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This study was supported by the European Commission's Seventh Framework program (grant no. 260895 [PreDicta] to H.L., T.V., and T.J.); Academy of Finland, Helsinki (grant no. 1257964 to K.H. and grant numbers 114034 and 132595 to T.J.); the Finnish Medical Foundation (to T.J.); the Sigrid Juselius Foundation, Helsinki (to M.S.-V., K.H., and T.J.); the Foundation for Pediatric Research, Helsinki (to T.J.); the TYKS Foundation, Turku (to H.L.); research funds from specified government transfers, Turku (grant no. 13032 to T.J. and grant no. 13933 to H.L.); the Allergy Research Foundation, Helsinki (to T.J. and H.L.); and the Helsinki University Research Fund (to M.S.-V.), all in Finland.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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<http://dx.doi.org/10.1016/j.jaci.2013.10.014>

Children with severe asthma have unique oxidative stress-associated metabolomic profiles

To the Editor:

Children with severe asthma are a challenging group of patients who are extremely difficult to treat. Although inhaled corticosteroids (ICS) are the cornerstone of asthma treatment, children with severe asthma have decreased responsiveness to high-dose corticosteroid therapy¹ associated with airway

TABLE I. Features of the subjects

	Mild-to-moderate asthma (N = 22)	Severe refractory asthma (N = 35)
Age (y)	12 (9-14)	13 (10-17)
Male	12 (55)	23 (66)
Nonwhite	15 (68)	33 (94)*
Asthma duration (y)	10 (4-12)	11 (9-15)†
BMI percentile		
<85th	8 (36)	15 (43)
85th-95th	7 (32)	9 (26)
>95th	7 (32)	11 (31)
Atopic history		
Allergic rhinitis	20 (91)	35 (100)
Atopic dermatitis	11 (50)	21 (60)
Asthma medical history		
Emergency room visit (previous year)	6 (27)	26 (74)†
Hospitalization (previous year)	3 (14)	12 (34)
Intubation (ever)	5 (23)	21 (60)†
Self-reported asthma symptoms at least weekly	7 (32)	32 (91)†
Asthma Control Questionnaire score	0.29 (0.04-0.64)	1.29 (0.86-2.54)†
Asthma Quality of Life Questionnaire score	6.02 (5.69-6.45)	5.06 (4.00-6.00)†
Serum IgE (kU/L)	78 (9-136)	193 (78-335)*
Exhaled nitric oxide (ppb)	24 (9-64)	31 (21-58)
Blood eosinophils (%)	3.8 (2.6-6.1)	5.2 (3.5-8.2)
Baseline spirometry		
FVC (% predicted)	112 (105-119)	100 (92-116)*
FEV ₁ (% predicted)	106 (91-108)	91 (78-100)†
FEV ₁ /FVC	0.83 (0.77-0.84)	0.74 (0.70-0.82)*
FEF ₂₅₋₇₅ (% predicted)	86 (77-95)	66 (53-77)*
FEV ₁ bronchodilator reversibility (%)	9 (5-12)	15 (8-25)*

Data represent the median (IQR) or the frequency (%).

BMI, Body mass index; FVC, forced vital capacity; IQR, interquartile range.

**P* < .05 versus nonsevere asthma.

†*P* < .01 versus nonsevere asthma.

inflammation and remodeling.² Given the limited efficacy of corticosteroids in severe asthma, our efforts have focused on the role of oxidative stress, namely, thiol redox disturbances, in the modulation of the disorder.³ Indeed, we have previously demonstrated greater oxidation and decreased concentrations of the thiols cysteine and glutathione in the airways⁴ and systemic circulation⁵ of children with severe asthma that were further associated with increased airway inflammation and impaired cellular function.⁶

Recognizing that the treatment of severe asthma might ultimately require novel approaches to restore corticosteroid sensitivity,¹ we performed metabolomic analyses of plasma samples from a highly characterized sample of children with severe asthma treated with high-dose ICS and long-acting beta agonists and children with mild-to-moderate asthma treated with ICS or ICS/long-acting beta agonist combination therapy. We report that children with severe asthma are differentiated by 2 metabolic pathways associated with oxidative stress: (1) the glycine, serine, and threonine metabolism pathway and (2) the N-acyl ethanolamine and N-acyltransferase pathway. These findings support the biological plausibility of oxidative stress as a contributory factor to corticosteroid insensitivity in severe asthma and potentially highlight novel therapeutic targets for further confirmation and study.

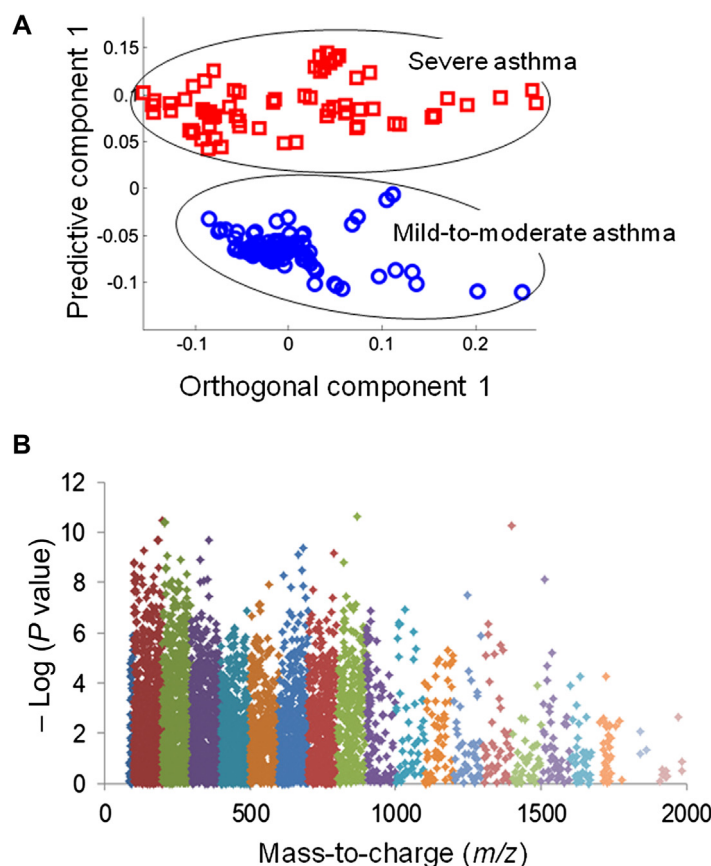


FIG 1. **A**, Analysis of identified m/z features by using partial least squares discriminant analysis with orthogonal signal correction demonstrates complete separation of the groups. Replicate values (averaged from triplicate analysis) are shown for each subject as a *circle* (mild-to-moderate asthma) or *square* (severe asthma). **B**, Manhattan plot demonstrating significantly different m/z features (individual points) between children with mild-to-moderate asthma and severe asthma.

