hydroxyphenylethyleneglycol	3
Degradation of aromatic compounds Dihydronaphthalene-1,2-diol 2-Hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa- 2,4-dienoate	2
Glutathione metabolism Cys-Gly gamma-Glutamylcystine	2

440

441

442

Figure Legends

Figure 1. Schematic design for biomarker discovery and algorithm development

Figure 2. (A) Manhattan plot between CS-R vs CS-NR using FDR $q=0.05$; 30 significant features using 8570 features used and (B) Hierarchical cluster analysis between CS-R vs. CS-NR using the significant features

Figure 3. KEGG pathway with 30 FDR $q=0.05$ significant features, a total 21 hits on KEGG human metabolic pathways

Figure 4. Two important urine biomarkers on the GSH pathway

Figure 5. The putative biomarkers to discriminate CS-R vs. CS-NR at Visit 2

Figure 6. Mummichog pathway analysis with significant metabolites to determine an important module between CS-R and CS-NR

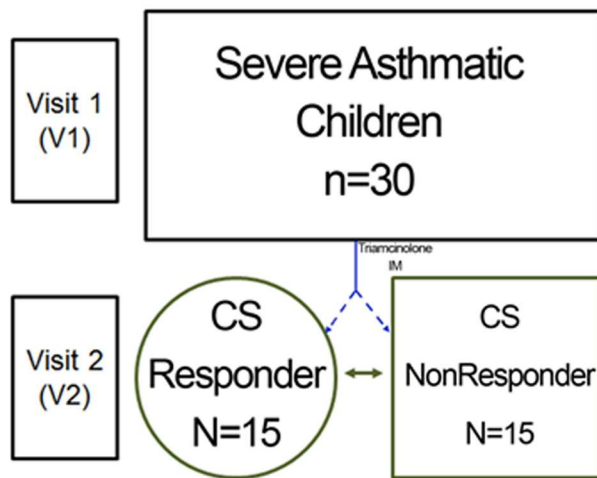
Figure E1. Orthogonal signal correction/Partial least square-discriminant analysis on Mild-to-Moderate and Severe Asthmatics Children.
Blue:Mild-to-Moderate asthmatics, Red: Severe asthmatics

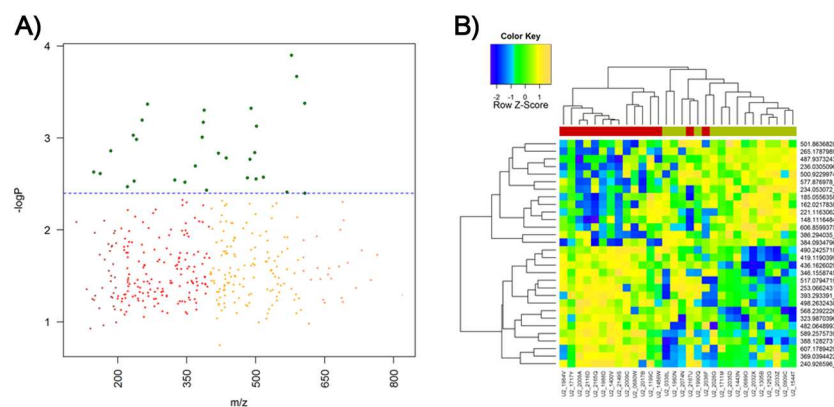
Figure E2. (Left) Manhattan plot between Mild-to-Moderate vs Severe Asthmatic children using FDR $q=0.05$; 119 significant features using 6963 features used and (Right) Hierarchical cluster analysis between Mild-to-Moderate vs Severe Asthmatic children using the significant features

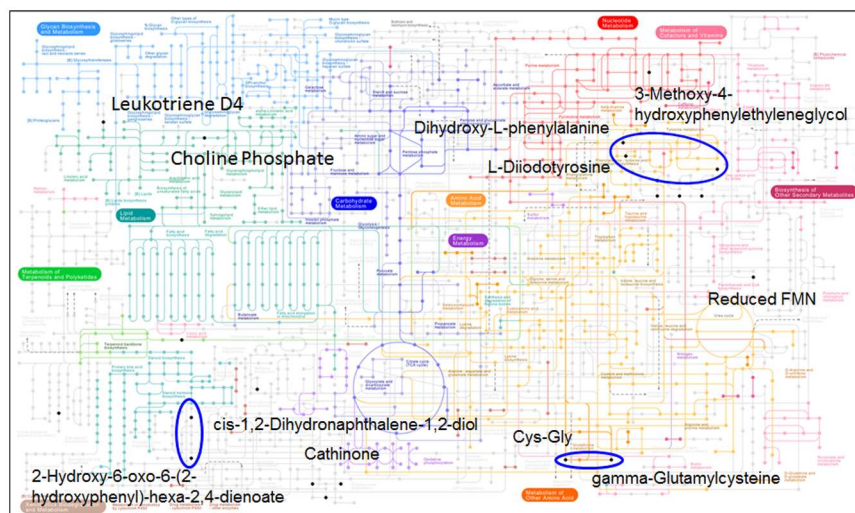
Figure E3. KEGG pathway with FDR $q=0.05$ significant features, a total 38 hits on KEGG human metabolic pathways between mild-to-moderate and severe asthmatic children