Enrolled children had confirmed asthma with a history of 12% or more reversibility in FEV₁ after bronchodilator (albuterol) administration or airway hyperresponsiveness to methacholine evidenced by a methacholine PC₂₀ value of 16 mg/mL or less. All children underwent comprehensive phenotypic characterization consisting of spirometry with bronchodilator reversibility, exhaled nitric oxide testing, and venipuncture for total serum IgE and whole blood eosinophils as described in the Online Repository at www.jacionline.org. Children who met criteria for severe asthma also underwent formal corticosteroid responsiveness testing with systemic triamcinolone acetonide (1 mg/kg intramuscular injection). Children with severe asthma were considered to have corticosteroid “responsiveness” if symptoms, lung function, bronchodilator reversibility, and exhaled nitric oxide values normalized 2 weeks after the intervention.⁷ Cut points for “normal” were defined as symptoms less than twice weekly, FEV₁ more than 80% predicted with FEV₁/forced vital capacity more than 0.85, bronchodilator reversibility less than 12% after albuterol administration, and exhaled nitric oxide values of 25 ppb or less. Plasma samples were obtained for metabolomic studies before triamcinolone administration to avoid carryover effects from the corticosteroid intervention.

Metabolic profiling was accomplished with HPLC whereby plasma was separated on a C₁₈ reverse-phase column and

analyzed by using Fourier transform ion cyclotron resonance mass spectrometry (see this article’s Online Repository at www.jacionline.org). Mass-to-charge (m/z) features were analyzed for each plasma sample on 2 separate occasions, in triplicate. Data from the 2 replicate analyses were averaged to obtain single m/z values for each subject. Metabolomic data were processed by using the R package apLCMS. False-discovery rate (FDR) analyses and orthogonal partial least squares discriminant analyses were applied to the m/z features to determine which discriminated significantly between the 2 groups. Pathway analysis was performed by using an in-house program that considers all common derivatives when comparing m/z values with the theoretical molecular weights of metabolites. Comparison was guided by a calibration curve on the Fourier transform ion cyclotron resonance mass spectrometer, which agrees with the inverse correlation between the mass spectrometer resolution and m/z . The Kyoto Encyclopedia of Genes and Genomes human metabolomic database was used for analyses of pathways and compounds, and pathways were examined in more detail with GeneGo software (Thomson Reuters, New York, NY) by using Enrichment analysis in Pathway Maps.

Features of enrolled children are given in Table I. By definition, children with severe asthma were treated with higher doses of ICS (median, 1000 vs 250 μ g fluticasone equivalent/day, $P < .001$), yet they had greater health care utilization for asthma as well as

increased airflow limitation and more impaired asthma control and quality of life (Table 1). In addition, no child with severe asthma displayed complete corticosteroid responsiveness per our composite definition (see Table E1 in the Online Repository at www.jacionline.org). Rather, 31 children (89%) had only partial responsiveness while 4 children (11%) had no discernible clinical response to systemic triamcinolone administration (see Table E2 in the Online Repository at www.jacionline.org).

The extracted metabolomic data consisted of 8953 high-resolution m/z features. Of these, 5629 had less than 5% missing values. Partial least squares discriminant analysis with orthogonal signal correction with autoscaling showed complete separation of the groups using predictive component 1 (y-axis) (Fig 1, A). The first orthogonal component accounted for 18% of the variation; after removal of the first orthogonal component, the first predictive component accounted for 93% of the remaining variation. To address the discriminatory features in greater detail, an FDR of 0.01 was used to identify features that differed significantly between groups. The Manhattan plot for significant m/z features identified by C_{18} column separation (1843 features) is shown in Fig 1, B. With an FDR of 0.01, the C_{18} features matched 164 metabolites in the Kyoto Encyclopedia of Genes and Genomes human metabolomics database and were distributed across the metabolic pathways (see Fig E1 in the Online Repository at www.jacionline.org). The metabolites identified were significantly associated with 2 metabolic pathways: (1) the glycine, serine, and threonine metabolism pathway (m/z : 9/123; $P = 3.485 \times 10^{-5}$) and (2) the N-acyl ethanolamine and N-acyltransferase pathway (m/z : 4/32; $P = 2.980 \times 10^{-4}$). Significant metabolites mapped into these pathways are shown in Tables E3 and E4 in the Online Repository at www.jacionline.org.

These preliminary observations from an unsupervised metabolomic analysis confirm that severe, corticosteroid refractory asthma in children is associated with a vast array of metabolic derangements that are most notably associated with oxidative stress. Glycine, serine, and threonine are closely related amino acids that share common biochemical pathways (see Fig E2 in the Online Repository at www.jacionline.org). Serine participates in the biosynthesis of purines and pyrimidines and is a precursor for the synthesis of phosphoglycerides, sphingolipids, and glycolipids. In addition, serine is a precursor to several amino acids including glycine and cysteine, which are essential components of the vital antioxidant glutathione.³ The fact that this pathway most strongly differentiated children with severe versus mild-to-moderate asthma is in keeping with our previous observations of altered cysteine and glutathione redox balance in this population.^{4,6} Similarly, N-acyl ethanolamine phospholipids, which are formed by membrane-associated N-acyltransferases, are metabolized to N-acyl ethanolamines in response to cellular injury⁸ and play important roles in signal transduction⁹ and cytoprotection against oxidative stress.¹⁰ However, the high number of carbon double bonds that we observed in the identified metabolites suggests that significant lipid peroxidation may be occurring in severe asthma. In keeping with this hypothesis, we have previously observed higher concentrations of lipid peroxidation biomarkers in children with severe asthma that were further associated with thiol redox disturbances.^{2,4}

Collectively, these observations from an unsupervised analytical approach support the biological plausibility of

oxidative stress as a contributory factor to corticosteroid refractory severe asthma in children. To our knowledge, this is the first study to undertake metabolomic analyses in this population. Important strengths of the study are the careful phenotyping of enrolled children and confirmation of corticosteroid insensitivity in children with severe asthma, suggesting that the observed differences cannot be solely attributed to corticosteroid adherence. However, the precise mechanisms underlying the metabolic differences in children with severe asthma are not yet known. It is also unclear whether similar differences would have been detected in airway versus plasma samples. Furthermore, the effect of the environment (ie, the "exposome") on our findings is not understood. Questions about confounding by indication, namely, asthma medication exposure and comorbid conditions such as obesity, sinus disease, and obstructive sleep apnea, which may accompany severe asthma, also remain and require further study. While independent replication and longitudinal validation of our results is essential and further studies with diseased and healthy controls are needed, these preliminary findings are intriguing and suggest that oxidative stress-related derangements in these metabolic pathways may be potential therapeutic targets for future study. However, we recognize that our statistical approach used very stringent thresholds for false discovery and we therefore submit that there may be other metabolic pathways with lower abundance metabolites that also warrant further study. Integration of various "omic" strategies through a systems biology approach is also necessary to ultimately unravel mechanisms of asthma and asthma severity.

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This work was funded by a grant from the NIH/National Institute of Nursing Research (RO1 NR012021), the National Center for Advancing Translational Sciences (UL1 TR000454), and the Children's Healthcare of Atlanta Center for Developmental Lung Biology.

Disclosure of potential conflict of interest: A. M. Fitzpatrick has consultant arrangements with MedImmune, the Merck Scientific Advisory Board, and the GlaxoSmithKline Scientific Advisory Board. The rest of the authors declare that they have no relevant conflicts of interest.

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<http://dx.doi.org/10.1016/j.jaci.2013.10.012>

Urinary metabolomic changes as a predictive biomarker of asthma exacerbation

To the Editor:

Exacerbations requiring hospitalization represent a high risk in patients with severe asthma. Predictive biomarkers of

exacerbations are needed but are not available. Oxidative stress resulting from pulmonary reactive oxygen species formation is involved in asthma, leading to physiologic damage.¹ Asthma exacerbation is associated with increased oxidative stress,² with increased levels of carbon monoxide, nitric oxide, nitrotyrosine, and H₂O₂ found in breath condensate. Aldehydes and alkanes, the end products of lipid peroxidation, are also known to be involved in asthma-related oxidative stress.³

Urine is a stable, noninvasively collectable body fluid with a complex metabolic composition, the profiling of which might have a role in asthma diagnosis and monitoring. The 2 techniques used in this study, comprehensive bidimensional gas chromatography coupled to mass spectrometry (GC × GC-ToFMS) and proton nuclear magnetic resonance (¹H-NMR) spectroscopy, allow the analysis of volatile and nonvolatile compounds, thus providing complementary information on the urinary metabolome.

The aim of this pilot study was to assess the urinary metabolic changes linked to asthma exacerbation. Aldehydes and alkanes were studied by using GC × GC-ToFMS analysis, and NMR was used to provide an overview of changes in major metabolites involved in central metabolic pathways.

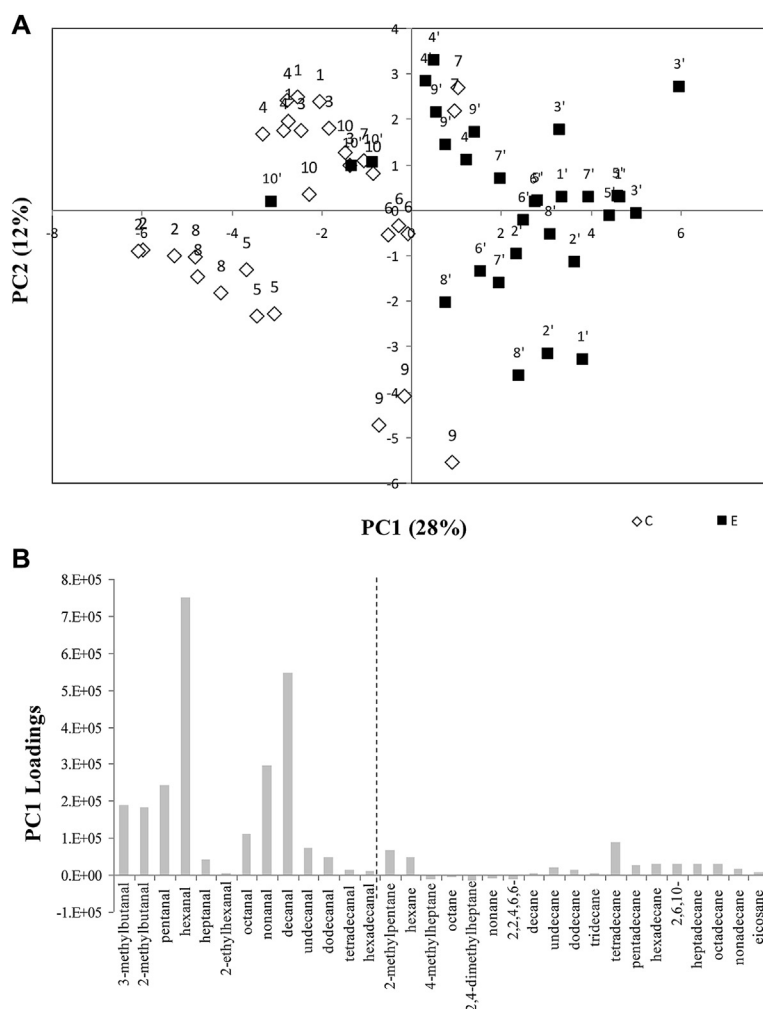


FIG 1. PCA applied to GC × GC peak areas of aliphatic aldehydes and alkanes detected in the urine of subjects in the stable state and under exacerbation conditions. **A**, PC1 versus PC2 score scatter plot in which *c* corresponds to the control condition (*open symbols*) and *e* corresponds to exacerbation (*solid symbols*). The numbers included close to the symbols correspond to the sample ID. **B**, PC1 loadings plot explaining the separation observed in the score map (positive loadings = compounds increased in exacerbated condition).