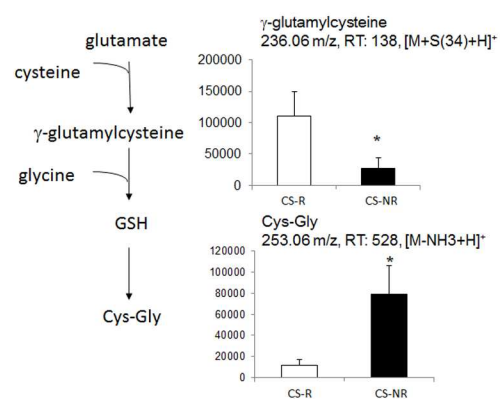
Figure E4. Bar graph of the compounds between mild to moderate and severe patients.

Severe Group

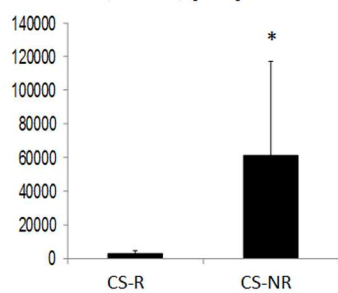




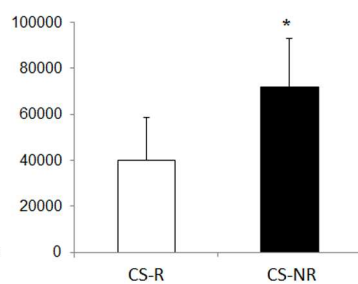




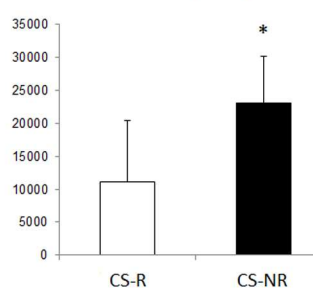
3,6-Dihydronicotinic acid,
126.05 m/z , RT: 106, $[M+H]^+$



3,4-dihydro-L-phenylalanine
236.0305 m/z , RT: 138, $[M+K]^+$



1,2-Dihydronaphthalene-1,2-diol
185.05 m/z , RT: 155, $[M+Na]^+$



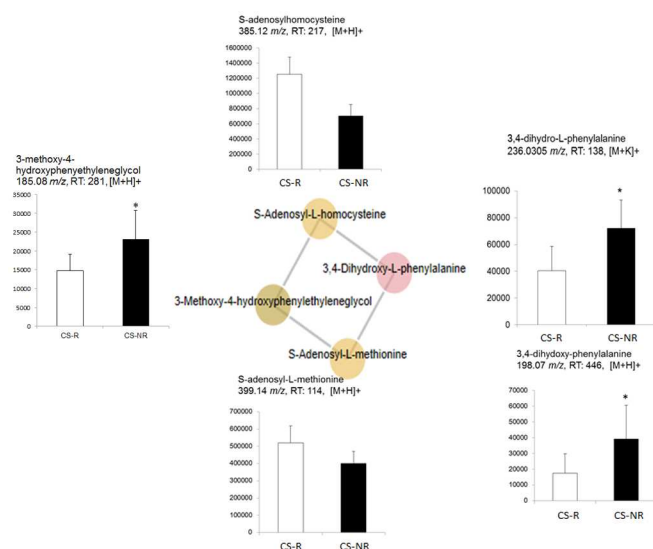


Figure E1. Orthogonal signal correction/Partial least square-discriminant analysis on Mild-to-Moderate and Severe Asthmatics Children.

Blue:Mild-to-Moderate asthmatics, Red: Severe asthmatics

Figure E2. (Left) Manhattan plot between Mild-to-Moderate vs Severe Asthmatic children using FDR $q=0.05$; 119 significant features using 6963 features used and (Right) Hierarchical cluster analysis between Mild-to-Moderate vs Severe Asthmatic children using the significant features

Figure E3. KEGG pathway with FDR $q=0.05$ significant features, a total 38 hits on KEGG human metabolic pathways between mild-to-moderate and severe asthmatic children

Figure E4. Bar graph of the compounds between mild to moderate and severe patients.