METHODS

Rationale for metabolomic analyses

Metabolomics is the study of metabolism at the global level whereby metabolites are assessed, identified, and quantified in biological samples through pattern recognition methods.^{E1-E5} As such, metabolomics is critical for the field of biomarker discovery and has considerable diagnostic potential for asthma. While previous studies (as summarized in the recent review by Adamko et al^{E6}) have focused on metabolomic biomarkers of asthma *susceptibility* (ie, how the metabolome of patients with asthma differs from that of healthy controls), no study to date has focused on metabolomic biomarkers of asthma *severity* in an exclusively asthmatic population. Given the high degree of morbidity associated with severe asthma,^{E7} we sought to compare metabolomic features in a highly characterized sample of children with severe and mild-to-moderate asthma with the intention of identifying differentiating pathways for further validation and study.

Subject recruitment and classification

Children aged between 6 and 17 years with physician-diagnosed persistent asthma treated with ICS or ICS/long-acting beta agonist combination therapy were recruited for this study from an outpatient asthma clinic at Emory University in Atlanta, Georgia. All enrolled children had a history of 12% or more reversibility in FEV₁ after bronchodilator (albuterol) administration or airway hyperresponsiveness to methacholine evidenced by a methacholine PC₂₀ value of 16 mg/mL or less. Children with comorbid disorders including chronic lung disease of prematurity, severe gastroesophageal reflux disease, aspiration disorders, or vocal cord dysfunction were excluded. Demographics of the enrolled subjects were reflective of the geographical area, which is ethnically diverse. 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 subjects aged 6 years and older. Although children aged 6 to 11 years provided verbal assent, written assent was obtained from children aged 12 to 17 years.

Subject characterization procedures

Subjects completed medical history questionnaires, including the Asthma Control Questionnaire^{E8} and the Asthma Quality of Life Questionnaire,^{E9} with technical assistance. Pulmonary function was measured at baseline and after receipt of 4 inhalations of albuterol sulfate (90 µg/actuation) using a calibrated spirometer (KoKo PDS, Ferraris, Louisville, Colo).^{E10} The best of 3 forced vital capacity maneuvers was interpreted according to population reference standards.^{E11} Participants were asked to withhold bronchodilator medication before the study visit (ie, 4 or more hours for short-acting bronchodilators and 12 or more hours for long-acting bronchodilators). Exhaled nitric oxide concentrations were determined by using online methods (NIOX MINO, Aerocrine, Morrisville, NC).^{E12} Venipuncture was also performed. Total serum IgE and whole blood eosinophil levels were determined by a local laboratory (Children's Healthcare of Atlanta, Atlanta, Ga). Plasma was obtained after centrifugation (10,000 rpm for 10 minutes), and 500 µL was reserved for metabolomic studies. Samples were snap frozen at -80°C and batched for analysis. Because children with severe asthma also underwent corticosteroid responsiveness testing, plasma samples obtained from the first (baseline) visit were used for metabolomic studies to minimize carryover effects from the triamcinolone intervention.

Severity determination and corticosteroid responsiveness testing

Participants were classified as having severe asthma if they met published criteria from the American Thoracic Society.^{E13} Thresholds for high-dose ICS were adjusted for the dosing ranges recommended for children by the National Heart, Lung and Blood Institute's Expert Panel Report (ie, ≥440 µg fluticasone equivalent/day for children <12 years and ≥880 µg fluticasone equivalent/day

for children ≥12 years).^{E14} Children not meeting criteria for severe asthma were classified as having mild-to-moderate asthma. Children who met criteria for severe asthma further underwent corticosteroid responsiveness testing with intramuscular triamcinolone acetone (1 mg/kg, 60 mg maximum dose) in which the clinical characterization procedures describe above were repeated 2 weeks following the initial dose. Children were considered to have complete corticosteroid responsiveness if symptoms, lung function, bronchodilator reversibility, and exhaled nitric oxide values were normal at the 2-week assessment point, consistent with the previous study of Bossley et al.⁷ Cut points for "normal" were as follows: (1) symptoms: less than twice weekly,^{E14} (2) lung function: FEV₁ more than 80% predicted with FEV₁/FVC more than 0.85,^{E14} (3) bronchodilator reversibility: less than 12% after albuterol administration,^{E10} and (4) exhaled nitric oxide values of 25 ppb or less.^{E12}

Metabolic profiling

Metabolic profiling was performed similar to the methods of Roede et al^{E15} with Fourier transform ion cyclotron resonance mass spectrometry. Using HPLC, plasma samples were separated on a C₁₈ reverse-phase column and the eluate was connected to a Thermo LTQ-FT mass spectrometer (Thermo Fisher Scientific, San Jose, Calif). Analyses were performed at a resolution of 50,000 with the wide range scan mode and 3 million ions per scan. The maximum ion injection time was 500 ms, and each plasma sample was analyzed on 2 separate occasions in triplicate with a 10 µL injection volume. Triplicate data were averaged, and the 2 replicate values were also averaged to obtain single data points for each subject.

Recognizing that metabolomic analyses may be subject to sample-to-sample and day-to-day variability, the consistency of our analytic approach was examined in a separate batch of 117 human plasma samples that were randomized for analysis and analyzed sequentially in triplicate. The results for total ion intensity showed that for individual samples, the average coefficient of variation for triplicates was 15.1%, with a median value of 9.1%. In addition, signal intensities for amino acids in the 117 samples were compared with measurements obtained by an independent laboratory and the results showed strong correlations for the amino acids tested, including methionine ($R = 0.97$, $P = 1.34 \times 10^{-74}$) and phenylalanine ($R = 0.97$, $P = 8.28 \times 10^{-73}$) (data not shown).

Metabolomic data were processed by using the R package *apLCMS* as previously described.^{E16} FDR analyses and orthogonal partial least squares discriminant analyses were applied to the 8953 identified *m/z* features from the C₁₈ column. FDR was performed by using MATLAB software (MathWorks, Natick, Mass). FDR adjustment is less conservative than the Bonferroni multiple comparison procedure and was used to determine which *m/z* discriminated significantly between the 2 groups.^{E17,E18} For orthogonal partial least squares discriminant analyses, variation not correlated to classification was removed with orthogonal signal correction using MATLAB using the mean center of each frequency as previously described.^{E19} Pathway analysis was performed by using an in-house program that considers all common derivatives as provided by Brown et al^{E20} when comparing *m/z* values with the theoretical molecular weights of metabolites. Comparison was guided by a calibration curve on the Fourier transform ion cyclotron resonance mass spectrometer, which agrees with the inverse correlation between the mass spectrometer resolution and *m/z*.^{E21} The Kyoto Encyclopedia of Genes and Genomes (KEGG) human metabolomic database was used for analyses of pathways and compounds. The KEGG human metabolic pathways contain 1486 metabolites that are accessible from the KEGG Web site (<http://www.kegg.jp/kegg/download>, accessed January 7, 2013). Visualization of the mapped compounds on the KEGG metabolic map was performed by using the "Color Pathway" tool (http://www.genome.jp/kegg/tool/color_pathway.html). Pathways were examined in more detail with the GeneGo software (Thomson Reuters, New York, NY) by using Enrichment analysis in Pathway Maps. This involves a functional analysis that scores and ranks the most relevant pathway maps by calculating the maximum $-\log(P \text{ value})$ for every *m/z* among all the experiments. The whole ontology is then sorted in decreasing order of those maximal *P* values.

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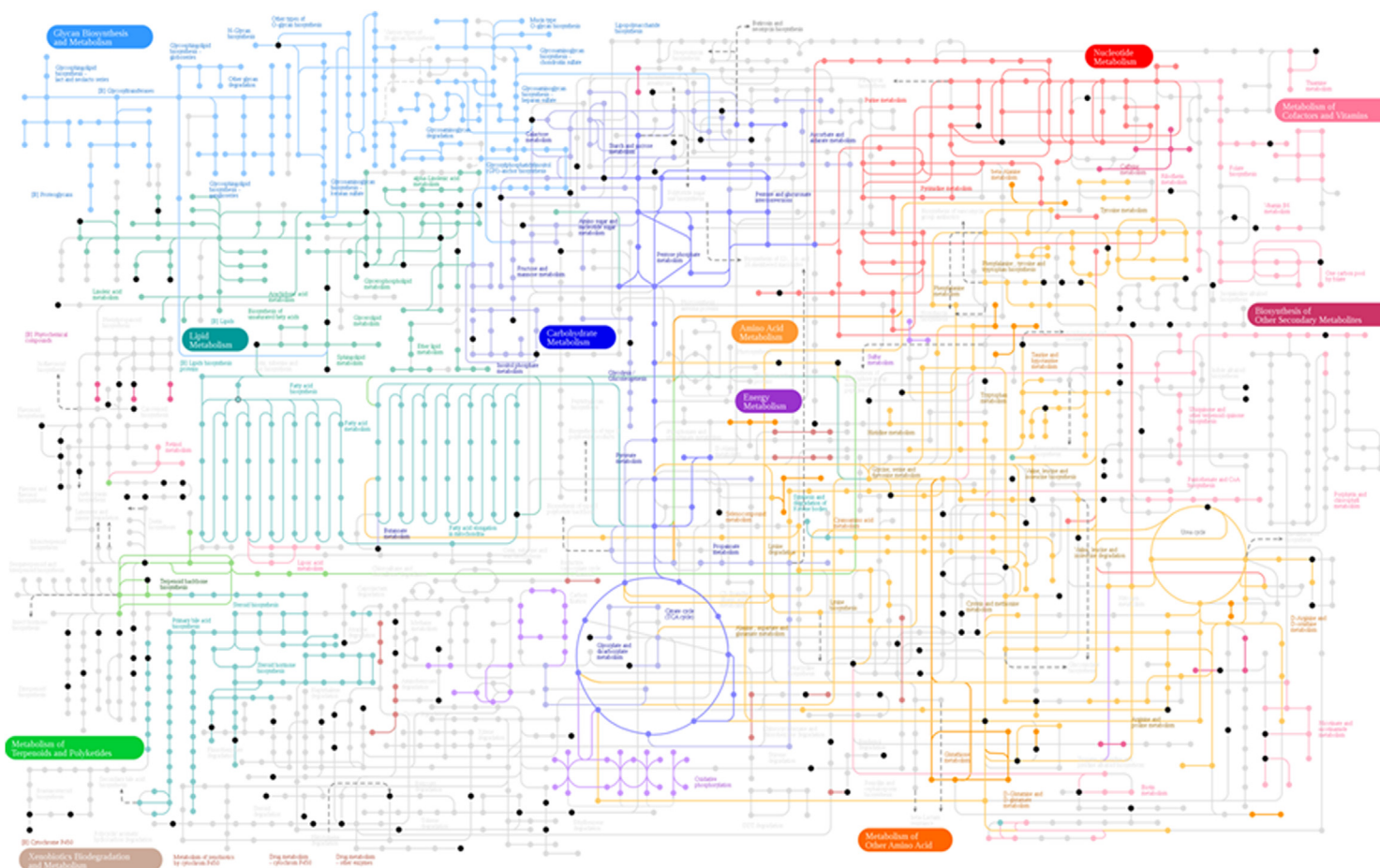


FIG E1. Distribution of 164 identified human compounds (FDR $q = 0.01$) across the KEGG metabolic pathway (accessible at <http://www.genome.jp.jp/kegg/>). Each *black dot* corresponds to an identified compound.

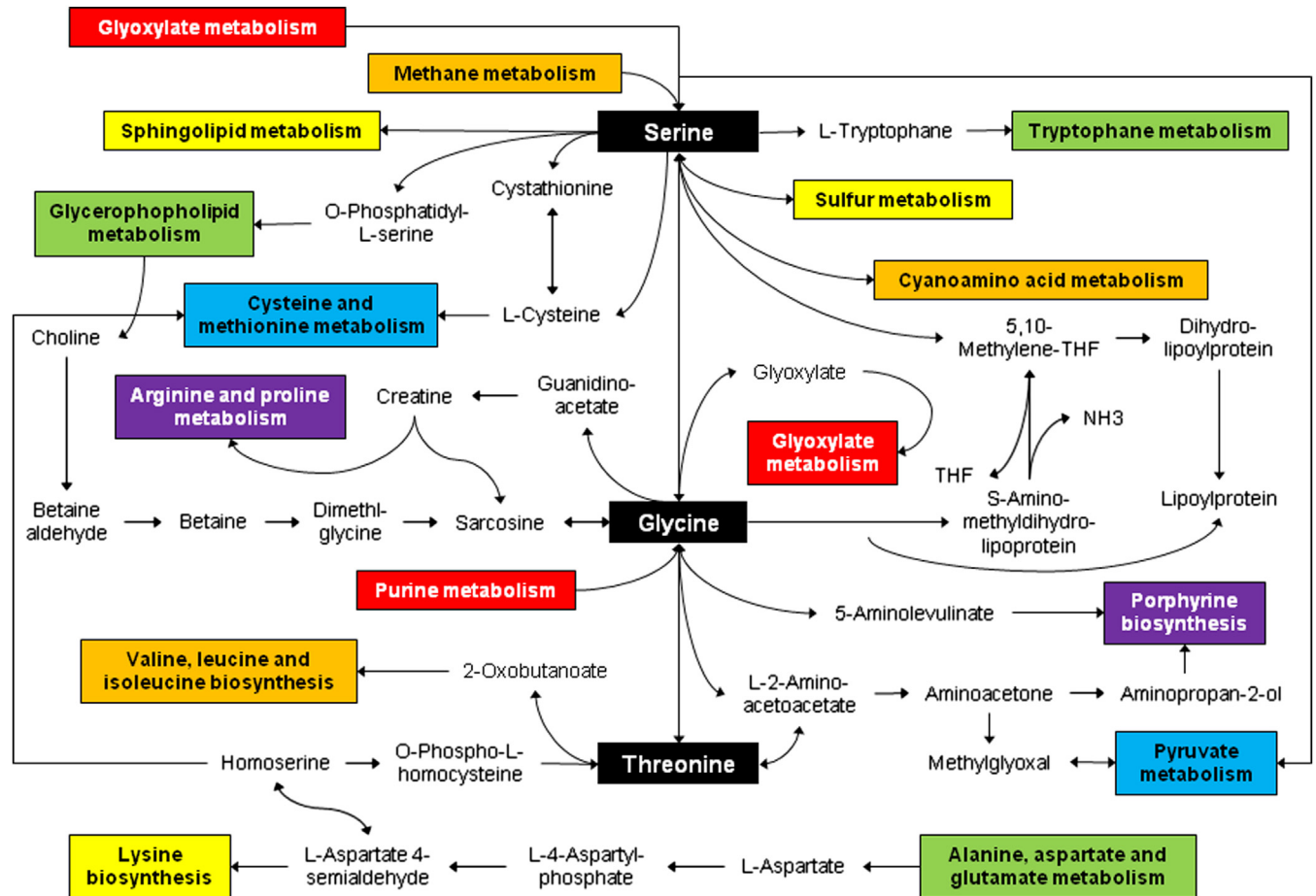


FIG E2. Serine, glycine, and threonine metabolism. THF, Tetrahydrofolate.

TABLE E1. Features of children with severe refractory asthma at baseline and 2 weeks after receipt of 1 mg/mL (60 mg maximum dose) of intramuscular triamcinolone acetonide

	Baseline (N = 35)	After triamcinolone (N = 35)
Self-reported asthma symptoms at least weekly	32 (91)	23 (66)*
Asthma Control Questionnaire score	1.29 (0.86-2.54)	1.09 (0.53-1.29)†
Asthma Quality of Life Questionnaire score	5.06 (4.00-6.00)	5.92 (4.61-6.63)*
Exhaled nitric oxide (ppb)	31 (21-58)	17 (13-28)*
Blood eosinophils (%)	5.1 (3.5-7.6)	2.1 (1.1-2.7)*
Baseline spirometry		
FVC (% predicted)	100 (92-116)	102 (90-116)
FEV ₁ (% predicted)	91 (78-100)	91 (80-98)
FEV ₁ /FVC	0.74 (0.70-0.82)	0.75 (0.71-0.81)
FEF ₂₅₋₇₅ (% predicted)	66 (53-77)	69 (51-81)
FEV ₁ bronchodilator reversibility (%)	12 (9-25)	15 (9-21)

Data represent the median (IQR). Plasma samples were collected for metabolomic studies at the baseline visit to minimize carryover effects from the triamcinolone administration.

FVC, Forced vital capacity; IQR, interquartile range.

* $P < .01$ versus baseline data.

† $P < .05$ versus baseline data.

TABLE E2. Features of children with severe refractory asthma at baseline and 2 weeks after receipt of 1 mg/mL (60 mg maximum dose) of intramuscular triamcinolone acetonide, according to triamcinolone responsiveness

	Baseline, all subjects (N = 35)	After triamcinolone				
		Symptom responders* (N = 15)	Lung function responders* (N = 5)	Bronchodilator reversibility responders* (N = 11)	Exhaled nitric oxide responders* (N = 22)	No response (N = 4)
Self-reported asthma symptoms at least twice weekly	32 (91)	0	4 (80)	6 (55)	10 (46)	4 (100)
Asthma Control Questionnaire score	1.29 (0.86-2.54)	0.57 (0.14-0.71)	0.79 (0.61-1.50)	0.71 (0.54-0.97)	0.64 (0.33-0.86)	1.86 (1.18-2.64)
Asthma Quality of Life Questionnaire score	5.06 (4.00-6.00)	6.34 (5.44-7.00)	5.61 (5.25-6.02)	5.92 (4.98-6.44)	6.25 (4.74-6.94)	5.03 (3.44-5.64)
Exhaled nitric oxide (ppb)	31 (21-58)	19 (13-25)	21 (13-36)	25 (16-35)	15 (11-20)	42 (29-49)
Blood eosinophils (%)	5.1 (3.5-7.6)	2.2 (1.1-4.0)	2.2 (0.8-2.5)	1.6 (1.0-2.2)	1.4 (1.0-2.7)	2.5 (2.2-3.3)
Baseline spirometry						
FVC (% predicted)	100 (92-116)	109 (84-121)	85 (81-91)	100 (90-114)	110 (97-117)	90 (78-117)
FEV ₁ (% predicted)	91 (78-100)	90 (81-100)	89 (82-95)	92 (88-99)	93 (87-99)	72 (65-107)
FEV ₁ /FVC	0.74 (0.70-0.82)	0.75 (0.65-0.79)	0.89 (0.87-0.93)	0.78 (0.74-0.89)	0.75 (0.70-0.79)	0.75 (0.67-0.80)
FEF ₂₅₋₇₅ (% predicted)	66 (53-77)	69 (44-77)	92 (77-109)	72 (64-102)	70 (53-84)	51 (33-81)
FEV ₁ bronchodilator reversibility (%)	12 (9-25)	15 (8-29)	7 (4-13)	7 (4-10)	15 (11-20)	27 (16-42)

Data represent the median (IQR). Plasma samples were collected for metabolomic studies at the baseline visit.

FVC, Forced vital capacity; IQR, interquartile range.

*Groups are not mutually exclusive, and so statistical comparisons were not performed.

TABLE E3. Significant metabolites (FDR $q = 0.01$) mapped into the glycine, serine, cysteine, and threonine metabolism pathway

Input mass	Adduct	KEGG ID	Mass	dppm*	Name	Formula
114.0657	[M+H-H ₂ O]+	C00300	131.0695	8	Creatine	C ₄ H ₉ N ₃ O ₂
116.9753	[M+H-2H ₂ O]+	C06368	151.9875	5	3-(Hydroxyhydroxyphosphoryl) pyruvate	C ₃ H ₅ O ₅ P
118.0857	[M+H]+	C00719	117.0790	4	Betaine	C ₅ H ₁₁ NO ₂
120.0649	[M+H]+	C00188	119.0582	4	L-threonine	C ₄ H ₉ NO ₃
186.0180	[M+H]+	C01005	185.0089	9	Phospho-L-serine	C ₃ H ₈ NO ₆ P
223.0745	[M+H]+	C00542	222.0674	0	Cystathionine	C ₇ H ₁₄ N ₂ O ₄ S
634.4498	[M+H]+	C00350	633.4370	8	Phosphatidylethanolamine (14:0/14:1)	C ₃₃ H ₆₄ NO ₈ P
646.4276	[M+H-2H ₂ O]+	C00350	681.4370	5	Phosphatidylethanolamine (18:4/14:1)	C ₃₇ H ₆₄ NO ₈ P
666.5237	[M+H-2H ₂ O]+	C00350	701.5359	0	Phosphatidylethanolamine (16:0/P-18:1)	C ₃₉ H ₇₆ NO ₈ P
684.4508	[M+Na]+	C00350	661.4683	9	Phosphatidylethanolamine (14:0/16:1)	C ₃₅ H ₆₈ NO ₈ P
698.4721	[M+H]+	C00350	697.4683	4	Phosphatidylethanolamine (15:0/18:4)	C ₃₈ H ₆₈ NO ₈ P
710.4696	[M+H]+	C00350	709.4683	8	Phosphatidylethanolamine (14:0/20:5)	C ₃₉ H ₆₈ NO ₈ P
710.4696	[M+Na]+	C00350	687.4839	4	Phosphatidylethanolamine (14:0/18:2)	C ₃₇ H ₇₀ NO ₈ P
718.4968	[M+H-H ₂ O]+	C00350	735.5050	7	Phosphatidylserine (16:0/16:0)	C ₃₈ H ₇₄ NO ₁₀ P
726.4826	[M+H-2H ₂ O]+	C00350	761.4996	5	Phosphatidylethanolamine (16:1/22:6)	C ₄₃ H ₇₂ NO ₈ P
736.4856	[M+H]+	C00350	735.4839	7	Phosphatidylethanolamine (14:0/22:6)	C ₄₁ H ₇₀ NO ₈ P
736.4856	[M+Na]+	C00350	713.4996	4	Phosphatidylethanolamine (14:0/20:3)	C ₃₉ H ₇₂ NO ₈ P
742.4831	[M+H-H ₂ O]+	C00350	759.4839	2	Phosphatidylethanolamine (18:3/20:5)	C ₄₃ H ₇₀ NO ₈ P
750.5120	[M+H]+	C00350	749.4996	6	Phosphatidylethanolamine (15:0/22:6)	C ₄₂ H ₇₂ NO ₈ P
768.5525	[M+H]+	C00350	767.5465	1	Phosphatidylethanolamine (16:1/20:3)	C ₄₃ H ₇₈ NO ₈ P
768.5525	[M+Na]+	C00350	745.5622	1	Phosphatidylethanolamine (14:0/22:1)	C ₄₁ H ₈₀ NO ₈ P
772.6155	[M+H-H ₂ O]+	C00350	789.6248	8	Phosphatidylethanolamine (15:0/24:0)	C ₄₄ H ₈₈ NO ₈ P
776.5132	[M+Na]+	C00350	753.5309	8	Phosphatidylethanolamine (22:4/15:0)	C ₄₂ H ₇₆ NO ₈ P
782.5674	[M+Na]+	C00350	759.5778	0	Phosphatidylethanolamine (15:0/22:1)	C ₄₂ H ₈₂ NO ₈ P
814.6144	[M+H-2H ₂ O]+	C00350	849.6248	2	Phosphatidylethanolamine (20:4/24:1)	C ₄₉ H ₈₈ NO ₈ P
836.5239	[M+Na]+	C00350	813.5309	4	Phosphatidylethanolamine (20:3/22:6)	C ₄₇ H ₇₆ NO ₈ P
842.6935	[M+H-H ₂ O]+	C00350	859.7030	8	Phosphatidylethanolamine (20:0/24:0)	C ₄₉ H ₉₈ NO ₈ P
844.6501	[M+H-2H ₂ O]+	C00350	879.6717	9	Phosphatidylethanolamine (22:4/24:0)	C ₅₁ H ₉₄ NO ₈ P
850.639	[M+H]+	C00350	849.6248	8	Phosphatidylethanolamine (20:4/24:1)	C ₄₉ H ₈₈ NO ₈ P
852.6389	[M+Na]+	C00350	829.6561	7	Phosphatidylethanolamine (18:1/24:0)	C ₄₇ H ₉₂ NO ₈ P
882.7014	[M+H]+	C00350	881.6874	7	Phosphatidylethanolamine (22:2/24:1)	C ₅₁ H ₉₆ NO ₈ P
902.6562	[M+Na]+	C00350	879.6717	5	Phosphatidylethanolamine (22:4/24:0)	C ₅₁ H ₉₄ NO ₈ P

*dppm refers to the difference in parts per million (ppm) between the observed metabolite and the mapped chemical ppm.

TABLE E4. Significant metabolites (FDR $q = 0.01$) mapped into the N-acyl ethanolamine and the N-acyltransferase pathway

Input mass	Adduct	KEGG ID	Mass	dppm*	Name	Formula
283.2621	[M+H] ⁺	C00712	282.2559	3	Oleic acid	C ₁₈ H ₃₄ O ₂
264.2676	[M+H-2H ₂ O] ⁺	C16512	299.2824	7	Palmitoyl ethanolamide	C ₁₈ H ₃₇ NO ₂
403.2642	[M+H-2H ₂ O] ⁺	C00416	438.2746	5	Lysophosphatidic acid (0:0/18:0)	C ₂₁ H ₄₃ O ₇ P
435.2543	[M+H] ⁺	C00416	434.2433	8	Lysophosphatidic acid (0:0/18:2)	C ₂₁ H ₃₉ O ₇ P
457.2293	[M+Na] ⁺	C00416	434.2433	7	Lysophosphatidic acid (0:0/18:2)	C ₂₁ H ₃₉ O ₇ P
634.4498	[M+H] ⁺	C00350	633.4370	8	Phosphatidylethanolamine (14:0/14:1)	C ₃₃ H ₆₄ NO ₈ P
646.4276	[M+H-2H ₂ O] ⁺	C00350	681.4370	5	Phosphatidylethanolamine (14:1/18:4)	C ₃₇ H ₆₄ NO ₈ P
666.5237	[M+H-2H ₂ O] ⁺	C00350	701.5359	0	Phosphatidylethanolamine (16:0/P-18:1)	C ₃₉ H ₇₆ NO ₇ P
684.4508	[M+Na] ⁺	C00350	661.4683	9	Phosphatidylethanolamine (14:0/16:1)	C ₃₅ H ₆₈ NO ₈ P
698.4721	[M+H] ⁺	C00350	697.4683	4	Phosphatidylethanolamine (15:0/18:4)	C ₃₈ H ₆₈ NO ₈ P
710.4696	[M+H] ⁺	C00350	709.4683	8	Phosphatidylethanolamine (14:0/25:5)	C ₃₉ H ₇₂ NO ₈ P
710.4696	[M+Na] ⁺	C00350	687.4839	4	Phosphatidylethanolamine (14:0/18:2)	C ₃₇ H ₇₀ NO ₈ P
726.4826	[M+H-2H ₂ O] ⁺	C00350	761.4996	5	Phosphatidylethanolamine (16:1/22:6)	C ₄₃ H ₇₂ NO ₈ P
736.4856	[M+H] ⁺	C00350	735.4839	7	Phosphatidylethanolamine (14:0/22:6)	C ₄₁ H ₇₀ NO ₈ P
736.4856	[M+Na] ⁺	C00350	713.4996	4	Phosphatidylethanolamine (14:0/20:3)	C ₃₉ H ₇₂ NO ₈ P
742.4831	[M+H-H ₂ O] ⁺	C00350	759.4839	2	Phosphatidylethanolamine (18:3/20:5)	C ₄₃ H ₇₀ NO ₈ P
750.5120	[M+H] ⁺	C00350	749.4996	6	Phosphatidylethanolamine (15:0/22:6)	C ₄₂ H ₇₂ NO ₈ P
768.5525	[M+H] ⁺	C00350	767.5465	1	Phosphatidylethanolamine (20:4/18:0)	C ₄₃ H ₇₈ NO ₈ P
768.5525	[M+Na] ⁺	C00350	745.5622	1	Phosphatidylethanolamine (14:0/22:1)	C ₄₁ H ₈₀ NO ₈ P
772.6155	[M+H-H ₂ O] ⁺	C00350	789.6248	8	Phosphatidylethanolamine (15:0/24:0)	C ₄₄ H ₈₈ NO ₈ P
776.5132	[M+Na] ⁺	C00350	753.5309	8	Phosphatidylethanolamine (15:0/22:4)	C ₄₂ H ₇₆ NO ₈ P
782.5674	[M+Na] ⁺	C00350	759.5778	0	Phosphatidylethanolamine (15:0/22:1)	C ₄₂ H ₈₂ NO ₈ P
814.6144	[M+H-2H ₂ O] ⁺	C00350	849.6248	2	Phosphatidylethanolamine (20:4/24:1)	C ₄₉ H ₈₈ NO ₈ P
836.5239	[M+Na] ⁺	C00350	813.5309	4	Phosphatidylethanolamine (20:3/22:6)	C ₄₇ H ₇₆ NO ₈ P
842.6935	[M+H-H ₂ O] ⁺	C00350	859.7030	8	Phosphatidylethanolamine (20:0/24:0)	C ₄₉ H ₉₈ NO ₈ P
844.6507	[M+H-2H ₂ O] ⁺	C00350	879.6717	9	Phosphatidylethanolamine (22:4/24:0)	C ₅₁ H ₉₄ NO ₈ P
850.6392	[M+H] ⁺	C00350	849.6248	8	Phosphatidylethanolamine (20:4/24:1)	C ₄₉ H ₈₈ NO ₈ P
8526389	[M+Na] ⁺	C00350	829.6561	7	Phosphatidylethanolamine (18:1/24:0)	C ₄₇ H ₉₂ NO ₈ P
882.7014	[M+H] ⁺	C00350	881.6874	7	Phosphatidylethanolamine (22:2/24:1)	C ₅₁ H ₉₆ NO ₈ P
902.6563	[M+Na] ⁺	C00350	879.6717	5	Phosphatidylethanolamine (22:4/24:0)	C ₅₁ H ₉₄ NO ₈ P

*dppm refers to the difference in parts per million (ppm) between the observed metabolite and the mapped chemical ppm.